LAT1 is associated with poor prognosis and radioresistance in head and neck squamous cell carcinoma

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Abstract. Head and neck squamous cell carcinoma (HNSCC) has been identified as the sixth most common disease in the world, and its prognosis remains poor. The basic treatment of HNSCC includes a combination of chemoradiation and surgery. With the advent of immune checkpoint inhibitors, the prognosis has improved; however, the efficacy of checkpoint inhibitors is limited. L-type amino acid transporter 1 (LAT1), an amino acid transporter, is highly expressed in a cancer-specific manner. However, to the best of our knowledge, LAT1 expression in HNSCC has not been determined. Therefore, the present study aimed to examine the role of LAT1 expression in HNSCC. A total of three HNSCC cell lines (Sa3, HSC2 and HSC4) were used to investigate the characteristics of LAT1-positive cells, including their ability to form spheroids, and their invasion and migration. The present study also examined LAT1 by immunostaining of biopsy specimens from 174 patients diagnosed, treated and followed-up at Akita University (Akita, Japan) between January 2010 and December 2019, and overall survival, progression-free survival and multivariate analyses were performed. The results demonstrated that LAT1-positive cells in HNSCC were an independent prognostic factor for overall survival and progression-free survival, and were resistant to chemoradiation. Therefore, JPH203, a LAT1 inhibitor, may be effective in treating chemoradiotherapy-resistant HNSCC and may improve the prognosis of patients with HNSCC.

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Abbreviations: HNSCC, head and neck squamous cell carcinoma; HR, hazard ratio; LAT1, L-type amino acid transporter 1; OS, overall survival; PFS, progression-free survival

Key words: HNSCC, LAT1, radioresistant, JPH203, prognostic factor

Introduction

Head and neck squamous cell carcinoma (HNSCC) has been identified as the sixth most common cancer in the world, with approximately 600,000 new cases diagnosed annually (1). Two-thirds of patients are diagnosed at an advanced stage, and the 5-year survival rate is 39-65%; thus, HNSCC has one of the worst prognoses compared to other cancer types (2). Worldwide, the standard treatments are surgery and chemoradiation (3-7). Unfortunately, recurrence and metastasis often occur even after these therapies. Recently, immune checkpoint inhibitors, such as nivolumab and pembrolizumab, have exhibited better performance than standard chemotherapy in the Phase III CHECK-MATE-141 and KEYNOTE-040 studies (8,9). However, the response rate was only about 15%, and the median overall survival was 1.4-2.4 months, which is deemed unsatisfactory (10).

The L-type amino acid transporter (LAT1, SLC7A5) has been attracting the recent attention as a therapeutic target. Among the 50 types of mammalian cell membrane amino acid transporters, the expression of the following transporters is upregulated in malignant tumors: LAT1 (SLC7A5) (11), LAT3 (SLC43A1) (12), ASCT2 (SLC1A5) (13), AT^{B0+} (SLC6A14) (14), and xCT (SLC7A11) (15). LAT1 forms a heterodimeric complex with CD98 heavy chain (CD98hc) (4F2hc, SLC3A2) (16) and transports large neutral amino acids, such as leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, methionine, and histidine. LAT1 is often upregulated in human cancer tissues, including colon, lung, prostate, gastric, breast, kidney, esophageal, and brain cancers. In non-small cell lung cancer, pancreatic cancer, brain tumor, prostate cancer, and breast cancer, high expression of LAT1 is associated with a poor prognosis, suggesting that the expression of LAT1 is related to cancer malignancy (17-22). LAT1 is upregulated in cancers, and its expression is highly specific to cancers. Inhibition of LAT1 often blocks the amino acid supply to tumor cells and evokes antitumor effects. Thus, LAT1 inhibitors are currently being developed as antitumor drugs. Intravenous administration of the LAT1 inhibitor, JPH203, inhibits tumor growth in nude mice (23).

Despite the high expression of LAT1 in various cancers, the function of LAT1 has not yet been elucidated in HNSCC.

Therefore, this study aimed to characterize LAT1 in HNSCC and to investigate the relationship between LAT1 and prognosis in clinical samples. If LAT1 is a prognostic factor, the use of JPH203 will be improved significantly. If LAT1 can be used as a prognostic factor, tailor-made treatment based on the patient's background can be implemented instead of the current standard of care, which includes platinum-based agents combined with radiation therapy and surgery. For refractory and recurrent tumors, JPH203 may be an alternative treatment to nivolumab and pembrolizumab.

Materials and methods

Cell culture. Sa3 (gingiva), HSC2 (oral), and HSC4 (tongue) cell lines were used. Cells were grown in RPMI1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Clontech Laboratories, Mountain View, CA) and 2 mM L-glutamine. The radioresistant cell lines were described previously (24).

Flow cytometry. For the analysis of the LAT1-positive fraction, cell pellets were incubated with FITC-conjugated SLC7A5/LAT1 antibody (BU53) (Novus Biologicals, Littleton). After washing with PBS twice, the cells were resuspended in 2 μg/ml propidium iodide/Hank's balanced salt solution and filtered through a cell strainer (BD Biosciences Discovery Labware, Bedford, MA). Flow cytometry was performed using a BD FACS Aria III instrument (Becton, Dickinson and Company Japan, Tokyo, Japan). To determine the negative fraction, FITC-conjugated mouse IgG2a isotype control (M2A) (Novus, Biologicals, Littleton, USA) was used.

Sphere formation assay. To avoid adhesion and subsequent development to non-CSCs, cells were cultured in serum-free semisolid medium. LAT1-positive and LAT1-negative cells ($1x10^3$ cells each) were seeded in 100 μl of PromoCell 3D Tumorsphere Medium XF (PromoCell GmbH, Heidelberg, Germany), containing 0.33% agar. The cells were then incubated for 14 days at 37˚C in a humidified atmosphere containing 5% CO_2.

Invasion assay. Invasion assays were performed using an 8 μm Boyden chamber (Falcon, USA). The filter was coated with 100 μl of Matrigel (1 mg/ml). The upper chamber was filled with 500 μl of serum-free RPMI 1640 medium with $5x10^3$ cells, whereas the bottom chamber was filled with 1,000 μl of 10%-FBS-supplemented RPMI 1640 medium. After 72 h of incubation, the upside of the filter was swabbed off and fixed with formalin. Cells were stained with hematoxylin-eosin and counted under an optical microscope (Olympus, Tokyo, Japan).

Wound healing assay. Cells were grown to confluency with growth medium in a 60-mm dish, and a straight line was drawn with a 200-μl pipet tip. After 24 h of incubation with serum-free RPMI medium, the cells were fixed in formalin, and the residual area of wound gap after migration was analyzed using ImageJ and compared to that of the corresponding initial wound gap (set at 1).

Table I. Patient characteristics.

Characteristic	Value
Mean age ± SD, years	65.4±10.6
Sex, n (male/female)	143/30
T category n (%)	
T1	16 (9.2)
T2	70 (40.5)
T3	37 (21.4)
T4	50 (28.9)
N category, n (%)	
N0	49 (28.3)
N1	17 (9.8)
N2	103 (59.5)
N3	4 (2.4)
M category, n (%)	
M0	173 (100.0)
Stage, n (%)	
I	11 (6.4)
II	27 (15.6)
III	19 (11.0)
IV	116 (67.0)
Tumor sites, n (%)	
Tongue	32 (18.5)
Nasopharynx	9 (5.2)
Oropharynx	55 (31.8)
Hypopharynx	63 (36.4)
Gingiva	14 (8.1)

Immunostaining. Immunohistochemical staining was performed on 173 preoperative untreated HNSCC biopsy specimens collected at our hospital from 2010 to 2019 for clinicopathological studies. The median follow-up period was 34 (range: 2-6) months. The anti-LAT-1 antibody was a monoclonal antibody purified from rabbit. Tissue sections (3 μm) were deparaffinized and treated with 3% hydrogen peroxide methanol for 10 min to inhibit endogenous peroxidase activity. After washing with the buffer, the sections were incubated with anti-LAT1 antibody (1:1,000; ab208776, Abcam, Cambridge, MA) for 90 min. The sections were then incubated with Dako EnVision+ System HRP and colorized with DAB (3,3-diaminobenzidine). The results were classified into four levels according to the degree of staining: score 0 (negative), score 1 (weak), score 2 (moderate), and score 3 (strong). A score of ≥2 was considered positive for LAT1 expression. The evaluation method was adopted from a study by Rietbergen *et al* (25). At least two skilled pathologists scored the staining while blinded to the clinical information.

Experiment using JPH203. JPH203 (Namiki, Tokyo, Japan) was used to inhibit LAT1 at a concentration of 100 μM, referring to the concentration used by Choi *et al* (26).

Table II. Univariate and multivariate analyses of OS and PFS.

A, 5-year OS

	Univariate analysis			Multivariate analysis		
Characteristic	HR	95% CI	P-value	HR	95% CI	P-value
Age (<65 vs. >65 years)	1.537	0.963-2.455	0.072	1.827	1.133-2.946	0.013[a]
Sex (female vs. male)	1.356	0.696-2.640	0.371	1.422	0.724-2.796	0.307
T category (T1-T2 vs. T3-T4)	1.954	1.221-3.128	0.005[b]	1.691	1.032-2.769	0.037[a]
N category (N0 vs. N1-N3)	1.845	1.046-3.253	0.034[a]	1.666	0.904-3.068	0.102
Stage (I-II vs. III-IV)	1.918	1.010-3.642	0.046[a]	NA	NA	NA
LAT1 (low vs. high)	1.710	1.013-2.887	0.045[a]	1.749	1.033-2.961	0.037[a]

B, 5-year PFS

	Univariate analysis			Multivariate analysis		
Characteristic	HR	95% CI	P-value	HR	95% CI	P-value
Age (<65 vs. >65 years)	1.046	0.700-1.564	0.825	1.200	0.792-1.816	0.390
Sex (female vs. male)	1.434	0.783-2.627	0.243	1.488	0.807-2.743	0.203
T category (T1-T2 vs. T3-T4)	1.503	1.003-2.254	0.048[a]	1.388	0.908-2.123	0.130
N category (N0 vs. N1-N3)	1.509	0.937-2.430	0.090	1.366	0.818-2.282	0.233
Stage (I-II vs. III-IV)	1.434	0.849-2.422	0.178	NA	NA	NA
LAT1 (low vs. high)	1.610	1.019-2.543	0.041[a]	1.640	1.035-2.597	0.035[a]

[a]P<0.05, [b]P<0.01. LAT1 was an independent prognostic factor for both OS and PFS. NA, not applicable; HR, hazard ratio; LAT1, L-type amino acid transporter 1; OS, overall survival; PFS, progression-free survival.

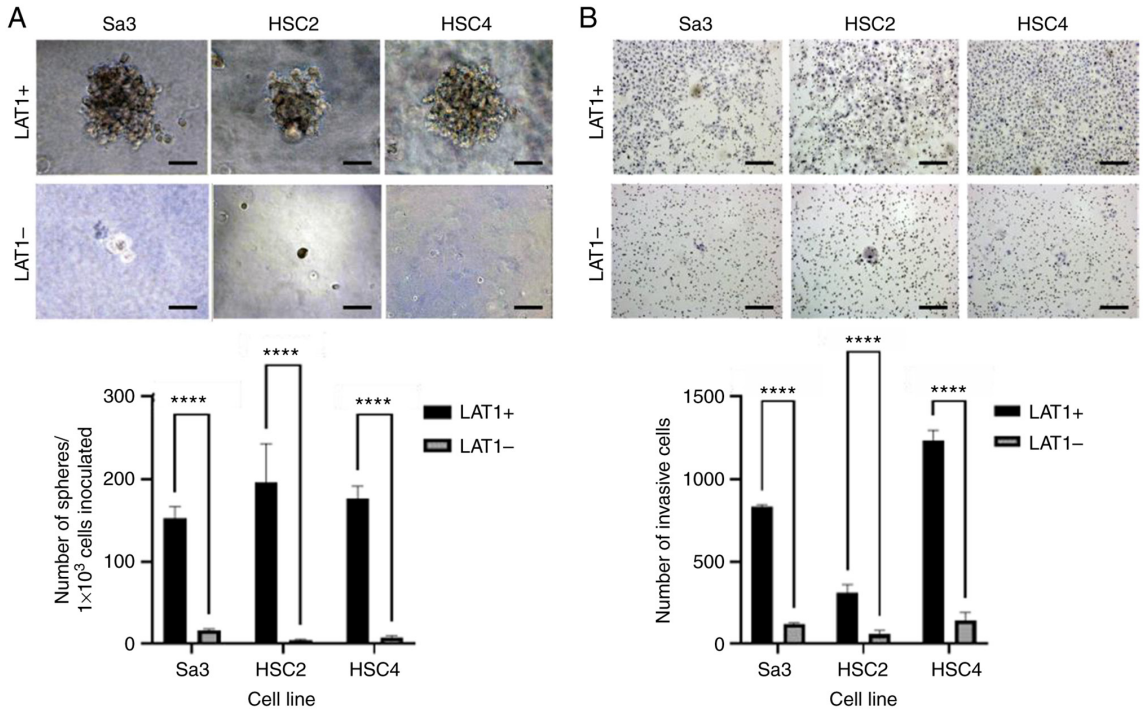

Figure 1. LAT1-positive cells exhibit enhanced spheroid formation and invasion. A total of three cell lines were separated into LAT1-positive and LAT1-negative cells using flow cytometry. (A) Spheroid formation was compared after 14 days of culture in serum-free semifluid medium. LAT1-positive cells were capable of forming more spheroids than LAT1-negative cells. LAT1-negative cells had little ability to form spheroids. Scale bar, 100 μm. (B) LAT1-positive cells were also more invasive than LAT1-negative cells. Scale bar, 200 μm. ****P<0.0001. LAT1, L-type amino acid transporter 1; LAT1+, LAT1-positive cell; LAT1-, LAT1-negative cell.

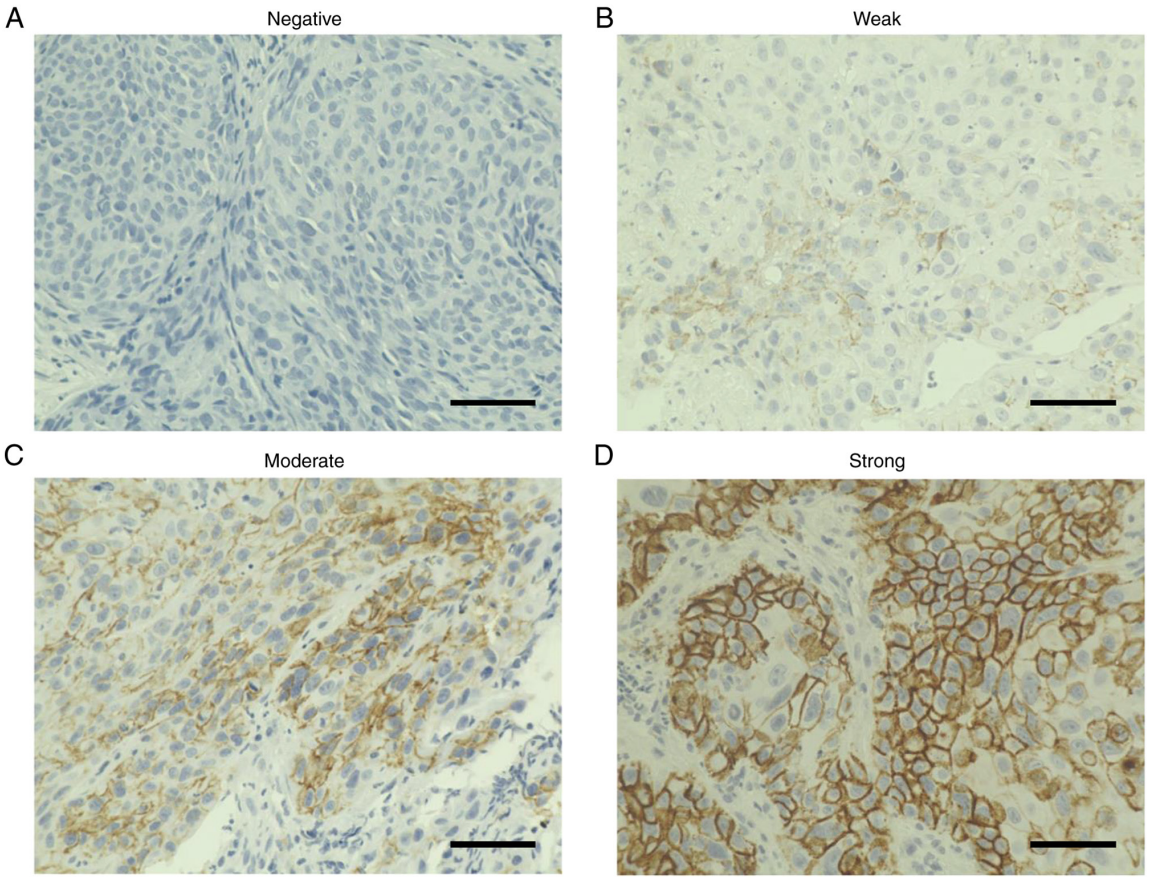

Figure 2. Immunostaining of L-type amino acid transporter 1 in biopsy specimens from patients with head and neck squamous cell carcinoma. Expression levels were determined by experienced pathologists as follows: (A) Negative, the proportion of positive cells is <10%; (B) weak, the proportion of positive cells is ≥10% and <25%; (C) moderate, the proportion of positive cells is ≥25% and <50%; (D) strong, the proportion of positive cells is ≥50%. Scale bar, 100 μm.

Statistical analysis. Significant differences in OS and PFS were assessed using the Kaplan-Meier method and log-rank test. The univariate and multivariate Cox proportional hazards modeling was used to evaluate prognostic significance. One-way ANOVA followed by Tukey's post hoc test was used to compare three unpaired groups, and unpaired Student's t-tests were used to compare two unpaired groups, respectively. All experiments were independently repeated at least three times. $P<0.05$ was considered significant.

Results

LAT1-positive cells in HNSCC have strong spheroid-forming and invasive potential. Three HNSCC cell lines, that is, Sa3, HSC2, and HSC4, were separated into LAT1-positive and -negative cells using a flow cytometer. The LAT1-positive cells had strong spheroid formation ability, whereas the LAT1-negative cells could hardly form spheroids (Fig. 1A). LAT1-positive cells from the three different cell lines also exhibited enhanced invasive ability (Fig. 1B).

Patients with LAT1-positive HNSCC have a poor prognosis and are refractory to chemoradiotherapy. According to TNM Classification of Malignant Tumours, 8th ed., Union for International Cancer Control (27), 78.0% of patients were at advanced stages (Table I). The intensity of LAT1 immunostaining of biopsy specimens was determined by skilled pathologists to be negative (Fig. 2A), weak (Fig. 2B), moderate (Fig. 2C), or strong (Fig. 2D). Specimens with negative and weak immunostaining were classified into the Low group, whereas specimens with moderate and strong immunostaining were classified into the High group. Of the 173 patients (Table I), 52 were in the Low group and 121 were in the High group. The 5-year OS was 56.8% in the Low group, whereas it was 45.3% in the High group (P=0.041) (Fig. 3A); moreover, PFS was 47.9% in the Low group and 36.2% in the High group (P=0.037) (Fig. 3B). Furthermore, multivariate analysis revealed that LAT1 was an independent prognostic factor for OS (Table IIA) and PFS (Table IIB) [OS, P=0.045, HR: 1.710, 95% confidence interval (95% CI): 1.013-2.887; PFS, P=0.037, HR: 1.749, 95% CI: 1.013-2.887].

In total, 85 of the 173 patients were treated with chemoradiation, including 36 patients in the Low group and 49 patients in the High group (Table III). The 5-year OS for patients treated with chemoradiation was 58.7% in the Low group and 33.0% in the High group (P=0.003) (Fig. 3C). The PFS for the Low group was found to be better than the prognosis for the High group (46.4% in the Low group vs. 28.4% in the High group, P=0.001) (Fig. 3D). The multivariate analysis showed that LAT1 was an independent prognostic factor for OS and PFS in patients who underwent chemoradiotherapy (OS: P=0.008, HR: 2.697, 95% CI: 1.292-5.464; PFS: P=0.017, HR: 2.124, 95% CI: 1.147-3.933) (Table IV). Surgical treatment was available for 88 patients (Table V), but there were no significant differences in terms of OS (53.5% for Low and 53.6% for High) (Fig. 3E) or PFS (50.0% for Low and 41.4% for High) (Fig. 3F), (Table VI).

Table III. Characteristics of patients treated by chemoradiotherapy.

Characteristic	Value
Mean age ± SD, years	65.6±9.1
Sex, n (male/female)	75/10
T category, n (%)	
T1	6 (7.1)
T2	35 (41.2)
T3	21 (24.7)
T4	23 (27.0)
N category, n (%)	
N0	13 (15.3)
N1	12 (14.1)
N2	56 (65.9)
N3	4 (4.7)
M category, n (%)	
M0	85 (100.0)
Stage, n (%)	
I	2 (2.4)
II	7 (8.2)
III	12 (14.1)
IV	64 (75.3)
Tumor sites, n (%)	
Tongue	2 (2.4)
Nasopharynx	9 (10.6)
Oropharynx	40 (47.0)
Hypopharynx	32 (37.6)
Gingiva	2 (2.4)

Radioresistant cells have an expanded LAT1-positive fraction and enhanced malignant potential. Because of the poor prognosis of LAT1-positive patients and their resistance to radiotherapy, we examined the expression of LAT1 in three cell lines, Sa3, HSC2, and HSC4, after irradiation with 60 Gy. The LAT1-positive fraction increased from 10-20% before irradiation to 60-80% after irradiation (Fig. 4A). After irradiation with 60 Gy, cells were separated into LAT1-positive and LAT1-negative cells using flow cytometry and cultured in serum-free semifluid medium. The LAT1-positive cells exhibited enhanced spheroid-forming and invasive abilities (Fig. 4B and C), indicating that radioresistant cells had high malignant potential.

LAT1 inhibition reduces the LAT1-positive fraction of normal and radioresistant cells and reduces the malignant potential. JPH203 was added to RPMI 1640 and incubated for 1 day, after which the LAT1-positive fractions were compared. Treatment with JPH203 reduced the fraction of LAT1-positive cells from 10-20% to 2-5% (Fig. 5A). In radioresistant cells (irradiated as described above), JPH203 reduced the LAT1-positive fraction from 80 to 20-40% (Fig. 5B). Thus, JPH203 reduced the LAT1-positive fraction in both the parental and radioresistant cells.

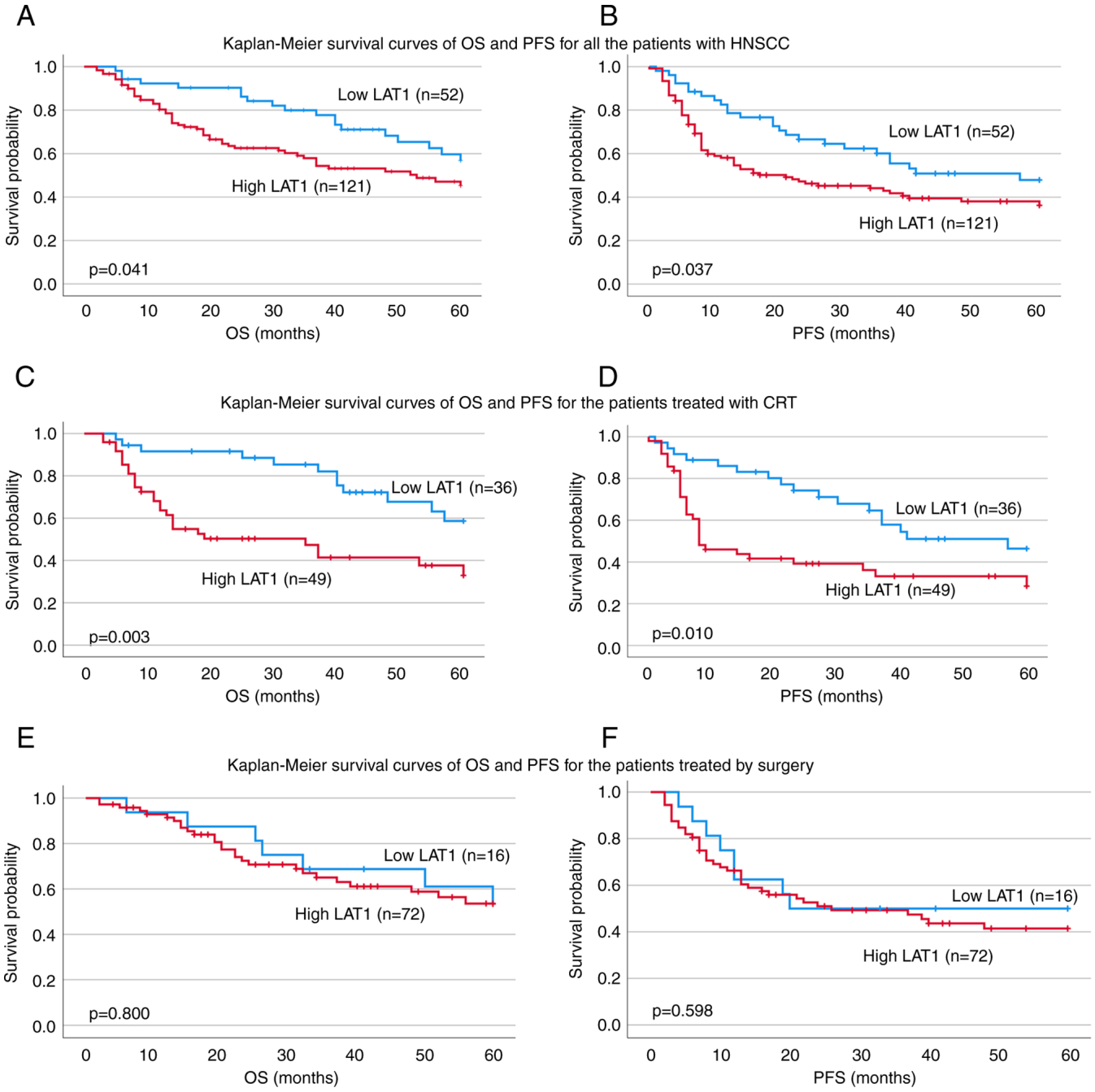

Figure 3. (A and B) Kaplan-Meier survival curves of OS and PFS for all patients with HNSCC. Patients with HNSCC (n=173) were classified into high (121 patients) and low (52 patients) LAT1 expression groups. (A) 5-year OS was 56.8% in the low group and 45.3% in the high group (P=0.041). (B) 5-year PFS was 47.9% in the low group and 36.2% in the high group (P=0.037). The high LAT1 group had a predominantly poor prognosis. (C and D) Kaplan-Meier survival curves of OS and PFS for 85 patients with HNSCC who underwent CRT. Patients were classified into high (49 patients) and low (36 patients) LAT1 expression groups. (C) 5-year OS was 58.7% in the low group and 33.0% in the high group (P=0.003). (D) 5-year PFS was 46.4% in the low group and 28.4% in the high group (P=0.010). These results indicated that high LAT1 expression conferred resistance to CRT. (E and F) Kaplan-Meier survival curves of OS and PFS for 89 surgically treated patients with HNSCC. Patients were classified into high (72 patients) and low (16 patients) LAT1 expression groups. (E) 5-year OS was 53.5% in the low group and 53.6% in the high group. (F) 5-year PFS was 50.0% in the low group and 41.4% in the high group. In surgically treated patients with HNSCC, neither OS nor PFS was associated with LAT1 expression. CRT, chemoradiotherapy; HNSCC, head and neck squamous cell carcinoma; LAT1, L-type amino acid transporter 1; OS, overall survival; PFS, progression-free survival.

Parental and radioresistant cells were separated into LAT1-positive and LAT1-negative cells and cultured in serum-free semifluid medium supplemented with JPH203. After JPH203 treatment, the spheroid formation ability was not significantly different between LAT1-positive and LAT1-negative cells (Fig. 5C and D) Similarly, no differences in invasive ability were detected between LAT1-positive and LAT1-negative cells after JPH203 treatment in both normal and radioresistant cells (Fig. 5E and F).

JPH203 inhibits the migratory ability of normal and radioresistant cells. Wound healing assays were performed in both parental and radioresistant cells. JPH203 was effective

Table IV. Univariate and multivariate analyses of OS and PFS.

A, 5-year OS

	Univariate analysis			Multivariate analysis		
Characteristic	HR	95% CI	P-value	HR	95% CI	P-value
Age (<65 vs. >65 years)	1.988	1.008-3.920	0.047[a]	1.707	0.852-3.418	0.131
Sex (female vs. male)	1.865	0.574-6.053	0.300	2.157	0.639-7.283	0.216
T category (T1-T2 vs. T3-T4)	2.798	1.438-5.443	0.002[b]	2.351	1.168-4.730	0.017[a]
N category (N0 vs. N1-N3)	1.624	0.635-4.156	0.311	0.896	0.314-2.560	0.838
Stage (I-II vs. III-IV)	3.360	0.808-13.963	0.095	NA	NA	NA
LAT1 (low vs. high)	2.695	1.361-5.336	0.004[b]	2.657	1.292-5.464	0.008[b]

B, 5-year PFS

	Univariate analysis			Multivariate analysis		
Characteristic	HR	95% CI	P-value	HR	95% CI	P-value
Age (<65 vs. >65 years)	1.331	0.744-2.382	0.336	1.160	0.634-2.123	0.630
Sex (female vs. male)	2.542	0.789-8.188	0.118	2.772	0.845-9.091	0.092
T category (T1-T2 vs. T3-T4)	1.925	1.080-3.430	0.026[a]	1.767	0.954-3.273	0.070
N category (N0 vs. N1-N3)	1.587	0.674-3.734	0.290	0.974	0.388-2.441	0.955
Stage (I-II vs. III-IV)	2.550	0.792-8.215	0.117	NA	NA	NA
LAT1 (low vs. high)	2.120	1.170-3.842	0.013[a]	2.124	1.147-3.933	0.017[a]

[a]P<0.05, [b]P<0.01. LAT1 was also an independent prognostic factor in chemoradiotherapy. NA, not applicable; HR, hazard ratio; LAT1, L-type amino acid transporter 1; OS, overall survival; PFS, progression-free survival.

Table V. Characteristics of patients treated by surgery.

Characteristic	Value
Mean age ± SD, years	65.2±11.9
Sex, n (male/female)	68/20
T category, n (%)	
T1	10 (11.4)
T2	35 (39.8)
T3	16 (18.2)
T4	27 (30.6)
N category, n (%)	
N0	36 (40.9)
N1	5 (5.7)
N2	47 (53.4)
N3	0 (0.0)
M category, n (%)	
M0	88 (100.0)
Stage, n (%)	
I	9 (10.2)
II	20 (22.7)
III	7 (8.0)
IV	52 (59.1)
Tumor sites, n (%)	
Tongue	30 (34.1)
Nasopharynx	0 (0.0)
Oropharynx	15 (17.0)
Hypopharynx	31 (35.3)
Gingiva	12 (13.6)

in inhibiting the migration in both the parental (Fig. 5G) and radioresistant cells (Fig. 5H).

Discussion

We demonstrated that LAT1 is strongly involved in sphere formation, invasion, and migration in HNSCC. Furthermore, patients with LAT1-positive specimens had a worse prognosis and were more resistant to chemoradiotherapy compared to patients with low LAT1 expression. JPH203, a LAT1 inhibitor, suppressed sphere formation, invasion, and migration in radioresistant cells.

We have previously reported that CD98hc is a marker for cancer stem cells in HNSCC (24), and similar reports have been published by other investigators (28,29). Since CD98hc binds to amino acid transporters in the light chain, LAT1-positive cells may have cancer stem cell characteristics. LAT1-positive cells can form spheres in serum-free semifluid medium, which is a characteristic of cancer stem cells (30). Although other stem cell markers, such as Oct3/4, Nanog, and SOX2, need to be investigated, LAT1 may be an important therapeutic target because it induces chemoradiotherapy resistance.

The mTOR signaling pathway plays an important role in invasion and migration. Amino acids, including leucine or amino acid prodrugs, are transported into cells by LAT1 and cause activation of mTORC1, resulting in enhanced invasion and migration (31). In our study, the enhanced invasion and migration of LAT1-positive cells may result from the activation of mTOR signaling.

According to the LAT1 immunostaining of HNSCC patient biopsies, high LAT1 expression was associated with poor prognosis and chemoradiotherapy resistance. In a previous report, high LAT1 expression was associated with an extremely poor prognosis in resected tongue cancer (32). However, in our study, no significant differences were detected in the LAT1 expression groups after surgical treatment. The lack of differences may be due to the staging based on clinical imaging diagnosis rather than pathological indicators and grouping head and neck cancers together. We believe that the ability to predict chemoradiotherapy resistance at the biopsy stage based on LAT1 expression is a significant finding of this study.

About half of HNSCC patients relapse after chemoradiotherapy or surgery, and immune checkpoint inhibitors have achieved some success. However, their efficacy is limited, and HNSCC remains a disease with a poor prognosis (33). Therefore, JPH203, a LAT1 inhibitor, is expected to be a new therapeutic agent. The expression of LAT1 increased from 10-20% to 60-80% after irradiation. Sphere formation, invasion, and migration are enhanced in LAT1-positive cells, even in radioresistant cell lines. The high fraction of LAT1-positive cells in resistant cell lines indicates high malignancy (34). Recurrent tumors may need to utilize more amino acids to survive and proliferate; however, the mechanism must be clarified.

JPH203 suppressed sphere formation, invasion, and migration in both the parental and radioresistant cells. After the addition of JPH203, the LAT1-positive cell fraction was noted to decrease to 2-5% in the parental cells and significantly decreased to 20-40% in the radioresistant cells. The expression of LAT1 was also suppressed by BCH, which is an inhibitor of LAT1 and LAT2. However, the expression of LAT1 is upregulated by feedback with prolonged exposure to JPH203 (35). In this study, the results were obtained after 24 h. The long-term expression of LAT1 requires further investigation.

In HNSCC, LAT1-positive cells are highly malignant and capable of sphere formation, invasion, and migration. Targeting these cells will improve the prognosis of HNSCC. Furthermore, LAT1 expression at the biopsy stage can be used to determine radiosensitivity. This will play an important role in designing tailor-made treatment strategies. For instance, patients with high LAT1 expression can undergo surgery first, whereas patients with low LAT1 expression can undergo chemoradiation first. JPH203 concomitant radiation therapy may be an alternative to platinum-based agents. JPH203 may also be an effective treatment for recurrent tumors that have become radioresistant, and the availability of other options, in addition to nivolumab and pembrolizumab, will improve the prognosis of HNSCC patients. In addition to treatment, ^{18}F-FAMT, a LAT1-selective amino acid PET, has been found to be effective in cancer diagnosis (36). In the HNSCC field, the function of LAT1 needs to be clarified urgently and actively applied in the future.

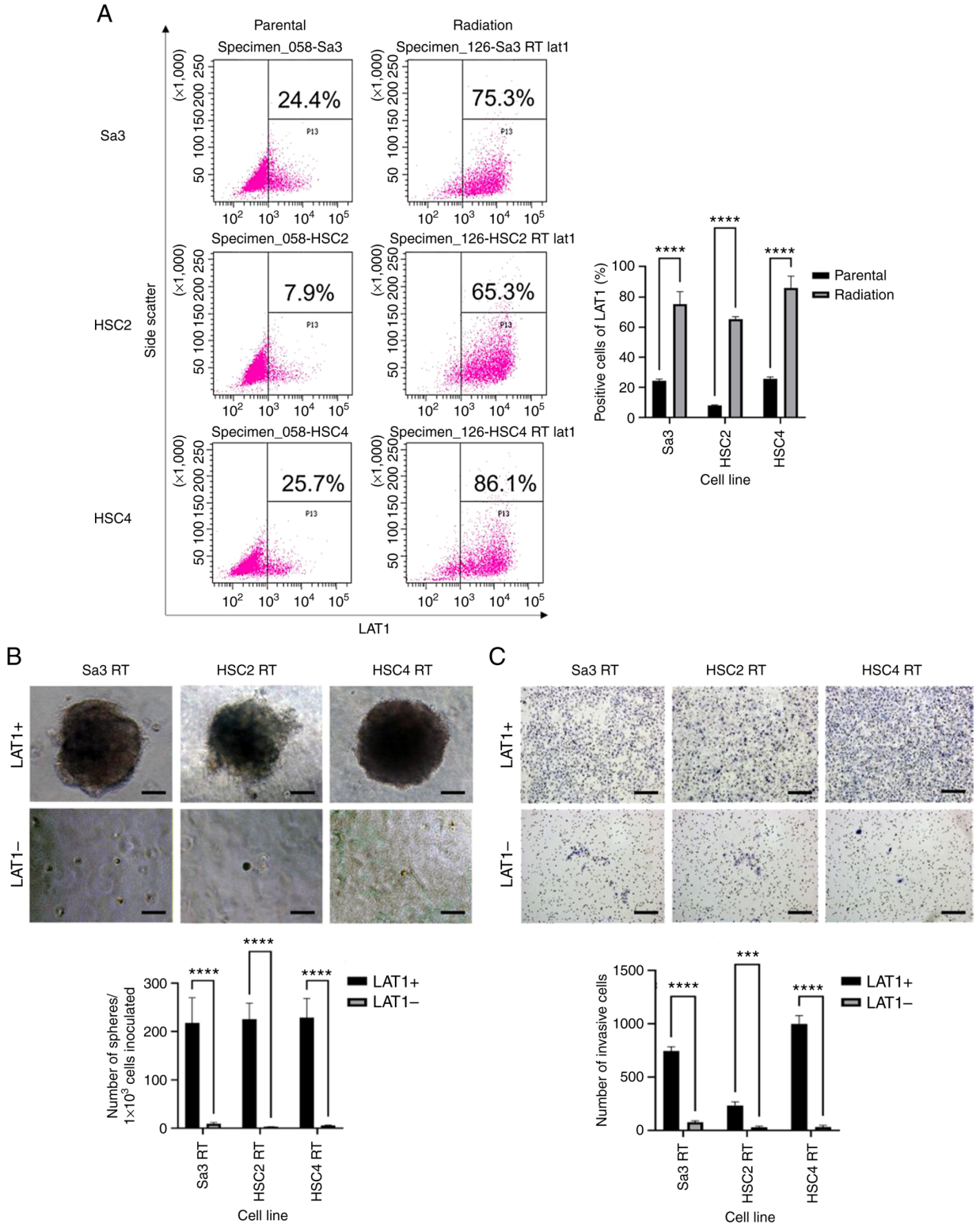

Figure 4. (A) Expanded LAT1-positive fractions in radioresistant cells. A total of three cell lines were irradiated with 60 Gy of radiation, and LAT1 expression was examined by flow cytometry. The positive rate of LAT1 increased from 10-20% to 60-80% after irradiation. It is unclear whether the LAT1-positive cells survived irradiation or the number of LAT1-positive cells increased after irradiation. (B) Spheroid formation in LAT1-positive radioresistant cells. Scale bar, 100 μm. (C) Invasiveness in LAT1-positive radioresistant cells. Scale bar, 200 μm. The present study examined the malignant characteristics in LAT1-positive and LAT1-negative radioresistant cells after 60 Gy irradiation. Note that the abilities of LAT1-positive cells to form spheroids and to invade to Matrigel were reinforced by acquisition of radioresistance. ***P<0.001, ****P<0.0001. LAT1, L-type amino acid transporter 1; LAT1+, LAT1-positive cell; LAT1-, LAT1-negative cell; RT, radiotolerant.

In conclusion, LAT1-positive cells in HNSCC are those with enhanced spheroid formation, invasion, and migration, as well as those in radioresistant cell lines. Immunostaining of HNSCC patient specimens showed that LAT1 is an independent prognostic factor and resistant to chemoradiotherapy. JPH203, a LAT1 inhibitor, could strongly suppress spheroid

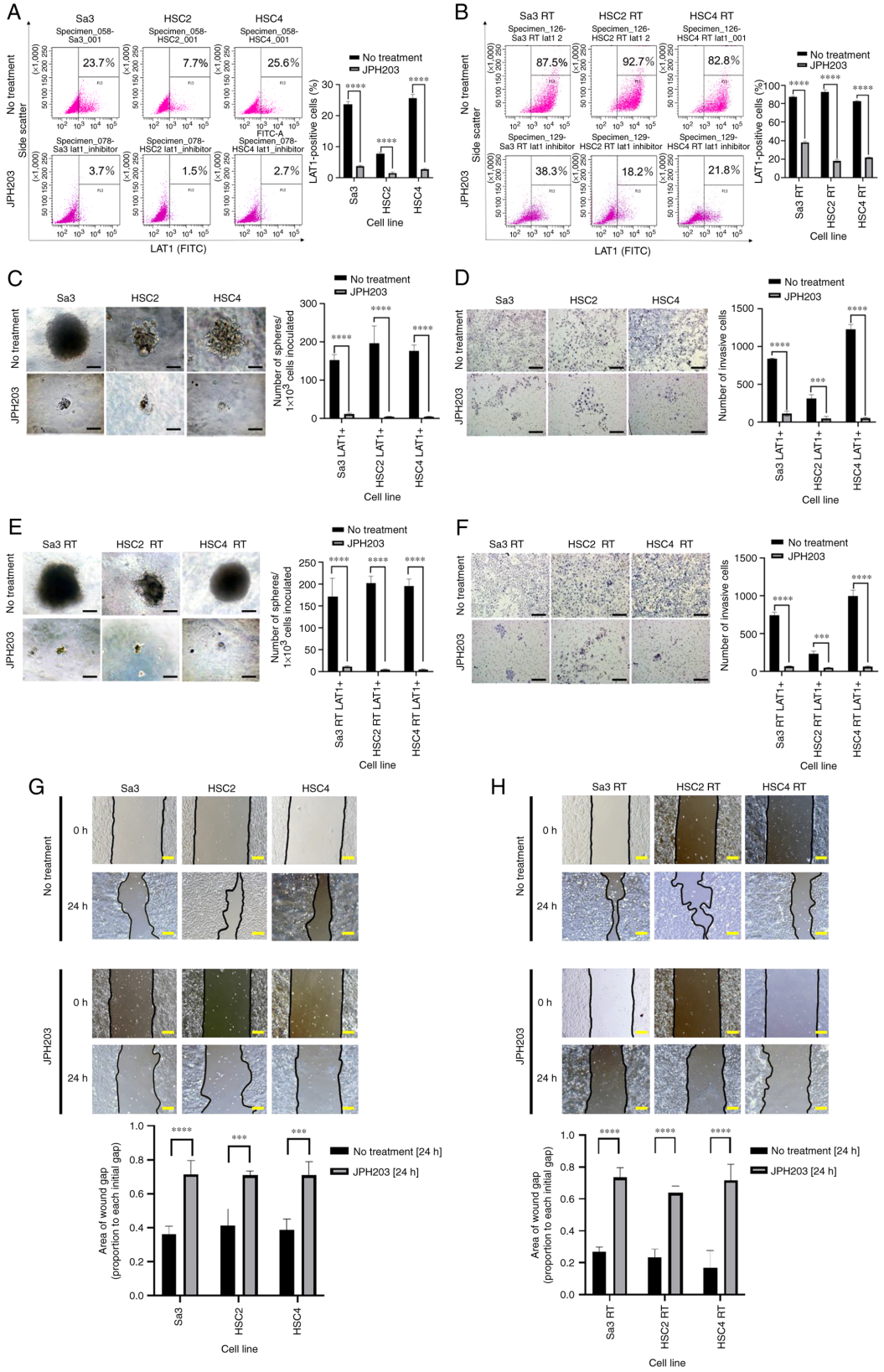

Figure 5. (A and B) Reduction in LAT1-positive cells in response to JPH203. Cells were incubated with JPH203 in RPMI 1640 medium for 24 h, and LAT1 expression was examined by flow cytometry. (A) In the parental cell lines, the LAT1-positive fraction decreased from 10-20% to 2-5%. (B) In the radioresistant cell lines, the LAT1-positive fraction decreased from 60-80% to 20-40%. (C-F) Inhibition of spheroid formation and invasiveness by JPH203 in both the LAT1-positive parental cells and radioresistant cells. (C) Spheroid formation and (D) invasion of the parental cell lines were examined after adding JPH203 to the culture medium. Spheroid formation and invasion of LAT1-positive cells were significantly suppressed. In the radioresistant cells, JPH203 was added to RPMI 1640 medium, and spheroid formation and invasion were examined. (E) Spheroid-formation and (F) invasion were significantly suppressed, indicating that JPH203 was effective against radioresistant cells. (G and H) In the presence of JPH203, wound healing was retarded in both parental and radioresistant cells compared with their corresponding untreated groups. Cells were grown to 90% confluency in a 60-mm dish, and a 500-μm-straight line was drawn using a 200-μl pipet tip to perform a 24-h wound healing assay. (C, E, and G) Scale bar, 100 μm. (D and F) Scale bar, 200 μm. (H) Scale bar, 120 μm. ***P<0.001, ****P<0.0001. LAT1, L-type amino acid transporter 1; LAT1+, LAT1-positive cell; RT, radiotolerant.

Table VI. Univariate and multivariate analyses of OS and PFS.

A, 5-year OS

Characteristic	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Age (<65 vs. >65 years)	1.150	0.587-2.257	0.683	1.501	0.728-3.093	0.271
Sex (female vs. male)	1.026	0.446-2.357	0.952	1.032	0.439-2.424	0.943
T category (T1-T2 vs. T3-T4)	1.314	0.667-2.586	0.430	1.036	0.483-2.225	0.927
N category (N0 vs. N1-N3)	1.897	0.906-3.973	0.089	2.133	0.913-4.984	0.080
Stage (I-II vs. III-IV)	1.447	0.675-3.103	0.342	NA	NA	NA
LAT1 (low vs. high)	1.113	0.484-2.561	0.801	1.102	0.474-2.564	0.822

B, 5-year PFS

Characteristic	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Age (<65 vs. >65 years)	0.807	0.454-1.435	0.466	0.941	0.497-1.780	0.851
Sex (female vs. male)	1.038	0.501-2.148	0.920	1.005	0.475-2.127	0.989
T category (T1-T2 vs. T3-T4)	1.164	0.656-2.064	0.604	1.001	0.527-1.901	0.998
N category (N0 vs. N1-N3)	1.474	0.806-2.696	0.208	1.427	0.707-2.880	0.322
Stage (I-II vs. III-IV)	1.139	0.609-2.128	0.684	NA	NA	NA
LAT1 (low vs. high)	1.224	0.572-2.620	0.603	1.180	0.545-2.555	0.674

In the surgical treatment group, there was no relationship between LAT1 and prognosis. NA, not applicable; HR, hazard ratio; LAT1, L-type amino acid transporter 1; OS, overall survival; PFS, progression-free survival.

formation, invasion, and migration of LAT1 positive cells. Therefore, JPH203 should also be used in the field of HNSCC, as LAT1 is a prognostic factor and can be used to predict therapeutic efficacy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YK and YO designed the outline of the study. HS, YK, MM, HH, SS, TY, MS and AI conducted the experiments and data analyses. YK, SH and YO confirmed the authenticity of all raw data. YK and YO interpreted the data and wrote the draft. YO revised the draft before the submission. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all patients. All procedures used in this research were approved by the Ethical Committee of Akita University Hospital (approval no. 2532; Akita, Japan). The study was performed according to the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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