Expression and Role of System L Amino Acid Transporters in Hypopharyngeal Cancer Cells

การแสดงออกและบทบาทของโปรตีนตัวขนส่งกรดอะมิโนระบบแอลในเซลล์มะเร็งช่องคอส่วนล่าง

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ABSTRACT

Treatment of hypopharyngeal squamous cell carcinoma (HSCC) is currently ineffective, therefore new therapeutic targets are urgently needed. Previous studies reported that system L amino acid transporter is upregulated in several types of cancers. Therefore, we aimed to investigate the expression pattern and role of system L amino acid transporter in HSCC cell line, namely FaDu cells. By quantitative real time PCR, we found that FaDu cells highly expressed system L1 amino acid transporter which is L-type amino acid transporter 1 (LAT1), system L2 amino acid transporter (LAT3) and system L1-associated chaperone 4F2 heavy chain (4F2hc or CD98). LAT1 interacted with 4F2hc via a disulfide bond and were co-localized predominantly at plasma membrane of FaDu cells. Inhibition of LAT1 mediated essential amino acids uptake by a specific system L inhibitor, BCH significantly suppressed cancer cell growth. In conclusion, it is proposed that LAT1 and 4F2hc could be novel therapeutic targets for HSCC.

บทคัดย่อ

การรักษาโรคมะเร็งช่องคอส่วนล่างในปัจจุบันยังมีประสิทธิภาพไม่เพียงพอ ทำให้ต้องมีการค้นคว้าหาเป้าใหม่สำหรับการรักษาเพิ่มเติมอย่างเร่งด่วน เพื่อให้การรักษามีประสิทธิภาพมากยิ่งขึ้น การศึกษาวิจัยที่ผ่านมาพบว่า มีการแสดงออกเพิ่มขึ้นของโปรตีนตัวขนส่งกรดอะมิโนระบบแอลในเซลล์มะเร็งหลายชนิด ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาการแสดงออกและบทบาทหน้าที่ของโปรตีนตัวขนส่งกรดอะมิโนระบบแอลในเซลล์มะเร็งช่องคอส่วนล่าง คือ เซลล์ FaDu การศึกษาการแสดงออกของยีนตัวขนส่งกรดอะมิโนระบบแอลโดยเทคนิคปฏิกิริยาลูกโซ่พอลิเมอเรสแบบเรียลไทม์พบว่า มีการแสดงออกของยีนตัวขนส่งกรดอะมิโนระบบแอล 1 (LAT1) ตัวขนส่งกรดอะมิโนระบบแอล 2 (LAT3) และตัวช่วยของตัวขนส่งกรดอะมิโนระบบแอล 1 คือ 4F2 heavy chain (4F2hc or CD98) สูง โปรตีน LAT1 จับกับโปรตีน 4F2hc ด้วยพันธะไดซัลไฟด์ และมีการแสดงออกมากที่ตำแหน่งเดียวกันบนเยื่อหุ้มเซลล์ของเซลล์ FaDu การยับยั้งการทำหน้าที่ขนส่งกรดอะมิโนชนิดที่จำเป็นเข้าเซลล์ของ LAT1 ด้วยตัวยับยั้งจำเพาะ BCH ลดอัตราการเจริญเติบโตของเซลล์มะเร็งอย่างมีนัยสำคัญ โดยสรุป LAT1 และ 4F2hc จึงเป็นโปรตีนที่สำคัญที่จะสามารถพัฒนาไปเป็นเป้าหมายใหม่สำหรับการรักษาโรคมะเร็งช่องคอส่วนล่างได้

Key Words: LAT1, 4F2hc, system L amino acid transporter, Hypopharyngeal squamous cell carcinoma

คำสำคัญ: LAT1 4F2hc โปรตีนตัวขนส่งกรดอะมิโนระบบแอล มะเร็งช่องคอส่วนล่าง

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Introduction

Hypopharyngeal squamous cell carcinoma (HSCC) is one type of head and neck cancer (HNCA) arising from the bottom part of pharynx to upper part of esophagus (Wycliffe et al., 2007). In Thailand, the highest incidences of HNCA is in southern area (Pruegsanusak et al., 2012). Among the types of head and neck cancers which are nasopharyngeal squamous cell carcinoma, oropharyngeal squamous cell carcinoma, and hypopharyngeal squamous cell carcinoma, HSCC is the most aggressive (Carvalho et al., 2005). Most of HSCC patients are diagnosed as an advanced stage, stage III and IV (Kuo et al., 2014). Similarly, new HSCC cases in Thailand are frequently diagnosed as a stage III (23 %) and IV (62 %) (Pruegsanusak et al., 2012). The risk factors of HSCC are known to be associated with smoking and drinking habit (Boute et al., 2014). The primary management of HSCC is remained to be chemoradiotherapy in order to preserve the adjacent organs. Unfortunately, it failed to improve long term survival of HSCC patients. The 5-year survival rates for Thai HSCC patients with stage I, II, III, and IV were 69.8%, 38.7%, 37.9%, and 21.5%, respectively (Pruegsanusak et al., 2012). This is largely due to the advanced stage of the disease at diagnosis. Moreover, the recurrence of HSCC after chemoradiotherapy was frequently reported (Lefebvre, 2010). Therefore, an additional therapeutic target, diagnostic biomarker as well as clinical markers to predict the prognosis after treatment are urgently needed for management of hypopharyngeal squamous cell carcinoma.

System L amino acid transporter provides the essential amino acids to transformed cells and that is crucial for their rapid growth and proliferation. System L amino acid transporter is classified into system L1 and system L2 (Weissbach et al., 1982). L-type amino acid transporter 1 (LAT1), the first isoform of system L1 amino acid transporter identified from C6 glioma cells, is a 12-membrane-spanning protein and mediates Na^+-independent transporting the essential amino acids, such as leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, methionine, and histidine (Kanai et al., 1998). For functional expression, LAT1 forms a heterodimeric complex via disulfide bond with 4F2 heavy chain (4F2hc or CD98). 4F2hc is necessary for LAT1 trafficking onto the plasma membrane (Yanagida et al., 2001). The transport activity is inhibited by specific system L-inhibitor, BCH (2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid). L-type amino acid transporter 2 (LAT2) is identified as the second isoform of system L1 amino acid transporter (Segawa et al., 1999). Similar to LAT1, LAT2 requires 4F2hc for plasma membrane routing and therefore its transport activity (Pineda et al., 1999). However, the amino acid substrates for LAT2 are not limited to only large neutral amino acids but including all neutral amino acids and the transport activity is inhibited by BCH (Segawa et al., 1999). Recently, system L2 amino acid transporters have been identified comprising of L-type amino acid transporter 3 (LAT3) and L-type amino acid transporter 4 (LAT4) (Babu et al., 2003; Bodoy et al., 2005). LAT3 and LAT4 mediate Na^+-independent leucine, phenylalanine, isoleucine, and valine transport and is inhibited by BCH, consistent with the properties of system L. Unlike system L1, the plasma membrane expression and transport activity of system L2 are not required an ancillary protein, 4F2hc and their transport activity is sensitive to inhibition of *N*-ethylmaleimide (NEM) (Babu et al., 2003; Bodoy et al.,

2005). Previous studies have demonstrated that LAT1 was up-regulated in several solid tumors such as brain, lung, liver, colon, and skin, to support continuous growth and proliferation, whereas barely detected in normal cells (Fuchs, Bode, 2005; Yanagida et al., 2001). The inhibition of LAT1 activity by BCH significantly suppressed the growth of several cancer cell lines like glioma, breast cancer, and prostate cancer cells (Nawashiro et al., 2006; Shennan, Thomson, 2008; Wang et al., 2011). These information illustrate the essential roles of LAT1 in promoting growth and proliferating of cancer cells. However, the functional expression of LAT1-4F2hc heterodimeric amino acid transporter in HSCC has not been reported. Therefore, the present study aimed to investigate the expression, functional activity and the impact of system L amino acid transporters on the malignant phenotype of HSCC cells.

We found that LAT1 and its associated protein, 4F2hc highly expressed in FaDu cells. LAT1 forms heterodimer with 4F2hc via disulfide bond and predominantly co-localized on plasma membrane. The inhibition of LAT1 activity by BCH reduced leucine uptake concomitant with suppression of cell growth. Collectively, these results suggest that LAT1 and 4F2hc may be developed as new molecular targets for hypopharyngeal squamous cell carcinoma treatment in the future.

Objectives of the study

(1) To determine the expression pattern and functional activity of system L amino acid transporter in human hypopharyngeal squamous cell carcinoma.

(2) To investigate roles of system L amino acid transporter on hypopharyngeal squamous cell carcinoma cell growth.

Methodology

Cell culture, antibodies, and reagents

Human hypopharyngeal squamous cell carcinoma cell (FaDu) was obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in Minimum Essential Media (MEM) (Invitrogen, Carlsbad, CA) containing 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, St. Louis, MO) and 1% antibiotic-antimycotic (Invitrogen, Carlsbad, CA). Cells were incubated at 37^{o}C in 5% CO_2 incubator. The following antibodies were used: anti-SLC7A5 (Abcam, Cambridge, MA); anti-CD98 (H-300) (Santa Cruz Biotechnology, Dallas, TX); HRP goat anti-rabbit IgG (H+L) antibody (Jackson ImmunoResearch Laboratories, West Grove, PA); goat anti-rabbit IgG (H+L) Alexa Fluor 488, goat anti-mouse IgG (H+L) Alexa Fluor 568, DAPI (Invitrogen, Carlsbad, CA). The following reagents were used; TRIzol reagent (Invitrogen, Carlsbad, CA); cDNA kit (Bio-Rad, Hercules, CA); SYBR kit (Biosystem Woburn, MA); Complete Mini EDTA-free (Roche, Mannheim, Germany); SuperSignal West Pico chemiluminescent (Thermo Scientific, Rockford, MD); MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St. Louis, MO); and [^{14}C]L-leucine (Perkin Elmer, Boston, MA).

Western blot analysis

Cells were cultured in a 60 mm dish at density 5 × 10^5 cells/dish and incubated at 37^{o}C with 5% CO_2 incubator for 24 h. Cells were lysed with modified RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and protease inhibitor cocktail). After 20 min incubation on ice, cells were centrifuged at 12,000 rpm for 20 min at

4°C. The supernatant was collected and the protein concentration was measured. Equal amount of protein samples were used for Western blot analysis. Protein was mixed with Laemmli's sample buffer with or without DTT and resolved by SDS-PAGE, subsequently transferred onto a nitrocellulose membrane by electro-blotting. Membranes were incubated with anti-SLC7A5 or anti-CD98 antibody overnight at 4°C, washed with TBST, and detection by incubating with HRP-conjugated goat anti-rabbit IgG for 1 h at room temperature. The signals were detected using the enhanced SuperSignal West Pico Chemiluminescent reagent.

Total RNA extraction and real time PCR quantification

FaDu cells were cultured in a 60 mm dish at density 5×10^5 cells/dish and incubated at 37°C with 5% CO_2 incubator for 24 h. Total RNAs were extracted from cells by using TRIzol reagent according to the recommendation of the manufacturer with a slight modification. cDNA synthesis was conducted by using iScript™ cDNA analysis kit according to the manufacturer's protocols. Quantitative real time PCRs of LAT1, LAT2, LAT3, LAT4, and 4F2hc were analyzed by using SYBR Green I dye with ABI PRISM 7500 Sequence Detection System and analysis software (Applied Biosystem). Primers were designed by using NCBI/Primer-Blast and 7 IDT Scitools Olioanalyzer 3.1. The primers used are summarized in table 1. The mRNA level of each gene was normalized to mRNA level of β-actin.

Table 1 The primers for quantitative real time PCR

Genes	Sequences
LAT1	FW: 5'-TTC GTC CAG ATC GGG AAG GGT GA-3'
	RW: 5'-TCC ATA GGC AAA GAG GCC GCT-3'
LAT2	FW: 5'-GGG AGC CCT CTG CTA TGC TGA AC-3'
	RW: 5'-GAA CCC AGC CAG TCC TCC GAA GA-3'
LAT3	FW: 5'-GCT GTG CTG GAG AAC CTC TT-3'
	RW: 5'-CTG AGC ACG AAG GAA CCA AT-3'
LAT4	FW: 5'-AAC CAA ACG CTC TCT CCG TGC T-3'
	RW: 5'-ACC GAA GGT CGC CGA ACA TGT-3'
4F2hc	FW: 5'-GGC GCA GAA GTG GTG GCA CA-3'
	RW: 5'-CCC CTT CAG ACC CGC CAG GT-3'
β-actin	FW: 5'-TAC CCT GGC ATT GCC GAC AGG A-3'
	RW: 5'-CTT GCG CTC AGG AGG AGC AAT GAT C-3'

Immunofluorescence microscopy

FaDu cells were grown on glass coverslips in a 24-well culture plate. At 50-60% confluence, cells were washed with cold PBS containing Ca^{2+}/Mg^{2+} (PBS^{++}), fixed with 4% paraformaldehyde (PFA) at room temperature for 20 min and permeabilized with permeabilizing buffer (0.3% TritonX-100 and 0.3% BSA in PBS^{++}) for 20 min. Cells were then washed and incubated with blocking solution (10% BSA in PBS^{++}) at room temperature for 1 h. Then, cells were incubated with rabbit anti-SLC7A5 and mouse anti-CD98 (4F2hc) antibodies for 24 h at 4°C. Cells were wash with PBS^{++} for 5 times and then incubated with Alexa Fluor® 488 goat anti-rabbit IgG (H+L) or Alexa Fluor® 568 goat anti-mouse IgG (H+L) and DAPI (nuclear marker) diluted in permeabilizing buffer for 1h. After washing with PBS^{++} for 5 times, the stained coverslips were mounted and visualized with confocal laser microscopy (Olympus FluoView FV10i; Olympus, Tokyo, Japan).

[^{14}C]L-leucine uptake measurement

Cells were seeded in 48-well plates and incubated at 37°C with 5% CO_2 incubator for 24 h. Cells were washed twice with D-PBS (137 mM NaCl, 3 mM KCl, 8 mM $Na_2HPO_4 \cdot 7H_2O$, 1 mM KH_2PO_4, 0.5 mM $MgCl_2 \cdot 6H_2O$, 1 mM $CaCl_2 \cdot 2H_2O$ and 5.6

mM D-glucose and adjusted pH to 7.4 with NaOH). Cells were incubated 10 min in D-PBS at 37°C, then further incubated with D-PBS containing [^{14}C]L-leucine (1 μM) in the presence or absence of BCH (10 mM) for 2 min. Uptake was stopped by removing the uptake solution and cells were washed with 500 μl of ice-cold D-PBS. Cells were solubilized with 10% SDS in 0.1 M NaOH and neutralized with 0.1 M HCl. The radio-activity was counted by scintillation spectrometry and expressed as % of uptake compared to cells without BCH [BCH(-)]. Three separate experiments were performed in order to confirm the reproducibility.

Cell proliferation

Cells were plated in 96-well plates in MEM medium containing 10% FBS for 24 h. Cells were cultured for 3 days in the presence or absence of BCH (40 mM). Cells viability was determined by MTT assay. Cells were incubated with 0.5% MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution at 37°C with 5% CO_2 incubator for 4 h. Then, the cultured medium was removed and 100% DMSO was added to solubilize the crystal before the measurement at $OD_{590\ nm}$ by Multiskan GO Microplate Spectrophotometer (Thermo Scientific). The result was calculated as the % of cell viability.

Statistical analysis

All data were expressed as mean and standard error of mean (mean ± S.E.M.). The statistical analysis was performed by using the statistical software package, GraphPad Prism version 5.0. The statistically significant differences groups were compared using Student's *t* test. Statistical significance was considered when $p < 0.05$.

Results

Expression of system L amino acid transporters in human hypopharyngeal squamous cell carcinoma (HSCC) cells

System L amino acid transporters are important for growth of several cancer cells. However, role of these transporters in HSCC is currently not known. We therefore examined the mRNA expression of system L1 (LAT1 and LAT2), system L2 (LAT3 and LAT4) amino acid transporters, and system L1 amino acid transporter associating protein, 4F2hc, in FaDu cells by quantitative real time PCR. As shown in Figure 1, FaDu cells expressed high mRNA levels of LAT1 and 4F2hc, the system L1 associating protein. Interestingly, the system L2 amino acid transporter,

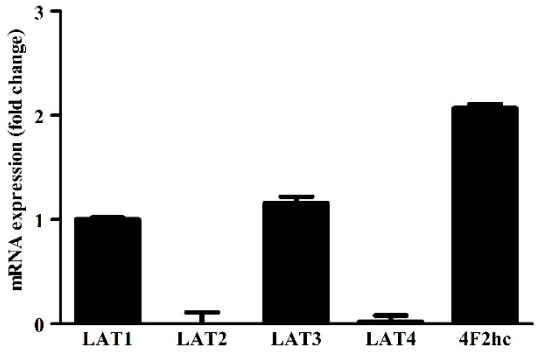

Figure 1 mRNA expression of system L1 and L2 amino acid transporters in HSCC cells. Total RNAs were isolated from FaDu cells and analyzed by quantitative real time PCR for the expression of system L1 amino acid transporters (LAT1 and LAT2), system L2 amino acid transporters (LAT3 and LAT4), and 4F2hc. The mRNA expression level of each gene was normalized by β-actin. Data is expressed as fold change compared with the expression level of LAT1 and presented as means ± S.E.M. (n = 3).

LAT3, was also detected compared to LAT1. On the other hand, the mRNA expression of LAT2 and LAT4, the second isoform of system L1 and L2, respectively, were barely detectible in this cell line compared to that of LAT1 and LAT3. Moreover, the expression of 4F2hc was almost 2 folds higher than LAT1 and LAT3, suggesting an addition role of 4F2hc in this cell line. The localization of LAT1 and 4F2hc were examined by confocal microscopic analysis. The expressions of LAT1 and 4F2hc were predominantly co-localized on the plasma membrane of FaDu cells (Figure 2). These results indicate that system L amino acid transporter may function to provide the essential amino acids into HSCC cells.

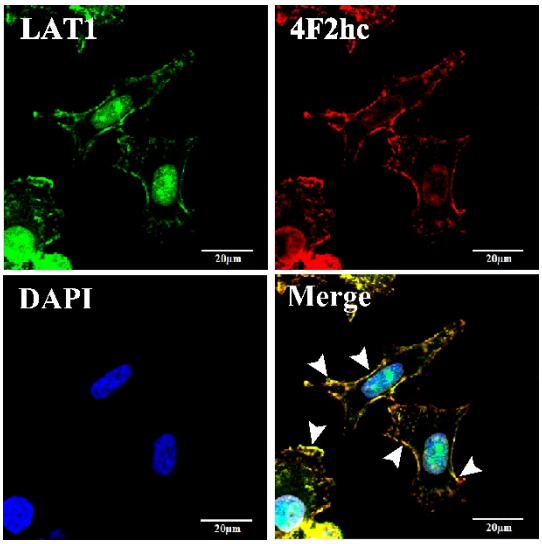

Figure 2 Co-localization of LAT1 and 4F2hc on the plasma membrane. FaDu cells were fixed and stained with anti-SLC7A5 (LAT1; green) and anti-CD98 (4F2hc; red) antibodies. DAPI was used as a nuclear marker (blue). Arrow heads indicate co-localization of LAT1 and 4F2hc. Scale bar = 20 μm.

LAT1 forms the heterodimeric complex with 4F2hc via disulfide bond in FaDu cells

The association between LAT1 and 4F2hc is necessary for plasma membrane trafficking and transport activity. We next examined the association between LAT1 and 4F2hc in FaDu cells by western blotting in the presence [DTT(+)] or absence [DTT (-)] of DTT with anti-SLC7A5 (LAT1) and anti-CD98 (4F2hc) antibodies. In the absence of DTT [DTT(-)], the LAT1-4F2hc heterodimer complex bands (125 kDa) were detected by using both antibodies (Figure 3). However, in reducing condition [DTT(+)], LAT1 and 4F2hc proteins were detected at 38 kDa and 85 kDa corresponding to their original molecular weight, respectively. Collectively, these data indicate that LAT1 forms a heterodimer complex with 4F2hc via disulfide bond and that necessary for functional activity on the plasma membrane of FaDu cells.

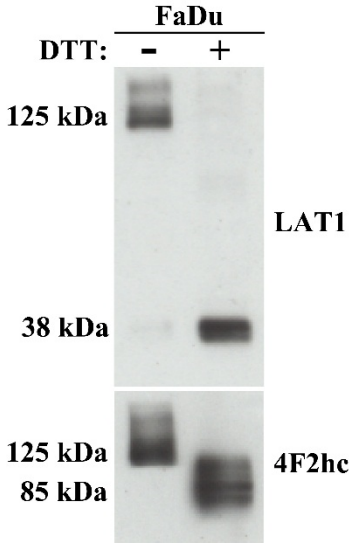

Figure 3 The interaction between LAT1 and 4F2hc via disulfide bond in HSCC cells. Western blot analysis was performed on FaDu cell lysates in the presence [DTT(+)] or absence [DTT(-)] of DTT using anti-SLC7A5 (LAT1) and anti-CD98 (4F2hc) antibodies.

System L amino acid transporters mediate leucine uptake into FaDu cells

We next determined the transport activity of system L amino acid transporters in FaDu cells by evaluation the [^{14}C]L-leucine uptake in the presence or absence of BCH, a system L-specific inhibitor. The [^{14}C]L-leucine uptake was significantly inhibited in the presence of 100 μM BCH (approximately 80-90%) ($p < 0.001$) in FaDu cells (Figure 4). This result suggests that the uptake of essential amino acid into HSCC cells is mediated by system L amino acid transporters.

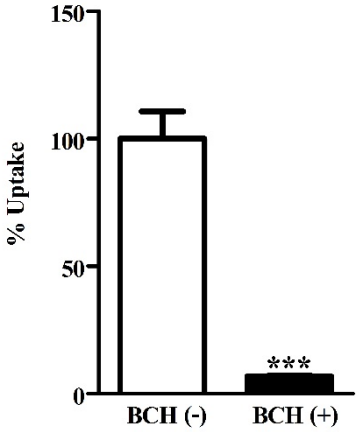

Figure 4 System L amino acid transporters mediate leucine transport in HSCC cells. The [^{14}C]L-leucine (1 μM) uptake in FaDu cells was measured in the presence or absence of 100 μM BCH for 2 min. The results are means ± S.E.M. (n = 3) and presented as % of uptake compared with control (absence of BCH). ***, $p < 0.001$ (Student's *t* test).

Inhibition of system L amino acid transporter activity suppresses FaDu cell growth

We next investigated whether the inhibition of the essential amino acid uptake into FaDu cells by system L amino acid transporters affect cell growth. FaDu cells were treated with 40 mM BCH, a competitive system L amino acid transporter, for 3 days. MTT assays were used for assessment the effect of BCH on cell viability. As shown in Figure 5, treatment with BCH significantly decreased cell growth at day 2 and day 3. Collectively, these results indicate a significant role of system L amino acid transporter on HSCC cell growth.

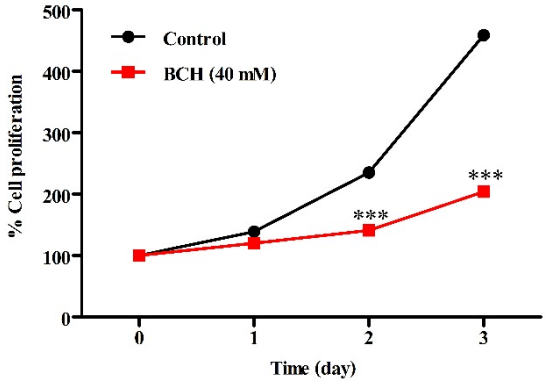

Figure 5 Inhibition of system L amino acid transporter activity suppresses HSCC cell growth. FaDu cells were cultured in the presence or absence of BCH (40 mM) for 3 days. Cell growth was assessed by MTT assays every 24 hours. The result is mean ± S.E.M. and presented as % cell proliferation. ***, $p < 0.001$ (ANOVA) compared with control (absence of BCH).

Discussion and Conclusion

Hypopharyngeal squamous cell carcinoma (HSCC) is highly aggressive type of head and neck cancer. Searching for therapeutic target for this cancer is urgently needed because of its insensitive to the current chemotherapeutic regimens. Upregulation of system L amino acid transporters in malignant

neoplasm has been illustrated in earlier studies. However, the expression pattern and transport activity of this transport system in hypopharyngeal squamous cell carcinoma cells is limited. Therefore, our present study aimed to investigate the expression and role of system L amino acid transporter in HSCC cells, namely FaDu cells. We found that cancer specific system L1 amino acid transporter, LAT1 and its chaperone 4F2hc were highly expressed. LAT1 forms a heterodimeric with 4F2hc via a disulfide bond and are predominantly co-localized on cell plasma membrane. The leucine transport in FaDu cells was inhibited by system L-specific inhibitor (BCH) and resulted in suppression of cancer cell proliferation. Herein, we demonstrate for the first time on the expression pattern and its role in hypopharyngeal squamous cell carcinoma cells. Therefore, it is proposed that system L amino acid transporter, especially LAT1-4F2hc heterodimeric amino acid transporter could be new therapeutic targets for hypopharyngeal squamous cell carcinoma.

Essential amino acids are necessary for growth and proliferation of both normal and malignant cells. We found that FaDu cells highly expressed LAT1-4F2hc heterodimeric amino acid transporter, a system L1 amino acid transporter that provides the essential amino acids into cells. Consistent with previous study, upregulation of LAT1 and 4F2hc has been observed in several solid tumor cells such as brain, lung, liver, colon, and skin (Fuchs, Bode, 2005). Recently, Toyoda et al., demonstrated that 86% and 83% of stage III/IV hypopharyngeal squamous cell carcinoma were positive staining for LAT1 and 4F2hc (Toyoda et al., 2014). However, the functional characteristic and role of system L on cancer cell proliferation has not been elucidated. In contrast to those of LAT1, FaDu cells expressed very low level of LAT2, the second isoform of system L1 amino acid transporter, known transporter that plays an important roles in normal cell functions (Segawa et al., 1999). Trafficking of LAT1 to plasma membrane required the association via the disulfide bond with an ancillary protein, 4F2hc (Kanai et al., 1998). Thus, we next examined the cellular localization of LAT1 and 4F2hc proteins in FaDu cells. The LAT1 and 4F2hc were predominantly co-localized on the plasma membrane of FaDu cells. Moreover, we also investigated the association between LAT1 and 4F2hc proteins in these cells by immunoblotting in the presence or absence of reducing agent, DTT. We found that LAT1 and 4F2hc proteins were detected at 125 kDa bands in the absence of DTT. In the reducing condition, however, LAT1 and 4F2hc were shifted to their original sizes at 38 kDa and 85 kDa, respectively. Collectively, our results indicate that LAT1 interacts with 4F2hc via disulfide bonds to form the system L1 heterodimeric amino acid transporter at the plasma membrane and mediates the amino acids uptake into FaDu cells.

LAT1/4F2hc heterodimeric amino acid transporter mediates the uptake of essential amino acids into cancer cells to promote their rapid growth and proliferation. Inhibition of the transport activity mediated by LAT1 by system L-specific inhibitor, BCH, suppressed growth of several cancer cells such as glioma, breast cancer, and prostate cancer cells (Nawashiro et al., 2006; Shennan, Thomson, 2008; Wang et al., 2011). However, BCH treatment has no effect on growth of normal epithelial cells with low LAT1 expression (Fan et al., 2010). Consistent with these studies, the leucine uptake into FaDu cells was

inhibited by BCH. Interestingly, growth of FaDu cells was significant suppressed after incubation with BCH suggesting the significant role of LAT1 on hypopharyngeal squamous cell carcinoma cells.

Normally, the level of intracellular leucine is detected by leucyl-tRNA synthetase (LRS) (Han et al., 2012). LRS will activate Rag GTPase when an increased in intracellular concentration of leucine. Rag GTPase will load RagB, the Rag heterodimer component which consists of RagA, RagB, RagC, and RagD with GTP and then enable it to directly interact with Raptor, the regulatory associated protein of mTOR. mTORC1 is then activated leading to promote cell growth and proliferation through phosphorylation of S6K1 (ribosomal S6 protein kinase 1) and 4EBP1 (eukaryotic initiation factor (eIF) 4F-binding protein 1) (Sancak et al., 2008). Indeed, inhibition of mTORC1 signaling pathway was associated with growth suppression of several type of cancer such as breast, renal, and colorectal cancer cells (Atkins et al., 2004; Gulhati et al., 2009; Yu et al., 2001). Therefore, most of cancer cells upregulate amino acid transporter in order to provide the sufficient intracellular concentration leucine. Our results suggest that the suppression of FaDu cell growth is due to the inhibition of LAT1/4F2hc mediated leucine uptake by BCH and then reduction the mTORC1 activity.

In addition, system L2 amino acid transporter LAT3 was also up-regulated as the similar level to that of LAT1. Inconsistent with these, Haase et al. have been demonstrated that FaDu cells expressed LAT4 at high level compared to LAT3 (Haase et al., 2007). This discrepancy might be affected by passage number and culturing condition that has been reported for the expression of P-glycoprotein efflux transporter (P-gp) in Caco-2 cells (Anderle et al., 1998; Siissalo et al., 2007). LAT3 was firstly identified by expression cloning from human hepatocarcinoma-derived cell line, FLC4 (Babu et al., 2003). shRNA mediated LAT3 knockdown resulted in growth retardation in prostate cancer cell lines (LNCaP and PC-3). However role of this transport system in these cancer cells is still unknown.

In conclusion, we have shown that hypopharyngeal squamous cell carcinomas cells expressed high level of system L1 amino acid transporter, LAT1 and its associated protein, 4F2hc. LAT1 associated with 4F2hc via a disulfide bond and was co-localized on cell surface. Inhibition of LAT1-4F2hc heterodimeric amino acid transporter mediated the leucine uptake into FaDu cells by system L-specific inhibitor, BCH resulted in suppression of cell proliferation. Therefore, it is proposed that system L1 amino acid transporters might be a novel molecular target for treatment of hypopharyngeal squamous cell carcinoma.

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