Received: 18 January 2021 | Revised: 5 May 2021 | Accepted: 18 May 2021

DOI: 10.1111/cas.14991

ORIGINAL ARTICLE

Cancer Science WILEY

Functional analysis of LAT3 in prostate cancer: Its downstream target and relationship with androgen receptor

Junryo Rii[1] | Shinichi Sakamoto[1] | Masahiro Sugiura[1,2] | Manato Kanesaka[1,2] | Ayumu Fujimoto[1] | Yasutaka Yamada[1] | Maihulan Maimaiti[3] | Keisuke Ando[1,4] | Ken Wakai[1,3] | Minhui Xu[5] | Yusuke Imamura[1] | Norihisa Shindo[6] | Toru Hirota[6] | Atsushi Kaneda[2] | Yoshikatsu Kanai[5] | Yuzuru Ikehara[3] | Naohiko Anzai[4] | Tomohiko Ichikawa[1]

[1]Department of Urology, Chiba University Graduate School of Medicine, Chiba, Japan

[2]Department of Molecular Oncology, Chiba University Graduate School of Medicine, Chiba, Japan

[3]Department of Tumor Pathology, Chiba University Graduate School of Medicine, Chiba, Japan

[4]Department of Pharmacology, Chiba University Graduate School of Medicine, Chiba, Japan

[5]Bio-system Pharmacology, Osaka University Graduate School of Medicine, Osaka, Japan

[6]Division of Experimental Pathology, Cancer Institute of the Japanese Foundation for Cancer Research, Tokyo, Japan

Correspondence
Shinichi Sakamoto, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba-City, Chiba 260-8670, Japan.
Email: rbatbat1@gmail.com

Funding information
Chiba University, Grant/Award Number: 20H03813 and 20K09555

Abstract

L-type amino acid transporter 3 (LAT3, *SLC43A1*) is abundantly expressed in prostate cancer (PC) and is thought to play an essential role in PC progression through the cellular uptake of essential amino acids. Here, we analyzed the expression, function, and downstream target of LAT3 in PC. LAT3 was highly expressed in PC cells expressing androgen receptor (AR), and its expression was increased by dihydrotestosterone treatment and decreased by bicalutamide treatment. In chromatin immunoprecipitation sequencing of AR, binding of AR to the SLC43A1 region was increased by dihydrotestosterone stimulation. Knockdown of LAT3 inhibited cell proliferation, migration, and invasion, and the phosphorylation of p70S6K and 4EBP-1. Separase (*ESPL1*) was identified as a downstream target of LAT3 by RNA sequencing analysis. In addition, immunostaining of prostatectomy specimens was performed. In the multivariate analysis, high expression of LAT3 was an independent prognostic factor for recurrence-free survival (hazard ratio: 3.24; $P = .0018$). High LAT3 expression was correlated with the pathological T stage and a high International Society of Urological Pathology grade. In summary, our results suggest that LAT3 plays an important role in the progression of PC.

KEYWORDS
amino acid transporter, androgen receptor, LAT3, prostate cancer, Separase

1 | INTRODUCTION

Prostate cancer (PC) is the second most common cancer among men worldwide.[1] The androgen receptor (AR) is crucial for not only the normal growth and maintenance of the prostate gland but also the development of PC.[2] Androgen deprivation therapy is regarded as the standard treatment for metastatic PC, and although it may show effectiveness initially, PC often develops resistance to the therapy

Abbreviations: AR, androgen receptor; ChIP-seq, chromatin immunoprecipitation sequencing; CRPC, castration-resistant prostate cancer; DHT, dihydrotestosterone; LAT3, L-type amino acid transporter 3; mTORC1, mammalian target of rapamycin complex 1; PC, prostate cancer; RNA-Seq, RNA sequencing.

Junryo Rii and Shinichi Sakamoto equally contributed to this work.

and progresses to fatal castration-resistant prostate cancer (CRPC).[3-8] Several schemes for the transition to CRPC have been postulated, and AR plays a central role in them.[9-12] Cancer cells require large amounts of amino acids for their survival, and their uptake into the cell is facilitated by amino acid transporters.[13,14] L-type amino acid transporter 3 (LAT3, *SLC43A1*) takes neutral amino acids, including leucine, isoleucine, valine, phenylalanine, and methionine, into cells.[15] LAT3 was originally identified as a gene *(POV1)* that was overexpressed in PC[16] and subsequently revealed to be an amino acid transporter.[17] Therefore, it may be possible that LAT3 plays an important role in PC growth through amino acid uptake. A recent study revealed that in PC, LAT3 coordinates mammalian target of rapamycin complex 1 (mTORC1), which requires intracellular leucine for its activation.[18] However, the function of LAT3 in the AR pathway and its downstream target gene are not well documented. In this study, we attempted to elucidate the function of LAT3 in the AR pathway and its downstream target gene. We also retrospectively investigated the LAT3 expression profile and its association with clinical parameters in PC specimens.

2 | MATERIALS AND METHODS

2.1 | Cell culture and transfection

The human PC cell lines LNCaP, PC3, and DU145 were obtained from the Cell Resource Centre for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan), and C4-2 was obtained from the American Type Culture Collection (Manassas, VA). Cell cultures were prepared in RPMI-1640 medium (FUJIFILM Wako) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin solution (FUJIFILM Wako). These cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. For steroid-free conditions, phenol red-free RPMI-1640 medium was used with 10% charcoal-stripped FBS (CSS). Mycoplasma contamination was tested using the CycleavePCR mycoplasma Detection kit (Takara Bio) to confirm that the cultures were mycoplasma-free. Cells were transfected with small interfering RNA (siRNA) using Lipofectamine™ RNAiMAX (Thermo Fisher Scientific) according to the manufacturer's instructions. The cells were harvested 72 hours after transfection.

2.2 | Reagents and antibodies

In this study, Lipofectamine™ RNAiMax Transfection Reagent, OPTI-MEM, siRNAs SiLAT3 (Stealth siRNAs: HSS112400 and HSS189324), SiAR (HSS100619 and HSS179972), and Stealth RNAi siRNA Negative Control Med GC Duplex #3 were obtained from Thermo Fisher Scientific. pIRESpuro-mSeparase-WT-6myc was kindly provided by Dr Toru Hirota, and pcDNA3.1(+)-LAT3 was a kind gift provided by Dr Naohiko Anzai. Anti-LAT3 (HPA018826) was obtained from Sigma. Anti-AR (AR441, ab9474) was obtained from Abcam. Anti-LAT1 (KE023) was obtained from Trans Genic Inc. Anti-AKT, anti-phosphorylated AKT (Ser473), anti-phosphorylated 4EBP-1, anti-phosphorylated p70s6k (Thr389, Ser371), anti-phosphorylated mTOR (Ser2448), anti-mTOR, anti-E-cadherin, anti-N-cadherin, anti-vimentin, and anti-slug were obtained from Cell Signaling Technology Inc. Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained from Ambion Inc. Dihydrotestosterone (DHT), bicalutamide, and MDV3100 were purchased from Sigma. Anti-Separase (WH0009700M1) was also obtained from Sigma, and the Separase-specific inhibitor Sepin-1 was obtained from AOBIOUS Inc. Mitomycin C solution was obtained from Nacalai tesque.

2.3 | Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RT-PCR was performed according to a protocol reported previously.[19] The *GAPDH* mRNA level was quantified for normalization control. The PCR primers used in this study are listed in Table S1.

2.4 | Western blotting

Protein extraction and Western blotting were performed as described previously.[14] The protein content was quantified using the bicinchoninic acid protein assay kit (Thermo Fisher Scientific), and protein samples (20 mg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a gel prepared from the TGX Fastcast Acrylamide kit (10%, Bio-Rad) according to the manufacturer's protocol. Block Ace (KAC Co., Ltd) was used for membrane blocking. Band signals were detected by the luminescent Image Analyzer LAS-4000 mini (Fujifilm). The original full images of membranes are shown in Figures S1-S3.

2.5 | Chromatin immunoprecipitation (ChIP) assay and ChIP sequencing (ChIP-seq) analysis

In this study, we utilized our previously deposited NGS (Next Generation Sequencing) data GSE122922 (GSM3488490-GSM3488508)[10] in Gene Expression Omnibus.

2.6 | Cell viability assay

Cells were seeded into 96-well plates (3000 cells/well), and viability was determined using Cell Counting Kit-8 (CCK8; Dojindo). At each time point, cells were incubated with CCK8 for 2 hours, and the absorbance was measured using Microplate Manager v6.0 (Bio-Rad). For assessing the Sepin-1 inhibitory effect, 24 hours after seeding, the medium was changed to medium containing Sepin-1 (for each concentration) or dimethyl sulfoxide (0.5%; vehicle control). The

IC_{50}, which is defined as the drug concentration at which cell viability is reduced by half, was determined by GraphPad Prism 7 (GraphPad Software) as described previously.[20] Three sets of independent experiments were performed for each time point.

2.7 | Cell migration and invasion assay

Falcon Cell Culture Insert (Corning Inc) was used for the migration assay, and Biocoat Matrigel invasion chamber (Corning Inc) was used for the invasion assay. The essential parts of the experiments have been described previously.[14] Briefly, 5000 cells were seeded into the upper chamber and incubated for 48 hours with serum-free medium. Cells were stained using Diff-Quik (Funakoshi). The cell numbers in five different fields at 100× magnification were determined under a microscope. Wound healing assay was performed as described previously.[21]

2.8 | RNA-seq analysis

Total RNA was isolated from LNCaP cells using the RNeasy Mini Kit (Qiagen). The detailed RNA-seq methods have been reported previously.[10] TopHat (v2.0.1) (https://ccb.jhu.edu/software/tophat/index.shtml) was used to align sequenced reads, and gene expression levels were quantified using Cufflinks (v2.2.1) (http://cole-trapnell-lab.github.io/cufflinks/). Gene ontology analysis was conducted using Metascape (https://metascape.org/gp/index.html#/main/step1).[22]

2.9 | Flow cytometric analysis

Cell cycle analysis was performed using an HS800 cell sorter (Sony) as described previously.[10] LNCaP cells were transfected with SiLAT3 for 72 hours and then analyzed.

2.10 | Analysis of clinical PC patient datasets

Two cohort datasets of clinical PC patient information were analyzed in this study. Datasets from Grasso et al (GSE35988)[23] and Luo et al[24] were obtained through Oncomine (https://www.oncomine.org/resource/main.html).

2.11 | PC tissue specimens

PC tissue specimens and clinical information were obtained from 95 patients who received radical prostatectomy at Chiba University Hospital between 2006 and 2011. Histopathological assessment of the tissues was conducted according to the World Health Organization classification of PC by the Department of Pathology, Chiba University Hospital. The clinical and pathological stages were determined according to the TNM classification of the International Union Against Cancer. The patient data are summarized in Table 1.

2.12 | Ethical approval and consent to participate

The present study was carried out in accordance with the Declaration of Helsinki and ethical standards that promote and ensure respect and integrity for all human subjects. The institutional review board of Chiba University Hospital approved this study (approval no. 914), and all patients provided written informed consent for participation before surgery.

2.13 | Immunohistochemistry (IHC)

The detailed IHC methods have been reported previously.[25] Anti-LAT3 antibody (1:200 dilution), anti-Separase antibody (1:100 dilution), anti-LAT1 antibody (1:100 dilution), and anti p-4EBP1 (1:400 dilution) were used. To quantify LAT3, LAT1, and Separase protein expression in these components, we applied the previously described IHC scoring method.[19] LAT3, LAT1, and Separase IHC scores

TABLE 1 Patient background

Characteristics	
No. patients	94
Median age at diagnosis (range), years	66 (50-75)
Median initial PSA (range), ng/mL	7.825 (0.62-39.06)
Median BMI (range)	23.6 (16.6-29.87)
Median TST (range), ng/dL	4.86 (0.42-10.98)
Pathological T stage, n (%)	
T2	58 (61.70)
T3	35 (37.23)
T4	1 (1.07)
ISUP grade, n (%)	
1	13 (13.83)
2	28 (29.79)
3	26 (27.66)
4	9 (9.57)
5	18 (19.15)
RM, n (%)	47 (50)
ly, n (%)	7 (7.45)
v, n (%)	3 (3.19)
pn, n (%)	59 (62.77)
No. recurrence, n (%)	31 (32.98)
RFS rate	3-year: 77.4%
	10-year: 63.8%

Abbreviations: BMI, body mass index; ISUP, International Society of Urological Pathology; ly, lymphatic invasion; pn, perineural invasion; PSA, prostate-specific antigen; RFS, recurrence-free survival; RM, resection margin; TST, testosterone; v, vascular invasion.

over the median scores were defined as positive. For immunostaining of p4EBP1, 10 cases were randomly selected from the LAT3 High and Low groups and stained for scoring. The expression levels were scored by two independent investigators (AF and JR) who were blinded to patient clinical and pathological information.

2.14 | Statistical analysis

Univariate and multivariate Cox proportional models were used to analyze statistical associations between clinical factors, pathological factors, IHC scores, and recurrence-free survival (RFS). RFS was defined as the period from prostatectomy to prostate-specific antigen recurrence. Survival curves were obtained using the Kaplan-Meier method, and differences in survival rates were compared using the log-rank test. The Wilcoxon signed-rank test and chi-square test were used to assess associations between the LAT3 or Separase IHC score and clinical factors. The unpaired Student's *t*-test was used for assessing differences between two groups. Statistical analyses were performed using JMP Pro version 13.0.0 (SAS Institute). Statistical significance was set at *P*-values below .05.

3 | RESULTS

3.1 | LAT3 expression and relationship with AR in PC cell lines and clinical PC datasets

First, we evaluated the expression of LAT3 in several PC cell lines. LAT3 was abundantly expressed in LNCaP and C4-2 cells that express AR, but it was hardly expressed in PC3 and DU145 cells that do not express AR (Figure 1A,B). In the publicly available clinical PC datasets, the *LAT3* mRNA levels were significantly elevated in PC tumors when compared with the normal controls, but there was no significant difference between CRPC and localized cancer (Figure S4A,B). In addition, LAT3 expression was also significantly increased in cases with amplified AR (Figure S4C). When cultured in CSS medium, there was little or no LAT3 expression in either cell line (Figure 1A,B). When LNCaP and C4-2 cells were cultured in CSS medium, stimulation by DHT (0.1 nmol/, 1 nmol/L, or 10 nmol/L) increased LAT3 expression in a dose-dependent manner (Figure 1C,D). The effect of DHT on LAT3 expression was suppressed by bicalutamide (10 μmol/L; Figure 1C,D). Similarly, AR expression was also reduced in CSS medium, but markedly increased by DHT administration, and this effect was suppressed by bicalutamide (Figure 1E,F).

3.2 | Effects of AR knockdown on LAT3 expression and LAT3 knockdown on the mTOR pathway

To further investigate the relationship between AR and LAT3, we performed an AR knockdown experiment using siRNA. AR knockdown significantly reduced LAT3 expression in LNCaP (Figure 2A,B) and C4-2 (Figure 2C,D) cells. Similarly, when the medium was changed to CSS for LNCaP cells, the expression of LAT3 decreased in a time-dependent manner (Figure 2E). In ChIP-seq of AR, the genome browser view showed binding of AR at the LAT3 locus in DHT-stimulated LNCaP cells (Figure 2F). To further investigate the regulation of LAT3 expression by AR, we performed motif analysis of the AR binding site in LAT3 and identified the AR half-site motif. (Figure S5). Next, we confirmed that SiLAT3 significantly reduced LAT3 expression (Figure 2G,H). Furthermore, LAT3 knockdown inhibited the phosphorylation of the mTOR, eukaryotic translation initiation factor 4EBP1, and ribosomal protein S6K1, but not the phosphorylation of Akt (Figure 2I). AR knockdown showed similar results (Figure S6).

3.3 | Inhibition of cell growth, migration, and invasion by LAT3 knockdown and rescue study of LAT3

We assessed the effect of LAT3 knockdown on the proliferation activities of PC cells. In LNCaP cells, LAT3 knockdown significantly suppressed the cell growth (Figure 3A), migration activity (Figure 3B), and invasion activity (Figure 3C) of the cells when compared with the negative control. In C4-2 cells under the castrated condition (in CSS medium), LAT3 knockdown did not decrease the cell growth (Figure 3D), migration activity (Figure 3E), or invasion activity (Figure 3F) of the cells. In contrast, when C4-2 cells were cultured under normal conditions (in FBS), LAT3 knockdown significantly inhibited the cell growth (Figure 3G), migration activity (Figure 3H), and invasion activity (Figure 3I) of the cells when compared with the negative control. Furthermore, wound healing assay was performed, and the same results were obtained by LAT3 knockdown. (Figure S7A-C). We next investigated the expression changes of EMT-related proteins (eg, E-cadherin, N-cadherin, slug, vimentin) by LAT3 knockdown (Figure S7D). Notably, the expression level of E-cadherin was upregulated, and N-cadherin, slug, and vimentin were suppressed by SiLAT3 (Figure S7D). In addition, under the condition that the expression of LAT3 was suppressed by MDV3100, an antiandrogenic drug, introduction of LAT3 significantly restored cell proliferation compared with cells transfected with control vector (Figure S8A). We confirmed that LAT3 transfection restored LAT3 protein expression in MDV3100-treated cells (Figure S8B). Collectively, these results indicated that LAT3 knockdown has inhibitory effects on cell growth, migration, and invasion in an androgen-dependent manner.

3.4 | Identification of LAT3 downstream target genes

To further elucidate the functional mechanism of LAT3 in PC, we extracted candidate LAT3 target genes by RNA-Seq analysis of LNCaP cells treated with SiLAT3. The genes downregulated by SiLAT3 were significantly associated with gene ontology

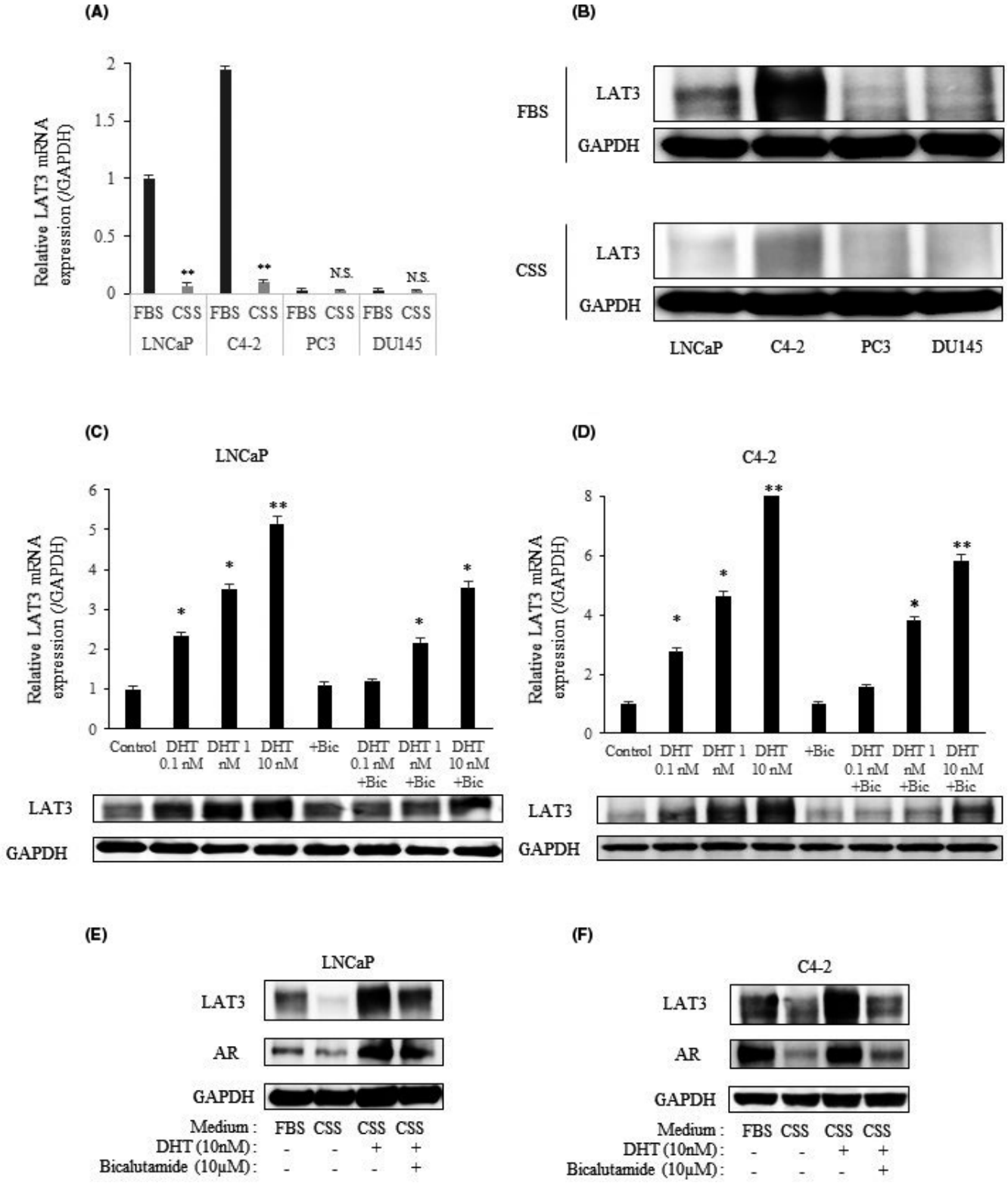


FIGURE 1 Quantification of L-type amino acid transporter 3 (LAT3) expression and regulation by androgen receptor (AR). A, *LAT3* mRNA levels of human prostate cancer (PC) cells in FBS or charcoal-stripped FBS (CSS) were analyzed by RT-PCR. B, LAT3 protein levels were analyzed by Western blotting. GAPDH was used as a loading control. C and D, Effect of dihydrotestosterone (DHT) and bicalutamide on LAT3 expression in LNCaP (C) and C4-2 (D) cells. E and F, Comparison of the LAT3 and AR protein expression levels in LNCaP (E) and C4-2 (F) cells treated with DHT and/or bicalutamide (Bic). Each bar represents the mean with the standard error of the mean (SEM). $^{*}P < .05$, $^{**}P < .01$, NS: not significant (unpaired Student's *t*-test)

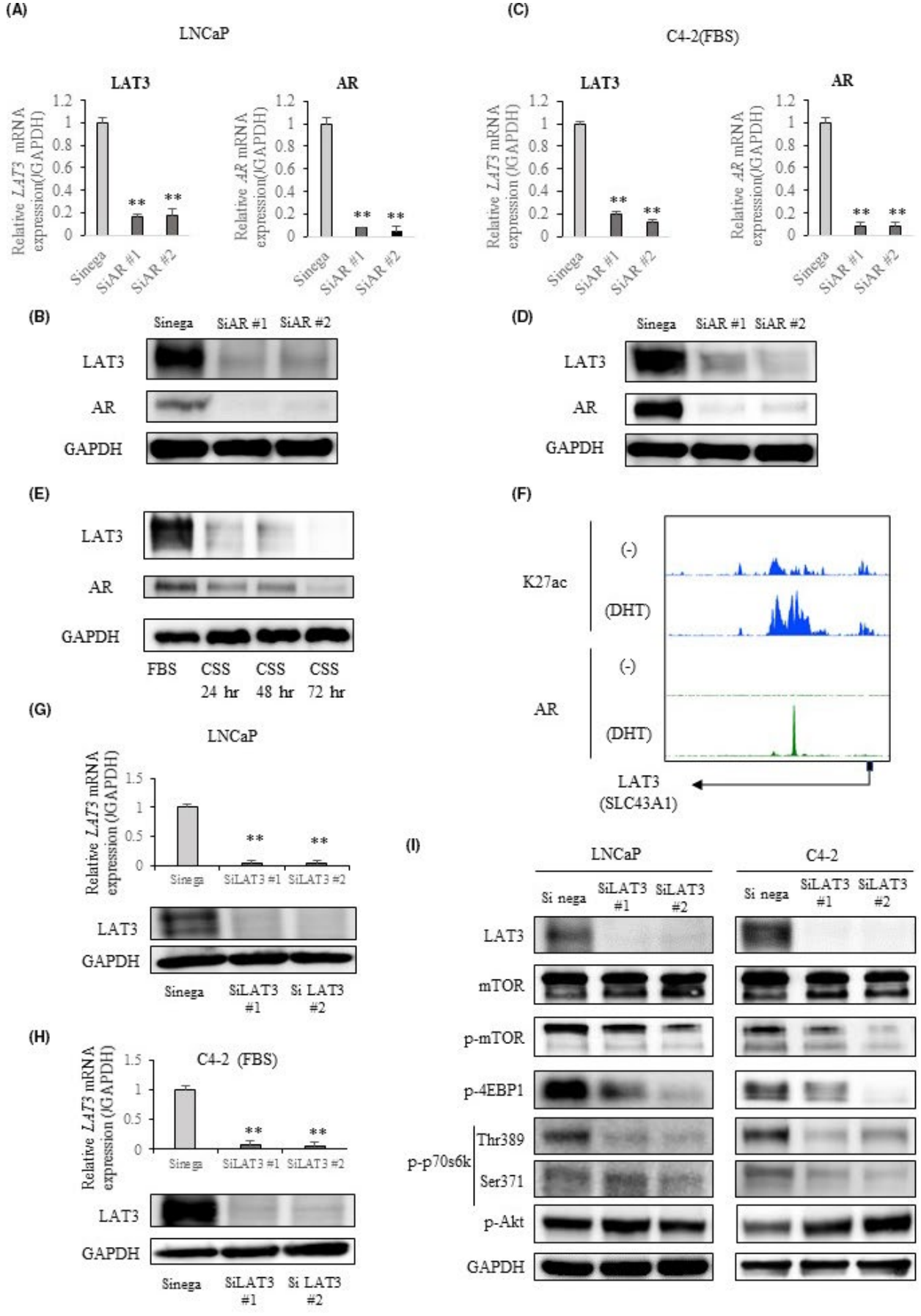

(A)
LNCaP
LAT3
AR
Relative LAT3 mRNA expression(/GAPDH)
Relative AR mRNA expression(/GAPDH)
Sinega
SiAR #1
SiAR #2
(C)
C4-2(FBS)
(B)
LAT3
AR
GAPDH
(D)
(E)
FBS
CSS 24 hr
CSS 48 hr
CSS 72 hr
(F)
K27ac
(-)
(DHT)
AR
LAT3 (SLC43A1)
(G)
LNCaP
Relative LAT3 mRNA expression (/GAPDH)
SiLAT3 #1
SiLAT3 #2
Si LAT3 #2
(H)
C4-2 (FBS)
(I)
LNCaP
C4-2
Si nega
mTOR
p-mTOR
p-4EBP1
p-p70s6k
Thr389
Ser371
p-Akt

FIGURE 2 Inhibition of L-type amino acid transporter 3 (LAT3) expression and the mTOR pathway by androgen receptor (AR) and LAT3 knockdown, and the chromatin immunoprecipitation sequencing assay of AR. A and C, Changes in *LAT3* and *AR* mRNA expression due to SiAR in LNCaP (A) and C4-2 (C) cells. B and D, Decreases in the LAT3 and AR protein expression due to SiAR in LNCaP (B) and C4-2 (D) cells. E, Changes in LAT3 and AR expression over time with charcoal-stripped FBS (CSS) administration. F, Epigenetic status around the *LAT3* locus in the genome browser view. AR binding to LAT3 (SLC43A1) in dihydrotestosterone (DHT)-stimulated LNCaP cells. G and H, Confirmation of the LAT3-knockdown effect of SiLAT3 in LNCaP (G) and C4-2 (H) cells. I, Effect of SiLAT3 on the phosphorylation status of the mTOR signaling pathway in LNCaP and C4-2 cells. GAPDH was used as a loading control. Each bar represents the mean with the SEM. Sinega, siRNA negative control, $^{*}P < .05$, $^{**}P < .01$, NS: not significant (unpaired Student's *t* test)

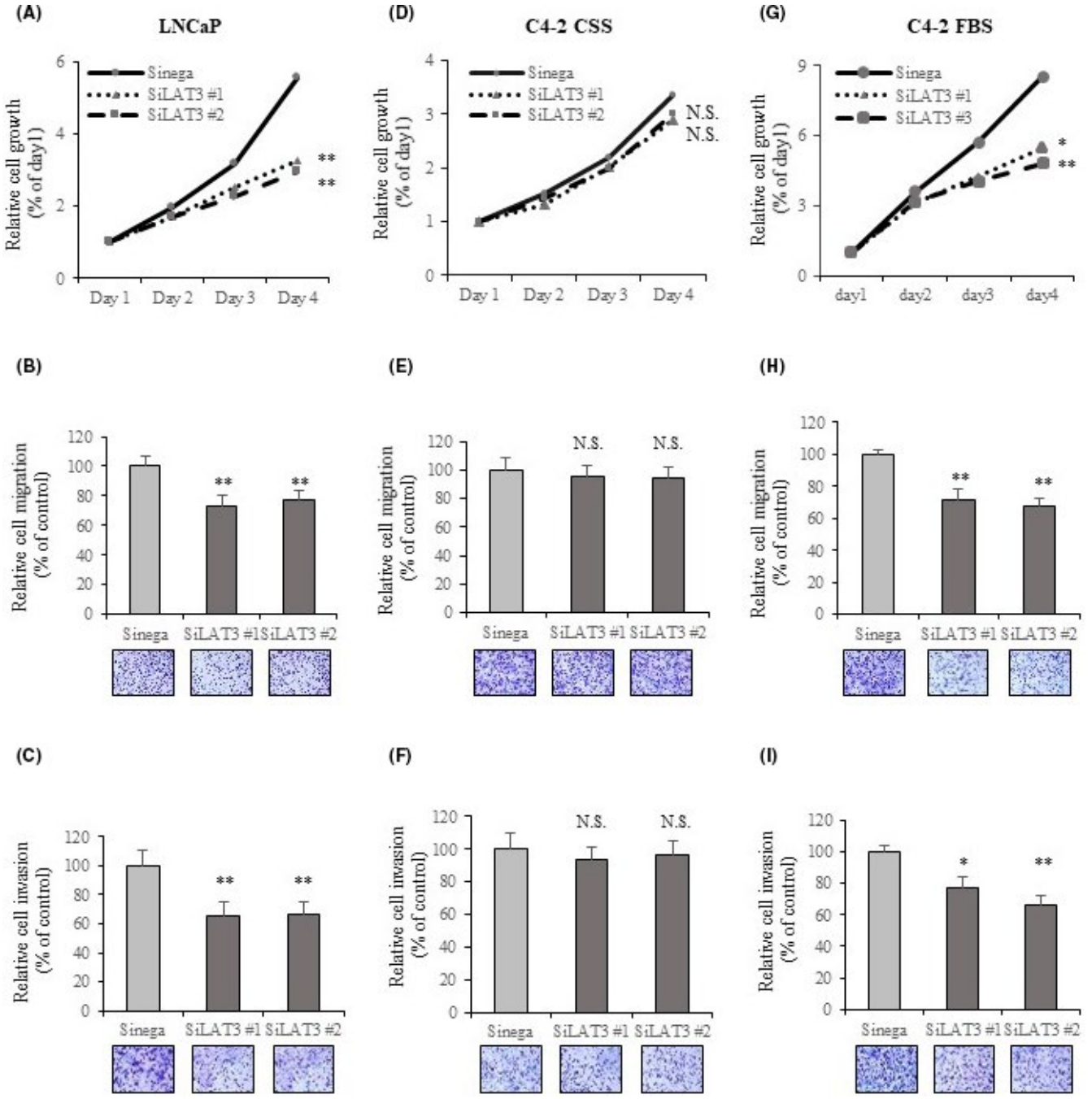


FIGURE 3 Effects of SiLAT3 on the cell viability, migration, and invasion of prostate cancer (PC) cells. A, D, G, SiLAT3 significantly reduced the cell proliferation in LNCaP cells (A) and C4-2 cells in FBS (G), but not C4-2 cells in charcoal-stripped FBS (CSS) (D). B, E, H, Transwell chamber migration assays were used to evaluate the migratory changes in LNCaP cells (B), C4-2 cells in CSS (E), and C4-2 cells in FBS (H). C, F, I, Matrigel invasion assays were used to evaluate the invasion activities of LNCaP cells (C), C4-2 cells in CSS (F), and C4-2 cells in FBS (I). Representative images of three independent experiments are shown. Each bar represents the mean with the SEM. Sinega, siRNA negative control, $^{**}P < .01$, NS: not significant (unpaired Student's *t*-test)

terms such as "cell cycle," "DNA replication," and "cell division" (Figure 4A). The top 10 genes that were downregulated by SiLAT3 are listed in Figure 4B. These genes were confirmed by RT-PCR (Figure 4C). Based on the basal expression levels and reproducibility, we selected Separase (*ESPL1*), which is known to play an important role in the cell cycle of cancer cells.[26,27] First, we confirmed that the expression of Separase was reduced by LAT3 knockdown (Figure 4D). In the cell cycle analysis, LAT3 knockdown significantly decreased the number of cells in the S and G2/M phases, suggesting cell cycle arrest (Figure 4E). We then tested the effect of Sepin-1, a selective Separase inhibitor,[28] on PC cell growth. The IC_{50} of Sepin-1 in LNCaP cells was 5.99 μmol/L (Figure 4F). Sepin-1 inhibited the proliferation of LNCaP cells in a dose-dependent manner (Figure 4G). Combined treatment with Sepin-1 and MDV3100 inhibited cell proliferation in an additive manner (Figure 4H). To confirm the additive effect of Sepin-1 and antiandrogenic drugs, proliferation studies were conducted using Sepin-1 and apalutamide, another antiandrogenic drug, which also inhibited cell proliferation in an additive manner (Figure S9A). Moreover, when cultured in CSS medium, Sepin-1 showed an inhibitory effect on proliferation of LNCaP cells (Figure S9B). In addition, introduction of both Separase and SiLAT3 significantly restored cell proliferation compared with cells transfected with SiLAT3 alone (Figure S8C). We confirmed that Separase transfection restored Separase protein expression in SiLAT3-treated cells (Figure S8D). These results suggested that LAT3 contributes to the cell cycle of PC through Separase.

3.5 | LAT3 and Separase expression in prostatectomy specimens and correlation with clinical parameters

To characterize the clinical significance of LAT3, we investigated LAT3 and Separase protein expression in prostatectomy specimens by IHC. The characteristics of all patients and the Kaplan-Meier curve for RFS are summarized in Table 1 and Figure S10, respectively. Both LAT3 and Separase tended to stain weakly in the cancerous areas with Gleason pattern 3 (Figure 5A-C and D-F) and strongly in the areas with Gleason pattern 4 (Figure 5G-I and J-L). LAT3 staining was observed at the cell membrane and cytoplasm (Figure 5C,I) while Separase staining was observed at the nucleus and cytoplasm (Figure 5F,L). Patients were divided into two groups based on the LAT3 and Separase IHC scores; the median intensity scores were 131 and 171.25, respectively, and 30 (31.91%) patients had high LAT3 and Separase expression while 29 (30.85%) patients had low LAT3 and Separase expression (Figure 5M). We then designed immunostaining for key genes in the mTOR pathway. Based on previous report,[29] we performed immunostaining for p-4EBP1 in prostatectomy specimens and investigated its association with LAT3 expression. As shown in Figure S11, comparing the LAT3 Low and High groups, the expression of p-4EBP1 was significantly higher in the High group ($P = .0228$; Figure S11M).

Next, we investigated the impact of LAT3 and Separase expression on postoperative recurrence. The high LAT3 expression group showed a significantly shorter RFS than the low LAT3 expression group ($P = .0041$; Figure 5N). Similarly, the Separase high expression group showed a shorter RFS, but the difference was not significant ($P = .0668$; Figure 5O). Combined analysis of LAT3 and Separase expression demonstrated that the group with high LAT3 and Separase expression had a shorter PFS than the group with low LAT3 and Separase expression ($P = .0018$; Figure 5P). Similarly, we also performed immunostaining for LAT1 to investigate its association with LAT3 expression and RFS. A total of 19 (20.21%) patients had high LAT3 and LAT1 expression, while 19 (20.21%) patients had low LAT3 and LAT1 expression (Figure S12A). The correlation between LAT3 and LAT1 expression was lower than that between LAT3 and Separase. We also examined the association between LAT1 expression and postoperative recurrence but found no association ($P = .8472$; Figure S12B).

Next, we investigated the clinical and pathological factors, including the LAT3 and Separase IHC scores, affecting the RFS after prostatectomy. In the univariate Cox proportional hazard model analysis, a high pT stage (hazard ratio [HR]: 7.04, $P < .0001$), high LAT3 score (HR: 3.24, $P = .0018$), positive resection margin (HR: 4.44, $P < .0001$), vascular invasion (HR: 6.46, $P = .0159$), and perineural invasion (HR: 2.93, $P = .0093$) were significantly associated with a shorter RFS. In the multivariate analysis, a high pT stage (HR: 4.10, $P = .0011$) and high LAT3 score (HR: 2.46, $P = .0234$) were independent predictive factors for a poor RFS (Table 2).

Finally, we examined the association between LAT3/Separase expression and clinical and pathological factors. High LAT3 expression was significantly associated with a high pT stage ($P = .0190$) and a high International Society of Urological Pathology (ISUP) grade ($P = .0053$; Table S2). High Separase expression was associated with a high ISUP grade ($P < .0001$; Table S3).

FIGURE 4 Identification of Separase (*ESPL1*) as a downstream target of L-type amino acid transporter 3 (LAT3). A, The genes downregulated by SiLAT3 were significantly associated with gene ontology (GO) terms such as "cell cycle," "DNA replication," and "cell division." B, The top 10 genes that were downregulated by SiLAT3 are listed. C, The mRNA expression level of downregulated genes was evaluated by RT-PCR. D, Western blotting results showing that the Separase protein expression level was decreased by SiLAT3. E, Cell cycle analysis. A remarkable decrease in the number of cells in the S phase and G2/M phase, and an increase of cells in the G0/G1 phase are seen. F, LNCaP cells were treated with various concentrations of Sepin-1, a selective Separase inhibitor, for 72 h. Viability curves are shown as a percentage of the control values, which were acquired from cells treated with dimethyl sulfoxide (0.5%). G, The relationship between the Separase inhibitor dose and LNCaP cell viability. H, Validation of the additive effects of Separase inhibitors and androgen receptor (AR) inhibitors on LNCaP cell proliferation. The data represent the results from three independent experiments. $^{*}P < .05$, $^{**}P < .01$ (unpaired Student's *t*-test)

(A)

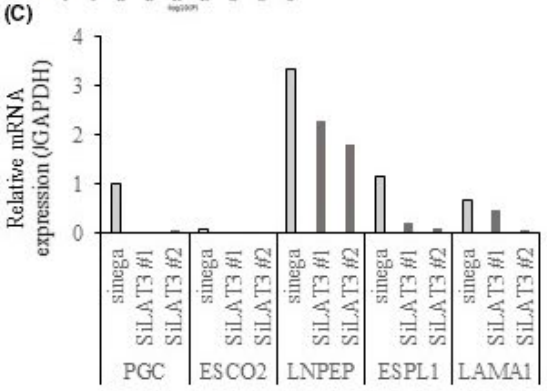
GO analysis in downregulated genes by SiLAT3

(B)

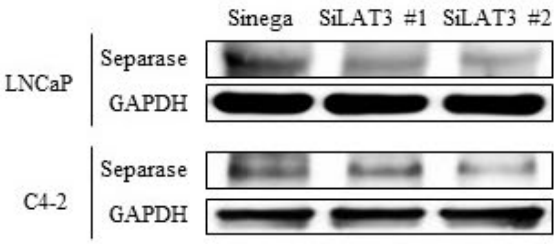
Downregulated gene list by SiLAT3

Gene name	Log
PGC	-2.74051
LAT3	**-2.58398**
CLEC7A	-2.4061
ESCO2	-2.35089
LNPEP	-2.31415
Separase	**-2.28594**
LAMA1	-2.20899
NMU	-2.0748
ACBD7	-1.99581
EFCC1	-1.99374

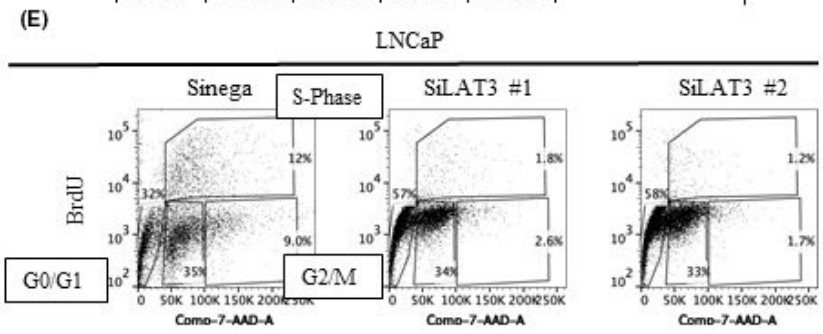
(C)

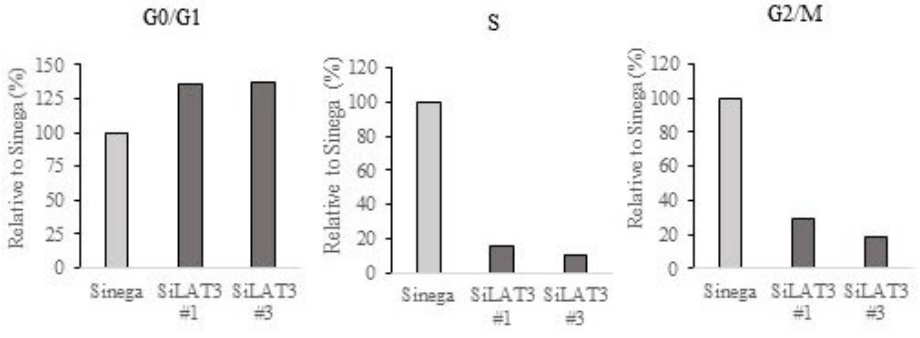


(D)

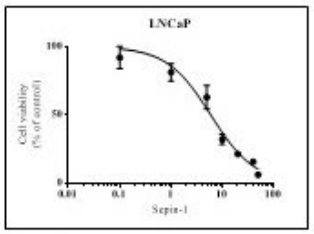


(E)



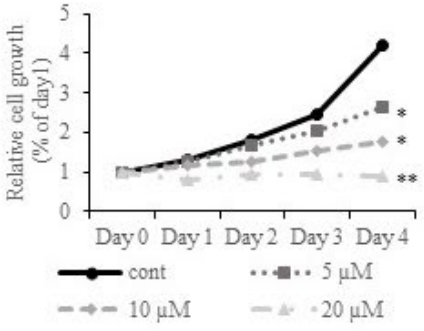


(F)

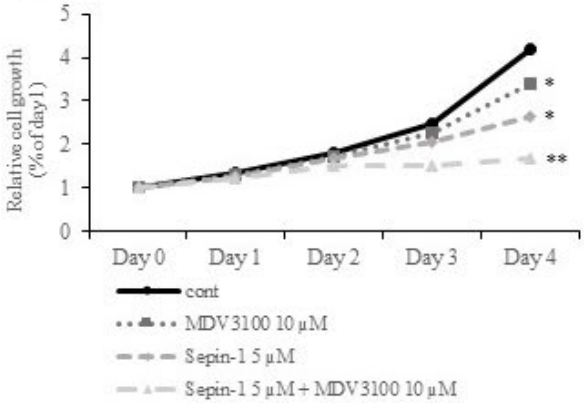


IC50 : 5.99μM ± 1.195 (95% CI)

(G)



(H)



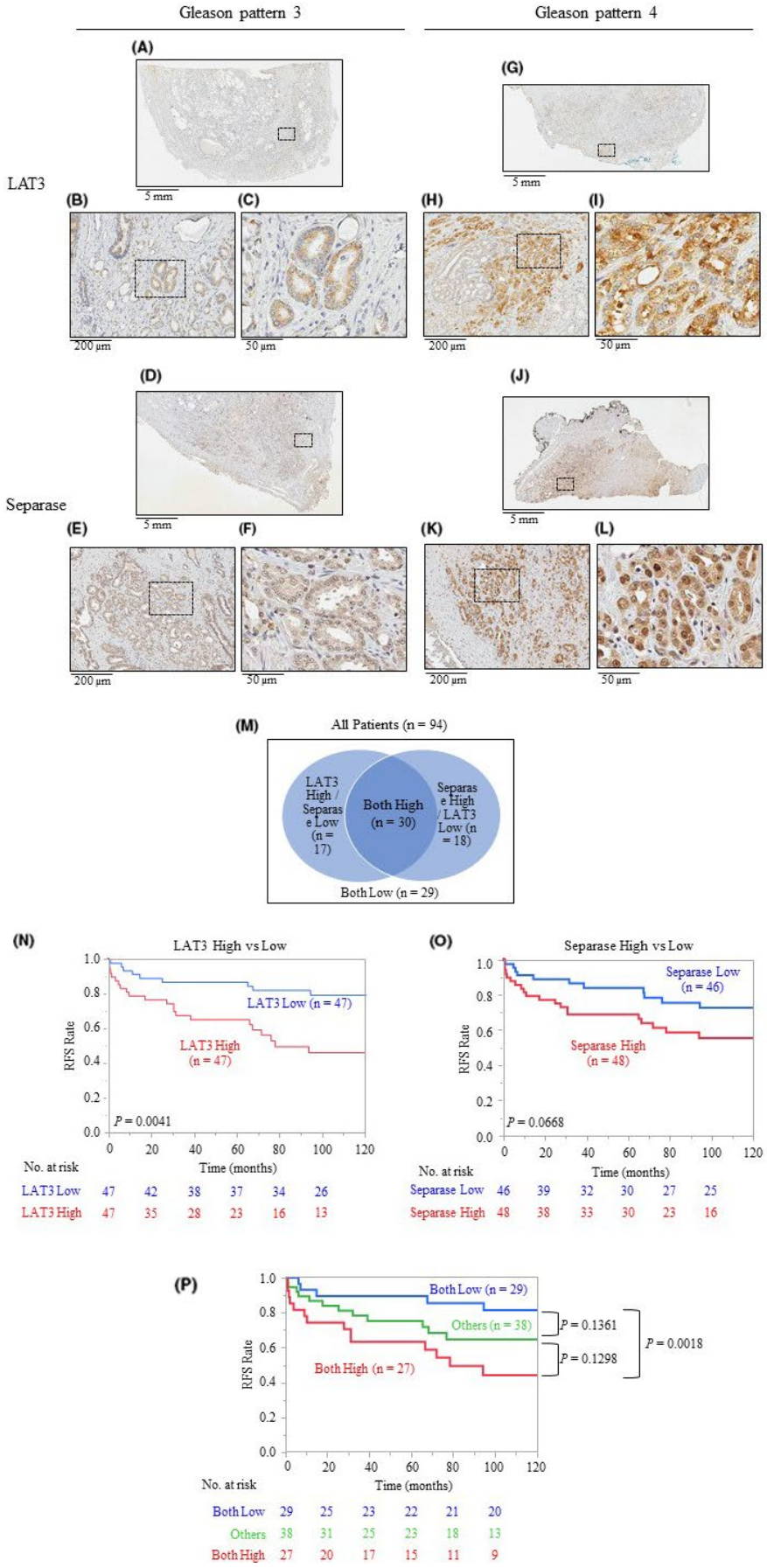


FIGURE 5 Immunostaining for L-type amino acid transporter 3 (LAT3) and Separase in prostatectomy specimens, and their relation to clinicopathological factors. A-L, Representative images of LAT3 immunostaining in areas with Gleason pattern 3 (A-C) and pattern 4 (G-I), and of Separase immunostaining in areas with Gleason pattern 3 (D-F) and pattern 4 (J-L). M, Venn diagram of the distribution of the number of patients with high and/or low LAT3 and Separase scores. N, Kaplan-Meier analysis of RFS according to high or low LAT3 scores. O, Kaplan-Meier analysis of recurrence-free survival (RFS) according to high or low Separase scores. P, Kaplan-Meier analysis of RFS according to high LAT3 and high Separase scores, low LAT3 and low Separase scores, or others. The *P*-value was calculated by the log-rank test

TABLE 2 Univariate and multivariate Cox proportional hazard models for RFS

	Univariate			Multivariate		
Variable	**HR**	**95% CI**	***P*-value**	**HR**	**95% CI**	***P*-value**
Age (over 66), years	1.04	0.51-2.19	.9234			
Initial PSA (over 7.825), ng/mL	1.44	0.71-3.01	.3102			
BMI (over 13.7)	1.07	0.52-2.37	.4316			
TST (over 4.3), ng/dL	1.24	0.60-2.52	.2530			
pT stage (3 or 4)	7.04	3.26-16.9	<.0001	4.10	1.72-10.8	.0011
GG (4 or 5)	2.07	0.99-4.19	.0529			
LAT3 score	3.24	1.53-7.46	.0018	2.46	1.13-5.80	.0234
Separase score	1.96	0.96-4.25	.0656			
RM	4.44	2.01-11.2	<.0001	1.69	0.65-4.83	.2907
ly	1.94	0.46-5.54	.3183			
v	6.46	1.52-18.8	.0159	3.83	0.84-12.8	.0769
pn	2.93	1.28-7.89	.0093	1.57	0.616-4.59	.3588

Abbreviations: BMI, body mass index; CI, confidence interval; GG, grade group; HR, hazard ratio; ly, lymphatic invasion; pn, perineural invasion; PSA, prostate-specific antigen; RFS, recurrence-free survival; RM, resection margin; TST, testosterone; v, vascular invasion.

4 | DISCUSSION

Our study had two major findings. Firstly, we analyzed the function of LAT3 in PC. We showed that the expression of LAT3 was regulated by AR, and knockdown of LAT3 suppressed cell proliferation, migration, and invasion in vitro. Moreover, high LAT3 expression was correlated with a short RFS after prostatectomy. Secondly, we identified Separase, a protein involved in the cell cycle, as a downstream target of LAT3. High expression of both LAT3 and Separase was correlated with a worse RFS after prostatectomy.

The association between AR, the mTOR pathway, and LAT3 has been reported previously by Wang et al[18] They showed that treatment with 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), a nonspecific inhibitor of the LAT family, caused cell cycle arrest in LNCaP cells and suppressed the phosphorylation of p70S6K, a member of the mTOR pathway. In our study, similar findings were obtained by the knockdown of LAT3 in LNCaP cells. Taken together, these results suggest that LAT3 may play an important role in AR-dependent PC. In addition, LAT3 has also been reported to be associated with epidermal growth factor[30] and oncogenic MYC,[31] and further studies are needed.

Recently, another amino acid transporter, LAT1, has been reported to be associated with a variety of cancers.[32] Similar to the present report, the correlation between LAT1 expression and prognosis has been investigated in PC,[14] pancreatic cancer,[33] and lung cancer.[34] Several groups, including ours, have reported that JPH203, a selective inhibitor of LAT1, interrupts the growth of cancer cells by blocking leucine uptake in various cancer types.[19,20,35,36] The first JPH203 phase I clinical trial in solid cancer patients was recently conducted.[37] The trial demonstrated modest side effects with long-term disease control in one bile ductal cancer patient.[37] We are currently planning a phase IIa clinical trial of JPH203 in CRPC patients in whom standard therapy failed. The relationship between cancer and amino acid transporters is expected to be increasingly studied in the future.

Separase is a protease that breaks down chromosomal cohesion during mitosis and plays an essential role in cell cycle progression.[38] Separase has been reported to be overexpressed in human bone, breast, and prostate cancer.[26] IHC of patient tissues showed that the expression of Separase was correlated with the prognosis in patients with breast cancer [39] and glioblastoma.[27] In addition, Sepin-1, an inhibitor of Separase, suppressed the cell proliferation, migration, and wound healing of breast cancer cells in vitro.[40] In this report, we identified Separase as a downstream target of LAT3; however, the mechanism by which LAT3 knockdown regulates Separase is unknown. Separase may represent a new biomarker or therapeutic target in PC as well as in other cancers.

The results of this study should be considered in light of several limitations. Firstly, no in vivo experiments were performed. We do not have the facilities to conduct in vivo experiments and are considering collaborating with other institutions. Instead, to obtain supportive evidence, we thoroughly studied the clinical significance of LAT3 in PC patients. Secondly, the study cohort used to investigate the LAT3 expression profile was quite small. We are planning a larger multi-institutional study to assess the prognostic value of LAT3 in PC patients. Thirdly, we analyzed the expression of LAT3 only in patients who had received a prostatectomy. We are currently planning a prospective clinical trial that includes patients with CRPC and neuroendocrine PC.

In summary, LAT3 expression was regulated by AR in PC cells, and high LAT3 expression correlated with a poor prognosis after prostatectomy. Knockdown of LAT3 suppressed the proliferative activities of PC cells and arrested the cell cycle. We identified Separase, which is involved in the cell cycle, as a downstream target of LAT3. LAT3 and Separase may serve as prognostic markers and therapeutic targets in hormone-sensitive PC.

ACKNOWLEDGEMENTS

The authors thank Dr. Osamu Ohara (Kazusa DNA Research Institute, Kisarazu, Japan) for the RNA-Seq analysis. The authors also thank Hisayo Karahi, Miyuki Yamaguchi, and Natsuko Kusama (experimental assistants, Chiba University) for their support of this study. The present work was supported by a Grant-in-Aid for Scientific Research (B) (grant #20H03813) to TI and a Grant-in-Aid for Scientific Research (C) (grant #20K09555) to SS.

DISCLOSURE

The authors declare no conflict of interest.

ORCID

Junryo Rii https://orcid.org/0000-0002-6093-3190
Shinichi Sakamoto https://orcid.org/0000-0002-7508-7521
Yasutaka Yamada https://orcid.org/0000-0002-0070-1590
Toru Hirota https://orcid.org/0000-0001-7064-9065
Atsushi Kaneda https://orcid.org/0000-0002-6980-5515
Yoshikatsu Kanai https://orcid.org/0000-0001-8126-286X

REFERENCES

1. Barsouk A, Padala SA, Vakiti A, et al. Epidemiology, staging and management of prostate cancer. *Med Sci*. 2020;8:28. https://doi.org/10.3390/medsci8030028
2. Shore ND, Morgans AK, Ryan CJ. Resetting the bar of castration resistance - understanding androgen dynamics in therapy resistance and treatment choice in prostate cancer. *Clin Genitourin Cancer*. 2020. https://doi.org/10.1016/j.clgc.2020.08.008
3. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. *Nat Rev Cancer*. 2001;1:34-45.
4. Yamada Y, Sakamoto S, Rii J, et al. How many bone metastases may be defined as high-volume metastatic prostate cancer in Asians: a retrospective multicenter cohort study. *Prostate*. 2020;80:432-440.
5. Yamada Y, Sakamoto S, Rii J, et al. Prognostic value of an inflammatory index for patients with metastatic castration-resistant prostate cancer. *Prostate*. 2020;80:559-569.
6. Ando K, Sakamoto S, Takeshita N, et al. Higher serum testosterone levels predict poor prognosis in castration-resistant prostate cancer patients treated with docetaxel. *Prostate*. 2020;80:247-255.
7. Rii J, Sakamoto S, Yamada Y, et al. Prognostic factors influencing overall survival in de novo oligometastatic prostate cancer patients. *Prostate*. 2020;80:850-858.
8. Sakamoto S, Maimaiti M, Xu M, et al. Higher serum testosterone levels associated with favorable prognosis in enzalutamide- and abiraterone-treated castration-resistant prostate cancer. *J Clin Med*. 2019;8:489.
9. Obinata D, Lawrence MG, Takayama K, et al. Recent discoveries in the androgen receptor pathway in castration-resistant prostate cancer. *Front Oncol*. 2020;10:581515.
10. Sugiura M, Sato H, Okabe A, et al. Identification of AR-V7 downstream genes commonly targeted by AR/AR-V7 and specifically targeted by AR-V7 in castration resistant prostate cancer. *Transl Oncol*. 2020;14:100915.
11. Sugiura M, Sato H, Kanesaka M, et al. Epigenetic modifications in prostate cancer. *Int J Urol*. 2021;28(2):140-149.
12. Sakamoto S. Current status of circulating tumor cell androgen receptor splice variant-7 in metastatic castration-resistant prostate cancer. *Ann Transl Med*. 2019;7:S375.
13. Bröer S. Amino acid transporters as targets for cancer therapy: why, where, when, and how. *Int J Mol Sci*. 2020;21:6156.
14. Xu M, Sakamoto S, Matsushima J, et al. Up-regulation of LAT1 during antiandrogen therapy contributes to progression in prostate cancer cells. *J Urol*. 2016;195:1588-1597.
15. Wang Q, Holst J. L-type amino acid transport and cancer: targeting the mTORC1 pathway to inhibit neoplasia. *Am J Cancer Res*. 2015;5:1281-1294.
16. Cole KA, Chuaqui RF, Katz K, et al. cDNA sequencing and analysis of POV1 (PB39): a novel gene up-regulated in prostate cancer. *Genomics*. 1998;51:282-287.
17. Babu E, Kanai Y, Chairoungdua A, et al. Identification of a novel system L amino acid transporter structurally distinct from heterodimeric amino acid transporters. *J Biol Chem*. 2003;278:43838-43845.
18. Wang Q, Bailey CG, Ng C, et al. Androgen receptor and nutrient signaling pathways coordinate the demand for increased amino acid transport during prostate cancer progression. *Cancer Res*. 2011;71:7525-7536.
19. Maimaiti M, Sakamoto S, Yamada Y, et al. Expression of L-type amino acid transporter 1 as a molecular target for prognostic and therapeutic indicators in bladder carcinoma. *Sci Rep*. 2020;10:1292.
20. Higuchi K, Sakamoto S, Ando K, et al. Characterization of the expression of LAT1 as a prognostic indicator and a therapeutic target in renal cell carcinoma. *Sci Rep*. 2019;9:16776.
21. Goto Y, Kojima S, Kurozumi A, et al. Regulation of E3 ubiquitin ligase-1 (WWP1) by microRNA-452 inhibits cancer cell migration and invasion in prostate cancer. *Br J Cancer*. 2016;114:1135-1144.
22. Zhou Y, Zhou B, Pache L, et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nat Commun*. 2019;10:1523.
23. Grasso CS, Wu YM, Robinson DR, et al. The mutational landscape of lethal castration-resistant prostate cancer. *Nature*. 2012;487:239-243.
24. Luo JH, Yu YP, Cieply K, et al. Gene expression analysis of prostate cancers. *Mol Carcinog*. 2002;33:25-35.
25. Imamura Y, Sakamoto S, Endo T, et al. FOXA1 promotes tumor progression in prostate cancer via the insulin-like growth factor binding protein 3 pathway. *PLoS One*. 2012;7:e42456.
26. Meyer R, Fofanov V, Panigrahi A, Merchant F, Zhang N, Pati D. Overexpression and mislocalization of the chromosomal segregation protein separase in multiple human cancers. *Clin Cancer Res*. 2009;15:2703-2710.
27. Mukherjee M, Byrd T, Brawley VS, et al. Overexpression and constitutive nuclear localization of cohesin protease Separase protein correlates with high incidence of relapse and reduced overall survival in glioblastoma multiforme. *J Neuro-Oncol*. 2014;119:27-35.
28. Zhang N, Scorsone K, Ge G, et al. Identification and Characterization of Separase Inhibitors (Sepins) for Cancer Therapy. *J Biomol screen*. 2014;19:878-889.
29. Chen X, Xiong X, Cui D, et al. DEPTOR is an in vivo tumor suppressor that inhibits prostate tumorigenesis via the inactivation of mTORC1/2 signals. *Oncogene*. 2020;39:1557-1571.
30. Zhang BK, Moran AM, Bailey CG, Rasko JEJ, Holst J, Wang Q. EGF-activated PI3K/Akt signalling coordinates leucine uptake by regulating LAT3 expression in prostate cancer. *Cell Commun Signal*. 2019;17:83.
31. Yue M, Jiang J, Gao P, Liu H, Qing G. Oncogenic MYC activates a feedforward regulatory loop promoting essential amino acid metabolism and tumorigenesis. *Cell Rep*. 2017;21:3819-3832.
32. Puris E, Gynther M, Auriola S, Huttunen KM. L-Type amino acid transporter 1 as a target for drug delivery. *Pharm Res*. 2020;37:88.
33. Kaira K, Arakawa K, Shimizu K, et al. Relationship between CD147 and expression of amino acid transporters (LAT1 and ASCT2) in patients with pancreatic cancer. *Am J Transl Res*. 2015;7:356-363.
34. Kaira K, Kawashima O, Endoh H, et al. Expression of amino acid transporter (LAT1 and 4F2hc) in pulmonary pleomorphic carcinoma. *Hum Pathol*. 2019;84:142-149.

35. Yun DW, Lee SA, Park MG, et al. JPH203, an L-type amino acid transporter 1-selective compound, induces apoptosis of YD-38 human oral cancer cells. *J Pharmacol Sci*. 2014;124:208-217.
36. Cormerais Y, Giuliano S, LeFloch R, et al. Genetic disruption of the multifunctional CD98/LAT1 complex demonstrates the key role of essential amino acid transport in the control of mTORC1 and tumor growth. *Cancer Res*. 2016;76:4481-4492.
37. Okano N, Naruge D, Kawai K, et al. First-in-human phase I study of JPH203, an L-type amino acid transporter 1 inhibitor, in patients with advanced solid tumors. *Invest New drugs*. 2020;38:1495-1506.
38. Zhang N, Pati D. Biology and insights into the role of cohesin protease separase in human malignancies. *Biol Rev Camb Philos Soc*. 2017;92:2070-2083.
39. Gurvits N, Löyttyniemi E, Nykänen M, Kuopio T, Kronqvist P, Talvinen K. Separase is a marker for prognosis and mitotic activity in breast cancer. *Br J Cancer*. 2017;117:1383-1391.
40. Zhang N, Pati D. Separase inhibitor Sepin-1 inhibits FoxM1 expression and breast cancer cell growth. *J Cancer Sci Ther*. 2018;10:517. https://doi.org/10.4172/1948-5956.1000517

SUPPORTING INFORMATION

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

How to cite this article: Rii J, Sakamoto S, Sugiura M, et al. Functional analysis of LAT3 in prostate cancer: Its downstream target and relationship with androgen receptor. *Cancer Sci*. 2021;112:3871–3883. https://doi.org/10.1111/cas.14991