# Impact of L-type amino acid transporter 3 on the prognosis of hepatocellular carcinoma

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**Abstract.** The aim of the present study was to investigate the impact of L-type amino acid transporter 3 (LAT3) expression on the prognosis of hepatocellular carcinoma (HCC). A total of 135 patients who had undergone initial hepatic resection for HCC at Tokushima University Hospital (Tokushima, Japan) were enrolled in the present study. Immunohistochemical analysis of LAT3 and phosphorylated AKT (p-AKT) was performed using resected specimens. Clinicopathological factors, including prognosis, were compared between the LAT3-high and -low expression groups. The results demonstrated that the LAT3-high group showed significantly higher protein induced by vitamin K absence-II levels (P=0.01) compared with the LAT3-low group. The LAT3-high group showed significantly worse prognosis compared with the LAT3-low group regarding cancer-specific survival and disease-free survival (P<0.05). Multivariate analysis revealed that high LAT3 expression and multiple tumors were independent prognostic factors for cancer-specific survival. Furthermore, the rate of p-AKT-positive cases was higher in the LAT3-high group than in the LAT3-low group. Overall, these findings suggested that LAT3 expression was associated with poor prognosis of HCC and high p-AKT expression.

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*Abbreviations:* LAT3, L-type amino acid transporter 3; HCC, hepatocellular carcinoma; p-AKT, phosphorylated AKT; PIVKA-II, protein induced by vitamin K absence-II; AFP, α-fetoprotein

*Key words:* LAT3, HCC, amino acid, leucine, p-AKT

## Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide, accounting for approximately 80-90% of primary liver cancer (1). Despite the progression in treatment strategies, the prognosis of HCC remains poor (2). Therefore, elucidation of the molecular mechanism of HCC progression may contribute to better prognosis.

L-type amino acid transporter 3 (LAT3) is a system L-amino acid transporter that transports neutral amino acids such as leucine, isoleucine, valine, phenylalanine, and methionine (3). System L-amino acid transporters transport large branched and aromatic neutral amino acids in almost all cell types independent of $Na^+$ (4). These transporters provide cancer cells with the essential amino acids required for protein synthesis and growth stimulation (5). The liver is the central organ for amino acid metabolism, and the importance of amino acid metabolism in HCC has been reported in recent years (6). Regarding cancer progression, LAT3 is highly expressed in primary and recurrent prostate cancer, and upregulated LAT3 levels result in increased intracellular leucine levels and subsequent cell proliferation in prostate cancer cells (7,8). However, the role of LAT3 in HCC remains unclear.

Multiple kinase signaling pathways including the PI3K/AKT pathway play central roles in the development of various cancers (9). Furthermore, it was reported that activation of PI3K/AKT signaling can affect the proliferation, invasion, and apoptosis of HCC cells (10). AKT is a serine/threonine protein kinase that plays a pivotal role in regulating diverse biological functions, including protein synthesis, cell metabolism, cell survival, and cell cycle progression, following its phosphorylation (11). Phosphorylated-AKT (p-AKT) was reported to stabilize LAT3 protein levels and subsequent cell proliferation in prostate cancer cell lines (12). Therefore, we hypothesized that high expression of LAT3 is associated with poor prognosis in HCC and that p-AKT activation is the mechanism of its malignant potential.

In the current study, we investigated the relationship between LAT3 expression and the clinicopathological factors of HCC including prognosis by immunohistochemical analysis, and investigated its mechanism with respect to p-AKT expression.

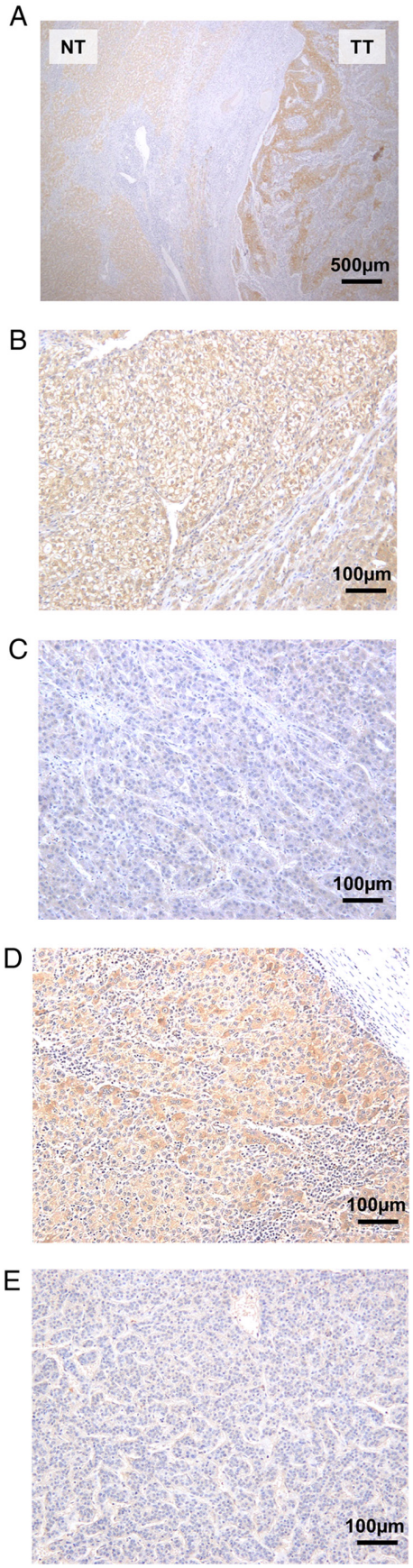


Figure 1. Representative images of immunohistochemical analysis. (A) LAT3 expression in TT and adjacent NT (magnification, x40; scale bar, 500 $\mu$m). (B) LAT3-high. (C) LAT3-low (magnification, x200; scale bar, 100 $\mu$m). (D) p-Akt positive. (E) p-Akt negative (magnification, x200; scale bar, 100 $\mu$m). LAT3, L-type amino acid transporter 3; NT, normal tissue; p-AKT, phosphorylated AKT; TT, tumor tissue.

Table I. Patient characteristics in the LAT3-high and LAT3-low expression groups.

| Variable | High (n=46) | Low (n=89) | P-value |
|---|---|---|---|
| Age, years | 66.6±10.5 | 68.4±9.9 | 0.515 |
| Sex (male/female) | 30/16 | 66/23 | 0.281 |
| HBV (-/+) | 30/16 | 70/19 | 0.096 |
| HCV (-/+) | 28/18 | 58/31 | 0.623 |
| Child-Pugh (5/>6) | 36/10 | 75/14 | 0.392 |
| Growth pattern (Eg/Ig) | 42/4 | 75/14 | 0.240 |
| Tumor size (<2/≥2 cm) | 13/33 | 19/70 | 0.375 |
| Tumor number (single/multi) | 37/9 | 75/14 | 0.578 |
| Differentiation (poorly/others) | 2/44 | 9/80 | 0.331 |
| Portal vein invasion (-/+) | 33/13 | 75/14 | 0.090 |
| Stage (I/II/III/IV) | 10/16/17/3 | 15/48/23/3 | 0.194 |
| AFP (<100/>100 ng/ml) | 31/15 | 69/20 | 0.208 |
| PIVKA-II (<400/>400 mAU/ml) | 25/21 | 67/22 | 0.014 |

Age is presented as the mean ± standard deviation and the unpaired Mann-Whitney U test was used. Other variables are presented as the number. Fisher's exact test was used for differentiation. $\chi^2$ test was used for other factors. LAT3, L-type amino acid transporter 3; HBV, hepatitis B virus; HCV, hepatitis C virus; Eg, expanding growth; Ig, invasive growth; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence.

## Materials and methods

*Patients*. One hundred thirty-five patients with histologically proved HCC who underwent hepatectomy at Tokushima University Hospital between January 2008 and December 2017 were enrolled in this study. In this patient cohort, median and range of age was 68 (33-88) years old. We included patients who: (a) had no history of treatment prior to surgery; and (b) had no extrahepatic metastasis. Pathological and morphological parameters and Japanese Tumor-Node-Metastasis stage were determined in accordance with the Liver Cancer Study Group of Japan (13). This study was approved by the institutional review board of our institute (no. 4144).

*Immunohistochemical analysis and evaluation*. Immunohistochemical analysis was performed in accordance with the protocol used in our department, which has been previously reported by Ishikawa *et al* (14). Briefly, sections were deparaffinized with xylene, followed by rehydration in a graded ethanol series. The sections were treated with 3% hydrogen peroxide in methanol for 10 min to quench the endogenous peroxidase activity. Antigen retrieval was performed by boiling in 10 mM citrate buffer (pH 6) using a microwave. After incubation with 1% bovine serum albumin to block nonspecific antibody binding, the sections were incubated with primary antibodies

Table II. Multivariate analysis of cancer-specific survival.

| Variable | 3-year survival rate, % | Univariate P-value | Hazard ratio (95% CI) | Multivariate P-value |
|---|---|---|---|---|
| Age (<70/≥70 years) | 90.1/87.4 | 0.372 | 1.880 (0.835-4.231) | 0.127 |
| Sex (male/female) | 90.2/86.3 | 0.945 | 0.919 (0.381-2.216) | 0.850 |
| Child-Pugh (5/>6) | 88.5/91.3 | 0.961 | 0.841 (0.297-2.381) | 0.744 |
| Growth pattern (Eg/Ig) | 90.1/80.8 | 0.201 | 1.549 (0.545-4.398) | 0.411 |
| Tumor size (<2/≥2 cm) | 89.7/87.6 | 0.351 | 1.537 (0.516-4.577) | 0.440 |
| Tumor number (single/multi) | 93.5/66.0 | <0.001 | 4.169 (1.686-10.310) | 0.002 |
| Differentiation (poorly/others) | 80.8/89.8 | 0.941 | 1.359 (0.302-6.113) | 0.689 |
| Portal vein invasion (-/+) | 92.2/76.6 | 0.025 | 2.295 (0.871-6.044) | 0.093 |
| AFP (<100/>100 mIU) | 91.5/81.9 | 0.539 | 0.539 (0.192-1.510) | 0.240 |
| PIVKA-II (<400/>400 mAU) | 93.1/80.7 | 0.040 | 1.233 (0.490-3.103) | 0.656 |
| LAT3 (low/high) | 94.2/78.8 | 0.020 | 2.558 (1.105-5.922) | 0.028 |

Eg, expanding growth; Ig, invasive growth; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence; LAT3, L-type amino acid transporter 3.

against rabbit monoclonal LAT3 antibody (1:100 dilution, NBP1-87332; Novus, CO, USA) and rabbit polyclonal phospho-AKT antibody (1:100 dilution, ab81283; Abcam, Tokyo, Japan) for 60 min at room temperature. After washing with phosphate-buffered saline, the sections were subjected to the Dako REAL EnVision/HRP detection system (Dako Corporation, Tokyo, Japan) for 60 min at room temperature. The peroxidase reaction was developed with 3,3'-diaminobenzidine as the chromogen. The sections were counterstained with 10% Mayer's hematoxylin, dehydrated in a graded series of ethanol, treated with xylene, and mounted in a synthetic resin. LAT3 expression scores were assessed based on the extent of obvious staining in the cell membrane or cytoplasm, as follows: 0, <5% of the tumor area stained; 1, 5-10% stained; 2, 11-25% stained; 3, 26-50% stained; and 4, ≥51% stained. Tumors in which the stained tumor cells were scored >1 were considered to show positive expression (15). Regarding p-AKT staining, when >10% of the tumor cells were stained in the cytoplasm and/or nucleus, the samples were considered positive (16). Microscopic observation was performed using an Olympus BX43 (Tokyo, Japan) with UPlanFL N objective lenses (4x/0.13, 10x/0.30, 20x/0.5 and 40x/0.75; Olympus) at room temperature, and then images were acquired using camera (DP27, Olympus) and cellSens Standard software (version 1.17.16030.0, Olympus). Representative images of positive and negative expression of LAT3 and p-AKT are shown in Fig. 1. Fig. 1A showed LAT3 expression in tumor tissue and adjacent normal tissue from the same patient, and LAT3 was highly expressed in tumor tissue compared with adjacent normal tissue.

*Statistical analysis*. The unpaired Mann-Whitney U test or the chi-squared test was used to compare clinicopathological factors between LAT3-high and -low expression groups. Cancer-specific survival and disease-free survival curves were obtained using the Kaplan-Meier method, and differences were compared using the log-rank test in univariate analysis. Multivariate analysis was conducted using the Cox proportional hazard regression model. For all statistical analyses, $P<0.05$ was considered significant. All statistical analyses were performed using JMP 8.0.1 statistical software (SAS Campus Drive, Cary, NC, USA).

## Results

*Clinicopathological factors*. On the basis of the immunohistochemical analysis, 135 patients were divided into LAT3-high (n=46) and LAT3-low (n=89) groups. Table I summarizes the clinicopathological factors of the patients in both groups. The LAT3-high group showed significantly higher levels of protein induced by vitamin K absence-II (PIVKA-II) ($P<0.05$). There were no significant differences in terms of age, sex, viral infection status, and other tumor factors between the two groups.

*Cancer-specific and disease-free survival*. The cancer-specific and disease-free survival rates were significantly worse in the LAT3-high group than in the LAT3-low group ($P<0.05$; Figs. 2 and 3). In the univariate analysis of cancer-specific survival, multiple tumors, portal vein invasion, PIVKA-II level (>400 mAU/ml) and high LAT3 expression were defined as prognostic factors. In the multivariate analysis, multiple tumors and high LAT3 expression were determined to be independent prognostic factors (Table II). In the univariate analysis of disease-free survival, multiple tumors, high PIVKA-II level (>400 mAU/ml) and high LAT3 expression were defined as prognostic factors. In the multivariate analysis, multiple tumors and high PIVKA-II level (>400 mAU/ml) were determined to be independent prognostic factors (Table III), but high LAT3 expression was not. Regarding recurrence, the rate of controllable recurrence with local therapy tended to be higher in the LAT3-high group than in the LAT3-low group ($P=0.06$) (Fig. 4).

Table III. Multivariate analysis of disease-free survival.

| Variable | 3-year survival rate, % | Univariate P-value | Hazard ratio (95% CI) | Multivariate P-value |
|---|---|---|---|---|
| Age (<70/≥70 years) | 67.2/73.0 | 0.915 | 0.986 (0.596-1.631) | 0.955 |
| Sex (male/female) | 67.3/76.1 | 0.142 | 0.679 (0.387-1.193) | 0.178 |
| Child-Pugh (5/>6) | 71.7/57.4 | 0.330 | 1.343 (0.692-2.608) | 0.384 |
| Growth pattern (Eg/Ig) | 70.2/62.5 | 0.945 | 0.779 (0.330-1.838) | 0.568 |
| Tumor size (<2/≥2 cm) | 68.5/69.0 | 0.101 | 0.570 (0.320-1.015) | 0.056 |
| Tumor number (single/multi) | 73.4/48.2 | 0.021 | 2.372 (1.192-4.720) | 0.014 |
| Differentiation (poorly/others) | 63.5/70.1 | 0.357 | 0.747 (0.225-2.481) | 0.634 |
| Portal vein invasion (-/+) | 69.1/73.5 | 0.906 | 0.954 (0.446-2.039) | 0.903 |
| AFP (<100/>100 mIU) | 68.4/73.9 | 0.172 | 0.555 (0.274-1.124) | 0.102 |
| PIVKA-II (<400/>400 mAU) | 77.7/52.1 | 0.024 | 2.340 (1.259-4.349) | 0.007 |
| LAT3 (low/high) | 72.7/64.2 | 0.049 | 1.471 (0.883-2.453) | 0.139 |

Eg, expanding growth; Ig, invasive growth; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence; LAT3, L-type amino acid transporter 3.

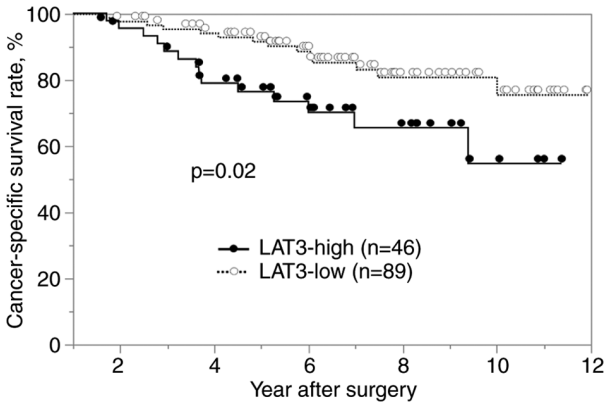


Figure 2. Cancer-specific survival rate in the LAT3-high and LAT3-low groups. The LAT-3 high group had a significantly worse prognosis compared with the LAT3-low group (P=0.02). LAT3, L-type amino acid transporter 3.

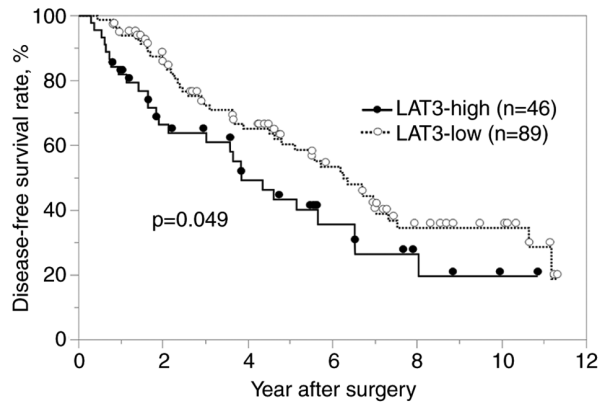


Figure 3. Disease-free survival rate in the LAT3-high and LAT3-low groups. The LAT-3 high group had a significantly worse prognosis compared with the LAT3-low group (P=0.049). LAT3, L-type amino acid transporter 3.

*p-Akt expression*. The LAT3-high group showed a significantly higher rate of p-AKT positivity compared with the LAT3-low

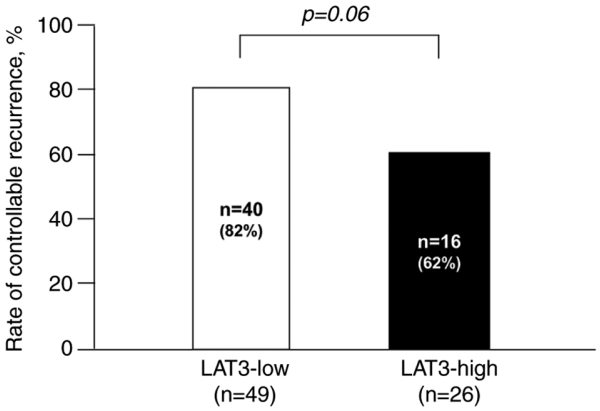


Figure 4. Rate of controllable recurrence in the LAT3-high and LAT3-low groups. Among recurrent cases, 40 out of 49 cases (82%) showed controllable recurrence in the LAT3-low group, while 16 out of 26 cases (62%) did in the LAT3-high group. The rate in the LAT3-high group tended to be lower than that in the LAT3-low group (P=0.06; $\chi^2$ test). LAT3, L-type amino acid transporter 3.

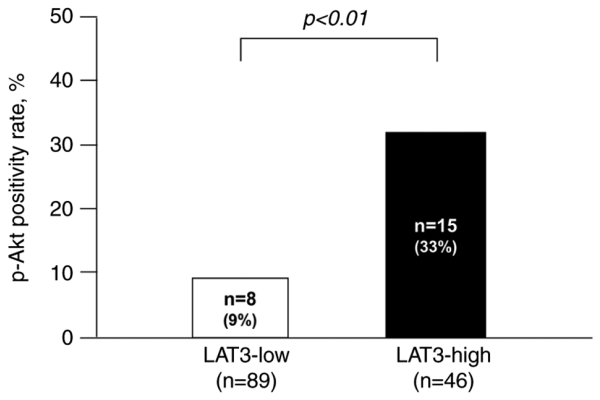


Figure 5. p-Akt positivity rate in the LAT3-low and LAT3-high groups. The LAT3-high group showed a significantly higher p-Akt positivity rate (15 cases; 33%) compared with the LAT3-low group (8 cases; 9%) (P<0.01; $\chi^2$ test). LAT3, L-type amino acid transporter 3; p-AKT, phosphorylated AKT.

group (P<0.01; Fig. 5).

## Discussion

The present study evaluated the clinical significance of LAT3 expression in HCC. The LAT family of proteins consists of four Na+-independent neutral amino acid transporters. The members of this family are divided into two sub-families: namely, SLC7 (LAT1 and LAT2) and SLC43 (LAT3 and LAT4) (3). In cancer progression, amino acid uptake is essential for cell proliferation. LAT1 is the most commonly known transporter regarding cancer and transports large neutral amino acids, such as leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, methionine, and histidine. However, LAT3 transports a narrow range of neutral amino acids, including leucine, isoleucine, valine, phenylalanine, and methionine (3). In particular, leucine plays pivotal roles in cancer progression, not only in protein synthesis but as a signaling factor (4). Compared with LAT1, LAT3 shows a more restricted expression pattern in some types of cancer, although reports are increasing about prostate cancer and leukemia. Rii *et al* (17) reported that high LAT3 expression was related to poor prognosis after prostatectomy, and knockdown of *SLC43A1*, which encodes LAT3, suppressed the proliferation of prostate cancer cells and arrested the cell cycle. Xu *et al* (18) showed that cellular proliferation and the mTOR pathway, which activates AKT, were significantly reduced when LAT3 was blocked. There is also a report of LAT3 expression in HepG2 HCC cells (19); however, there is no report regarding LAT3 expression in clinical HCC samples. To our knowledge, this is the first report about LAT3 correlated with prognosis in HCC.

LAT3 is an amino acid transporter that uptakes neutral amino acids, most of which are essential amino acids. Among these amino acids, leucine has been reported to promote myofibroblast differentiation via the AKT signaling pathway (20). Because the AKT signaling pathway is correlated with cell survival, cell cycle progression, and apoptosis inhibition (11), AKT signaling was investigated in this study. Zhang *et al* (12) reported that LAT3 protein levels were increased when AKT was phosphorylated, and that AKT and LAT3 were co-localized on the plasma membrane in prostate cancer cell lines. They confirmed that activated PI3K/AKT signaling regulates leucine transport through LAT3 in prostate cancer. In this study, the LAT3-high expression group showed a significantly higher rate of p-AKT positivity. Therefore, LAT3 expression might accelerate the malignant potential of HCC via AKT signaling. In this study, the LAT3-high group showed significantly higher PIVKA-II levels. PIVKA-II is commonly known as a prognostic factor in HCC (21). In fact, the LAT3-high group showed a worse cancer-specific survival rate compared with the LAT-3-low group. Although there was no significant difference in the disease-free survival rate between the two groups, more aggressive recurrence tended to occur in the LAT3-high group. This may have promoted the worse cancer-specific survival in the LAT3-high group. Thus, high LAT3 expression seemed to be related to the malignant potential of HCC.

In terms of LAT inhibitors, JPH203 has already been reported to inhibit LAT1, and clinical trials are ongoing in various cancers, including biliary and breast cancer (22). Regarding LAT3, ESK246 from *Pittosporum* has been reported to inhibit LAT-mediated leucine transport in prostate cancer cells compared with leucine analog (23). This inhibitor has potential as a new anti-HCC agent.

This study has several limitations. First, we only investigated LAT3 and p-AKT expression in resected specimens by immunohistochemistry. In this study sample, number of specimens which was available for RT-PCR was small. We plan to perform an *in vivo* study using an HCC cell line with an inhibitor such as ESK246. Second, this was a single-center study and the number of cases was relatively small. Thus, prospective studies in larger patient populations are necessary in the future.

In conclusion, LAT3 expression was associated with the poor prognosis of HCC and higher expression of p-AKT.

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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

BS and SY designed the experiments, acquired, analyzed and interpreted the data, and drafted and revised the manuscript. TI, YS, CT and HT acquired and analyzed the data, and revised the manuscript. YM and MS designed the experiments and revised the manuscript. All authors have read and approved the final manuscript. BS and SY confirm the authenticity of raw data.

## Ethics approval and consent to participate

The present study was approved by the ethics committee of Tokushima University Hospital (Tokushima, Japan; no. 4144). The requirement for informed consent was waived, and an information disclosure statement was uploaded onto the homepage of our hospital website for opt-out.

## Patient consent for publication

An information disclosure statement that the text, data and images are published, and they will be freely available on

the internet and may be seen by the general public was uploaded onto the homepage of the hospital website for opt-out.

## Competing interests

MS declares receiving an unrestricted research grant from Bayer Yakuhin, Co. Ltd., Japan. All other authors declare that they have no competing interests.

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