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Relationship between [^{14}C]MeAIB uptake and amino acid transporter family gene expression levels or proliferative activity in a pilot study in human carcinoma cells: Comparison with [^{3}H]methionine uptake

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ABSTRACT

Introduction: To clarify the difference between system A and L amino acid transport imaging in PET clinical imaging, we focused on the use of α-[*N*-methyl-**^{11}C**]-methylaminoisobutyric acid ([**^{11}C**]MeAIB), and compared it with [*S*-methyl-**^{11}C**]-L-methionine ([**^{11}C**]MET). The aim of this study was to assess the correlation of accumulation of these two radioactive amino acid analogs with expression of amino acid transporters and cell proliferative activity in carcinoma cells.

Methods: Amino acid uptake inhibitor studies were performed in four human carcinoma cells (epidermal carcinoma A431, colorectal carcinoma LS180, and lung carcinomas PC14/GL and H441/GL) using the radioisotope analogs [^{3}H]MET and [^{14}C]MeAIB. MeAIB was used to inhibit the A system and 2-amino-2-norbornane-carboxylic acid (BCH) was used to inhibit the L system. The carcinoma gene expression levels of a number of amino acid transporters were measured by microarray and quantitative polymerase chain reaction. Carcinoma proliferative activity was assessed using accumulation of [methyl-^{3}H]-3'-deoxy-3'-fluorothymidine ([^{3}H]FLT).

Results and conclusion: [^{14}C]MeAIB uptake occurred principally via a Na^{+}-dependent A type mechanism whereas [^{3}H]MET uptake occurred predominantly via a Na^{+}-independent L type mechanism although other transporters were also utilized depending on cell type. There was no correlation between [^{3}H]MET uptake and total system L amino acid transporter (LAT) expression. In contrast, [^{14}C]MeAIB uptake strongly correlated with total system A amino acid transporter (SNAT) expression and proliferative activity in this preliminary study using four human carcinoma cell lines. Carcinoma proliferative activity also correlated with total SNAT expression.

Advances in Knowledge and Implications for Patient Care: Because there is a significant correlation between the accumulation of [^{14}C]MeAIB and the gene expression level of total SNAT as well as the accumulation of [^{3}H]FLT, it is suggested that use of the analog [^{11}C]MeAIB in PET may provide an indication of tumor cell proliferative activity. [^{11}C]MeAIB is therefore expected to be very useful in PET imaging.

1. Introduction

2-[^{18}F]fluoro-2-deoxy-D-glucose ([^{18}F]FDG), an analog of glucose, is the most commonly used radiopharmaceutical in positron emission tomography (PET)-CT imaging [1]. PET-CT imaging is based on the preferential uptake of [^{18}F]FDG in tumor cells as compared to normal cells, because glucose metabolism is increased in tumor cells. [^{18}F]FDG PET-CT has been found to be useful in lesion detection and characterization, evaluation of tumor stage, assessment of treatment response and detection of recurrent disease [2,3]. However, the specificity of this technique is low in patients with active infections and inflammatory diseases (because of high FDG uptake in macrophages) and in the brain (because of high background FDG uptake) [4,5]. Therefore, the development of post-FDG radiopharmaceuticals is needed.

After glucose transport, amino acid transport is another important pathway in cellular energy metabolism. Therefore, natural or artificial amino acid analogs have been widely studied clinically as potential

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post-FDG radiopharmaceuticals for PET imaging; one of the most important radiolabeled amino acids in this regard is [*S*-methyl-^{11}C]-L-methionine ([^{11}C]MET) [6,7]. Since both amino acid transport and protein synthesis rates are enhanced in tumors, [^{11}C]MET has been as widely used in brain tumor imaging as *O*-(2-[^{18}F]-fluoroethyl)-L-tyrosine ([^{18}F]FET) [8,9].

Numerous amino acid transporters have been identified at the molecular level and have been characterized in mammalian cells [10–12]. The main transport systems for the uptake of neutral amino acids are the A, L, and alanine-serine-cysteine (ASC) amino acid transport systems. System L amino acid transporters are Na^+ independent, and are the main transport mechanism for methionine, tyrosine, phenylalanine, and their analogs such as FAMT (3-fluoro-α-methyl-tyrosine) [13–16]. The system A and ASC amino acid transporters are Na^+-dependent, however, compared to the system L transporters, their involvement in the transport by radiolabeled amino acids in nuclear medicine has not been studied in detail.

The artificial amino acid radiopharmaceutical α-[*N*-methyl-^{11}C]-methylaminoisobutyric acid ([^{11}C]MeAIB) is a promising specific substrate of system A amino acid transport. Compared with [^{11}C]MET, [^{11}C]MeAIB is metabolically stable [17] and it has been studied both pre-clinically and clinically. For example, [^{11}C]MeAIB has been shown to be useful in the measurement of amino acid uptake into skeletal muscle and in the diagnosis of malignant lymphoma and head and neck cancers [18–20]. In our institute, [^{11}C]MeAIB PET has proven useful in the diagnosis of chest diseases, especially in the differential diagnosis between sarcoidosis and metastasis [21].

Previous studies in carcinoma cells have shown that there is a high correlation between both MET and FAMT uptake and the gene expression levels of system L amino acid transporters [15,16,22]. However, the relationship between the accumulation of radiolabeled amino acids and the gene expression levels of system A amino acid transporters has not been examined.

In this study, we explored the amino acid transport systems in four different human carcinoma cell lines using α-[1-^{14}C]-methylaminoisobutyric acid ([^{14}C]MeAIB) as a substrate and comparing it to [*S*-methyl-^{3}H]-L-methionine ([^{3}H]MET) and [methyl-^{3}H]-3'-deoxy-3'-fluorothymidine ([^{3}H]FLT). We elected to use ^{14}C or ^{3}H–labeled amino acid analogs because both [^{11}C]MeAIB and [^{11}C]MET have a very short half-life (20 min). We also investigated the gene expression profiles of numerous amino acid transporters in these four types of human carcinoma cell lines using microarray analysis. Following on from this initial screen we used quantitative reverse transcription polymerase chain reaction (qRT-PCR) to characterize the expression of the mRNAs encoding SNAT1, SNAT2, SNAT4, ASCT1, ASCT2, y^+LAT1, y^+LAT2, LAT1, LAT2, LAT3, LAT4, and 4F2hc. We then examined the correlation between accumulation of [^{3}H]MET and the quantitative mRNA expression of total system L transporter (total LAT = LAT1 + LAT2 + LAT3 + LAT4) and total system A amino acid transporters (total SNAT = SNAT1 + SNAT2 + SNAT4) in these human carcinoma cell lines. Finally, we examined the correlation between accumulation of [^{3}H]FLT uptake and [^{14}C]MeAIB uptake as well as the correlation between [^{3}H]FLT uptake and the quantitative gene expression of total SNAT transporters.

2. Materials and methods

2.1. Radiolabeled amino acid analogs and amino acid transport inhibitors

Because of the short half-life of ^{11}C (20 min), radiolabeled amino acid analogs with much longer half-lives were used instead. ^{14}C–labeled MeAIB ([^{14}C]MeAIB, 37 kBq/ml), ^{3}H–labeled MET ([^{3}H]MET, 18.5 kBq/ml) and ^{3}H–labeled FLT ([^{3}H]FLT, 18.5 kBq/ml) were obtained from American Radiolabeled Chemicals Inc. (St Louis, Missouri, USA). MeAIB (α-methylaminoisobutyric acid), a specific inhibitor of system A, and BCH (2-amino-2-norbornane-carboxylic acid), a specific inhibitor of system L, were acquired from Sigma-Aldrich Japan KK (Tokyo, Japan).

2.2. Cell culture

Cell line studies were performed using a modification of the methods described by Shikano et al. and Nakajima et al. [23,24], as follows. A431 and LS180 cell lines were purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). The cultured human tumor cell lines H441 and PC14 were obtained from the University of Texas, MD Anderson Cancer Center, Houston, TX, USA. All cells were cultured in 150 mm cell culture dishes (Becton Dickinson, New Jersey, USA)in a 5% CO_2 humidified atmosphere at 37 °C with specific media as follows; A431 cells were maintained in Dulbecco's modified Eagle"'s medium (DMEM; Sigma-Aldrich, Japan) containing high glucose supplemented with 10% fetal bovine serum (FBS) and 3.7 g/L $NaHCO_3$, H441 cells were maintained in RPMI-1640 medium (Sigma-Aldrich, Japan) supplemented with 10% FBS, 1% sodium pyruvate (Sigma-Aldrich, Japan) and 2.0 g/L $NaHCO_3$, PC14 cells were maintained in DMEM/Nutrient Mixture F-12 Ham (DMEM/F12; Sigma-Aldrich, Japan) supplemented with 10% FBS and 1.2 g/L $NaHCO_3$, and LS180 cells were maintained in minimum essential medium Eagle (MEM; Sigma-Aldrich, Japan) supplemented with 10% FBS, 1% sodium pyruvate and 2.2 g/L $NaHCO_3$. Sub-culturing was performed every five days using 0.02% EDTA and 0.05% trypsin. For amino acid uptake experiments, cells were seeded into a 24-well Multiwell Plate (Becton Dickinson, New Jersey, USA) at a density of 5×10^5 cells/well and were used 24 h after plating.

2.3. Measurement of [^{14}C]MeAIB, [^{3}H]MET and [^{3}H]FLT transport in human carcinoma cells

For transport studies in a sodium-containing medium, phosphate-buffered saline (PBS) pH 7.4 (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM K_2HPO_4, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$) was used. For sodium-free transport studies, the NaCl and Na_2HPO_4 in PBS were replaced with the same concentrations of choline-Cl and K_2HPO_4, respectively. After removal of the culture medium, the 24-well Multiwell Plate was washed once with 5 mL of incubation medium for 10 min at 37 °C. The cells were then incubated with 500 μL/well of incubation medium containing [^{14}C]MeAIB (37 kBq/mL) and [^{3}H]MET (18.5 kBq/mL) or [^{3}H]FLT (18.5 kBq/mL) for 10 min at 37 °C. For the experiment involving amino acid transport, inhibitors were added to a final concentration of 1 mM, and the cells were then incubated for 10 min at 37 °C with [^{14}C]MeAIB (37 kBq/mL) and [^{3}H]MET (18.5 kBq/mL) or [^{3}H]FLT (18.5 kBq/mL). After incubation with the radiolabeled amino acid analog, the medium was aspirated and the monolayers were rapidly rinsed twice with 500 μL of ice-cold incubation medium. Cells were solubilized in 500 μL of 0.1 N NaOH, and the radioactivity (either ^{3}H and ^{14}C) of an aliquot (400 μL) was measured by addition of Clear-Sol II (Nacalai Tesque Inc., Kyoto, Japan) and scintillation counting using an LSC-5100 liquid scintillation counter (Hitachi Aloka Medical, Ltd., Tokyo, Japan).

To characterize relative contributions of each type of transport system to overall amino acid analog uptake, we performed inhibition experiments with inhibitors as described above. BCH was used as a system L inhibitor and MeAIB was used as a system A inhibitor. To calculate the relative contributions of amino acid transporter systems, we used the methods reported by Shikano et al. [23] and Nakajima et al. [24]. In brief, uptake of [^{3}H]MET and [^{14}C]MeAIB in the absence of inhibitors was used as the control (100%). System A uptake was calculated as [control uptake in Na^+-PBS] – [uptake in the presence of MeAIB in Na^+-PBS]. Uptake by system ASC and/or other systems (for example IMINO, B^0, G-like, N, and y^+L which cannot be individually assessed yet) was calculated as [uptake in the presence of MeAIB in Na^+-PBS] – [control uptake in Na^+ free-PBS]. System L uptake was calculated as [control uptake in Na^+ free-PBS] – [uptake in the presence of BCH in Na^+ free-PBS].

2.4. DNA microarray and quantitative real-time PCR

DNA microarray and quantitative real-time PCR were performed using the method s of Okudaira et al. [25] and Yoshimoto et al. [26] The quality of RNA preparations was assessed using RIN (RNA Integrity Number) software tool on an Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). The expression levels of neutral amino acid transporters in the four types of human carcinoma cells were analyzed using a DNA microarray (Agilent Technologies). The values are expressed as relative quantities to a universal reference RNA (Stratagene Products Division, Agilent Technologies).

mRNA levels of neutral amino acid transporters were determined by conducting qRT-PCR assays. Amplification and real-time fluorescence detection were performed using a model Mx3005P Real Time QPCR system (Stratagene Products Division, Agilent Technologies). All data were normalized using the geometric mean of β-actin and GAPDH. All reactions were performed in triplicate.

3. Results

3.1. Uptake of [^{14}C]MeAIB, [^{3}H]MET and [^{3}H]FLT transport in human carcinoma cells

As shown in Fig. 1 for all four carcinoma cell lines the uptake of [^{3}H]MET under control conditions (Na^+-PBS) was found to be about 3–5 times higher than the uptake of either [^{14}C]MeAIB. The uptake of [^{14}C]MeAIB relative to [^{3}H]FLT varied between the different cell lines with [^{3}H]FLT showing greater uptake than [^{14}C]MeAIB in A431 and PC14 cells and [^{14}C]MeAIB showing greater uptake than [^{3}H]FLT in H441 and LS180 cells.

3.2. Competitive inhibition studies using [^{3}H]MET and [^{14}C]MeAIB

The majority of [^{3}H]MET transport appeared to occur in a Na^+-independent manner in all four carcinoma cell lines (Fig. 2). The inhibitor BCH caused a large significant inhibition of this Na^+-independent transport indicating the importance of system L. Although, the system A inhibitor MeAIB showed significant inhibition of Na^+-dependent transport in H441, PC14, and LS180 cells, the absolute magnitude of inhibition was very small relative to the magnitude of inhibition seen with BCH. The contribution of system A to MET uptake is therefore small relative to the contribution of system L. By comparing the uptake in MeAIB treated cells in the presence of Na^+ with uptake in control medium lacking Na^+ the relative contribution of system ASC and/or other systems to MET uptake can be calculated. The ASC system was found to have a significant contribution to MET transport in PC14 and LS180 cells but not in A431 or H441 cells, although as for system A, the relative magnitude was less than system L.

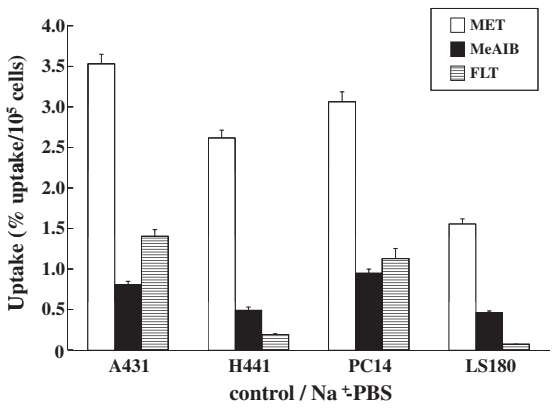

Fig. 1. Uptake of [^{14}C]MeAIB, [^{3}H]MET, and [^{3}H]FLT by four human carcinoma cell lines. Uptake of radiolabeled amino acid analogs in the presence of Na^+ was measured 10 min after addition to four different carcinoma cell lines. Data are expressed as %ID/10^5 cells. Error bars represent S.D. (n = 4). ID injected dose.

In contrast, in all four carcinomas, the majority of [^{14}C]MeAIB uptake was Na^+ dependent (Fig. 3) and as expected there was a large and significant inhibition of this Na^+ dependent uptake of [^{14}C]MeAIB by MeAIB indicating the importance of system A. The system L inhibitor had either no or minimal effect on the remaining Na^+-independent transport.

3.3. Relative contribution of amino acid transport systems for MET and MeAIB in human carcinomas

The relative contributions of amino acid transport systems A, L, and, ASC/other to the uptake of [^{3}H]MET and [^{14}C]MeAIB in the four types of human carcinomas were estimated using the modified methods of Shikano et al. [23] and Nakajima et al. [24] and the results are shown in Table 1. Although the dominant uptake mechanism for [^{3}H]MET in human carcinomas was through system L amino acid transport, system A, ASC, or other Na^+-dependent system(s) also played a role. The dominant uptake mechanism of [^{14}C]MeAIB in human carcinomas was Na^+-dependent and occurred via system A.

3.4. Correlation between [^{3}H]MET and [^{14}C]MeAIB uptake and transporter gene expression or [^{3}H]FLT in human-derived tumor cells

With regard to the contribution of system L amino acid transport to the Na^+-dependent uptake of [^{3}H]MET the A431 and H441 cells had the highest system L contribution (76.3%and 48.9% respectively) (Table 1). When the expression of numerous sodium-dependent and -independent amino acid transporters were analyzed using microarray gene expression profiling, as well as by qRT-PCR, the expression of the system L transporter LAT1 and the coupling factor 4F2hc were found to be abundantly expressed in both A431 and H441 cells compared to the PC14 and LS180 cells (Tables 2 and Table 3). Based on the results of the qRT-PCR analysis, the expression of LAT1 varied across the four different carcinoma cell lines (Table 3). When the [^{3}H]MET uptake in the presence of Na^+ for all four carcinomas was plotted against total LAT (system L) expression no correlation was found (Fig. 4A). On the other hand, there was a significant correlation between accumulation of [^{14}C]MeAIB and total SNAT (system A) gene expression in the carcinoma cells (Fig. 4B). Moreover, there are significant correlations between accumulation of [^{3}H]FLT and accumulation of [^{14}C]MeAIB as well as between [^{3}H]FLT accumulation and gene expression level of the system A amino acid transporter SNAT in the carcinoma cells (Fig. 5C and D). For [^{3}H]MET, the R^2 value between accumulation of [^{3}H]FLT and accumulation of [^{3}H]MET was lower compared with [^{14}C]MeAIB (Fig. 5A and C). There was no correlation between accumulation of [^{3}H]FLT and gene expression of the total system L amino acid transporter (LAT) expression (Fig. 5B).

3.5. Statistical analysis

Data are presented as means and ± SDs. *P* values were calculated using a two-tailed paired Student *t* test for comparison between two groups. A *P* value less than 0.05 was considered significant.

4. Discussion

The amino acid analog MeAIB is an inhibitor and specific substrate for system A amino acid transport and the main transport mechanism for MeAIB uptake is thought to occur via system A [27–32]. A study in Chinese hamster ovary (CHO) cells showed that more than 90% of MeAIB transport occurred through system A [9,33]. A study in cultured human erythroleukemic (K562) cells using radiolabeled [^{14}C]MeAIB also gave the same result [34]. Given all of data, it is thought that system A is the principal [^{11}C]MeAIB transport pathway in in vivo human PET

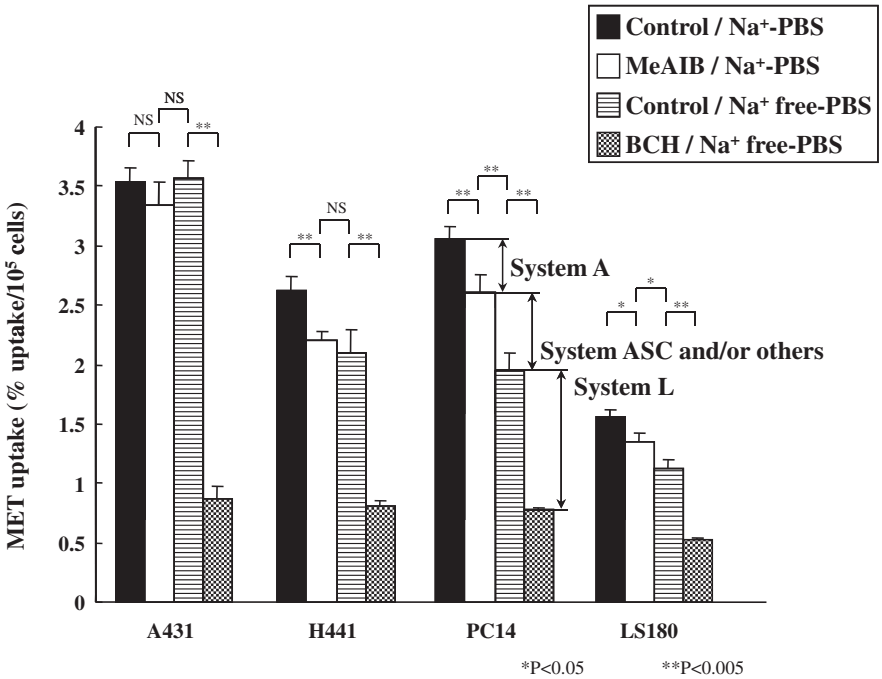

Fig. 2. Effect of Na^+ and selective amino acid transport inhibitors on the uptake of [^{3}H]MET by four human carcinoma cell lines. Uptake of [^{3}H]MET by four different cell lines was measured under the following conditions; (i) in the presence of Na^+ (Na^+-PBS) and either the absence or presence of the transport inhibitor MeAIB at a final concentration of 1 mM (Control/Na^+-PBS and MeAIB/Na^+-PBS respectively) and (ii) in the absence of Na^+ (Na^+-free PBS) and either the presence or absence of the transport inhibitor BCH at a final concentration of 1 mM (Control/Na^+ free-PBS and BCH/Na^+ free-PBS respectively). As shown for the PC14 cell line, system A uptake was calculated as [control uptake of [^{3}H]MET in Na^+-PBS] – [uptake of [^{3}H]MET in the presence of MeAIB in Na^+-PBS]. Uptake by system ASC and/or other systems was calculated as [uptake of [^{3}H]MET in the presence of MeAIB in Na^+-PBS] – [control uptake of [^{3}H]MET in Na^+ free-PBS]. System L uptake was calculated as [control uptake of [^{3}H]MET in Na^+ free-PBS] – [uptake of [^{3}H]MET in the presence of BCH in Na^+ free-PBS]. Data are expressed as %ID/10^5 cells. Error bars represent S.D. ($n = 4$). *$P < 0.05$ and **$P < 0.005$, NS not significant. ID injected dose.

studies [19,20], although no human PET study has been conducted in the presence of amino acid transport inhibitors. In our study using four different types of human carcinoma cells we have shown that system A contributes more than 70% to [^{14}C]MeAIB uptake. In contrast the uptake of [^{3}H]MET was less specific; although system L was the predominant mechanism there were contributions of both system A and

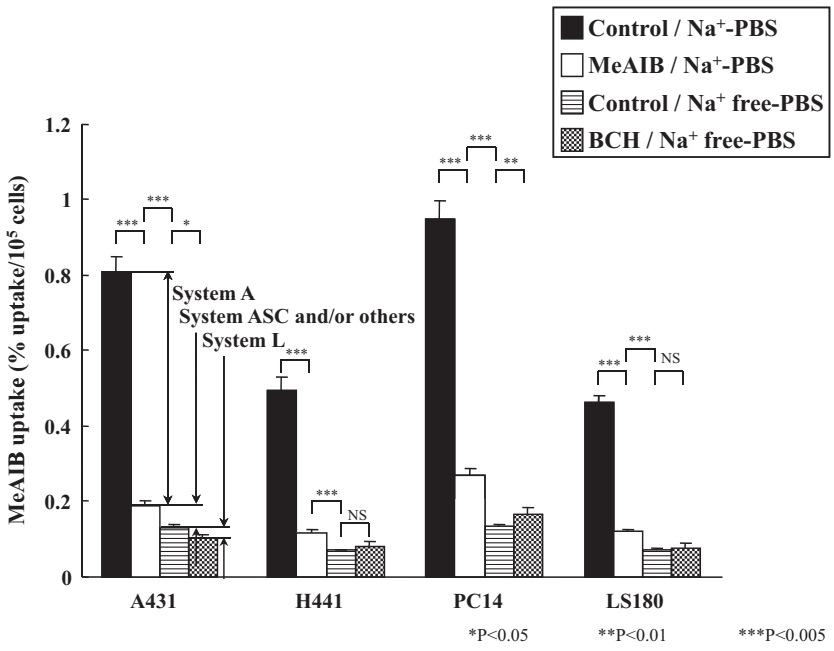

Fig. 3. Effect of Na^+ and selective amino acid transport inhibitors on the uptake of [^{14}C]MeAIB by four human carcinoma cell lines. Uptake of [^{14}C]MeAIB by four different cell lines was measured under the following conditions; (i) in the presence of Na^+ (Na^+-PBS) and either the absence or presence of the transport inhibitor MeAIB at a final concentration of 1 mM (Control/Na^+-PBS and MeAIB/Na^+-PBS respectively) and (ii) in the absence of Na^+ (Na^+-free PBS) and either the presence or absence of the transport inhibitor BCH at a final concentration of 1 mM (Control/Na^+ free-PBS and BCH/Na^+ free-PBS respectively). As shown for the A431 cell line, system A uptake was calculated as [control uptake of [^{14}C]MeAIB in Na^+-PBS] – [uptake of [^{14}C]MeAIB in the presence of MeAIB in Na^+-PBS]. Uptake by system ASC and/or other systems was calculated as [uptake of [^{14}C]MeAIB in the presence of MeAIB in Na^+-PBS] – [control uptake of [^{14}C]MeAIB in Na^+ free-PBS]. System L uptake was calculated as [control uptake of [^{14}C]MeAIB in Na^+ free-PBS] – [uptake of [^{14}C]MeAIB in the presence of BCH in Na^+ free-PBS]. Data are expressed as %ID/10^5 cells. Error bars represent S.D. ($n = 4$). *$P < 0.01$, **$P < 0.05$, ***$P < 0.005$, NS not significant. ID injected dose.

Table 1
Relative functional contributions of different amino acid transport systems in four human carcinoma cells.

	Cells	System A	System ASC and/or others	System L
[^{3}H]MET	A431	–	–	76.3%
	H441	15.8%	–	48.9%
	PC14	15.0%	21.2%	38.5%
	LS180	13.7%	13.7%	38.5%
[^{14}C]MeAIB	A431	76.8%	7.1%	3.6%
	H441	76.1%	9.8%	–
	PC14	71.5%	14.4%	–
	LS180	73.9%	10.5%	–

Percentage of control.

system ASC and the relative contributions of all systems varied between the different cell lines (Table 1). Stability studies have shown that more than 95% of [^{11}C]MeAIB remained unchanged in human plasma 30 min after administration. Since it is an unnatural amino acid, it also cannot be used for protein synthesis [17]. Therefore, [^{11}C]MeAIB imaging may more accurately represent the uptake of amino acid transporter system A compared to [^{11}C]MET. Since [^{11}C]MeAIB has higher selectivity for system A compared to [^{11}C]-2-aminoisobutyric acid ([^{11}C]AIB, another system A substrate) [27,31], [^{11}C]MeAIB is expected to be very useful in system A amino acid transport PET imaging. We have previously reported the utility of [^{11}C]MeAIB in the diagnosis of chest diseases, especially in the differential diagnosis between sarcoidosis and metastasis [21]. [^{11}C]MeAIB has comparable sensitivity to [^{18}F]FDG PET for the diagnosis of prostate cancer [35] and the tumor/non-tumor ratio obtained with [^{11}C]MeAIB precisely distinguishes the malignant group from the benign group in patients with brain tumors [36].

Table 2
Microarray gene expression profiling of sodium-dependent and -independent amino acid transporters in four human carcinoma cell lines.

	System	Transporter	A431	H441	PC14	LS180
Sodium dependent	A	SNAT1	1.233	0.938	0.687	0.383
			1.148	1.128	0.553	1.022
		SNAT2	0.448	0.319	0.835	0.190
			1.022	0.654	1.740	0.382
		SNAT4	0.148	0.298	0.143	0.491
			0.286	0.587	0.268	0.389
		SNAT6	3.860	1.407	2.324	0.252
	GLY	GlyT1	5.004	4.089	1.305	0.938
		GlyT2	0.575	0.693	0.836	0.470
	ASC	ASCT1	1.355	3.487	0.432	1.036
			0.644	1.360	0.174	0.395
		ASCT2	3.536	1.860	0.896	2.097
	N	SNAT3	0.285	0.100	0.254	0.249
		SNAT5	10.941	5.275	0.019	0.037
	β	Taut	0.348	0.157	0.829	0.398
			1.102	0.723	2.296	2.622
	B^0	B^0AT	0.047	0.023	0.023	1.302
			0.080	0.164	0.074	0.850
	y^+L	y^+LAT1	0.167	0.184	36.885	46.218
		y^+LAT2	1.882	0.572	2.559	3.589
			0.618	1.012	1.235	0.694
			0.942	0.931	1.381	1.220
Sodium independent	L	LAT1	2.700	7.329	0.244	1.197
			4.981	11.428	0.166	1.693
		LAT2	0.540	2.500	0.014	1.309
		LAT3	0.193	0.669	0.753	5.427
		LAT4	0.106	0.182	0.057	0.569
	T	TAT1	0.116	3.366	0.332	0.159
	B^0	BAT1	0.166	0.337	0.156	0.227
	asc	asc1	0.065	0.134	0.059	0.086
Coupling factor	L, y^+L, asc, X-C	4F2hc	4.682	9.485	0.637	1.946
			4.646	9.681	0.560	1.939

Table 3
Absolute quantification of sodium-dependent and -independent amino acid.

	System	Transporter	A431	H441	PC14	LS180
Sodium dependent	A	SNAT1	1.341	0.917	0.767	0.915
		SNAT2	0.407	0.306	0.814	0.203
		SNAT4	0.001	0	0	0.004
	ASC	ASCT1	0.110	0.236	0.034	0.083
		ASCT2	1.452	0.659	0.300	0.893
	y^+L	y^+LAT1	0	0	0.066	0.084
		y^+LAT2	0.038	0.016	0.055	0.119
Sodium independent	L	LAT1	2.384	5.688	0.064	0.768
		LAT2	0.040	0.179	–	0.111
		LAT3	0	0.006	0.007	0.051
		LAT4	0.005	0.008	0.002	0.037
System L cofactor	4F2hc	4F2hc	4.337	10.085	0.508	2.171

Copy/10 copies of housekeeping gene.

On the other hand, the main transport mechanism of [^{3}H]MET in tumor cells has been reported to be system L [21,35], which is consistent with our study results. The radiolabeled amino acid [^{11}C]MET has the [^{11}C] group incorporated into the methyl group attached to the sulfur. [^{11}C]MET is used at a lower rate in protein synthesis compared with other [^{11}C] labeled amino acids such as [1-^{11}C]-L-methionine which has the label in the carboxyl terminal. Therefore, [^{11}C]MET tumor uptake represents a combination of amino acid transport and intracellular metabolism [37]. However, since radiosynthesis of [^{11}C]MET is extremely simple and easy, in clinical practice, [^{11}C]MET-PET, together with [^{18}F]FET, has been widely used in brain tumor imaging [8,9].

We investigated the correlation between accumulation of [^{3}H]MET and [^{14}C]MeAIB in four types of human carcinoma cells and gene expression, which led to the conclusion that there was no correlation between [^{3}H]MET uptake and LAT expression (Fig. 4A). A weak relationship between [^{3}H]MET uptake and total LAT expression has previously been reported [22,26]. The reasons for this lack of correlation are not completely clear but could perhaps be related to the fact that LAT1 biology is complex and involves a number of other proteins. One such protein is the amino acid transporter activating factor 4F2hc with which LAT1 forms disulfide-linked heterodimers thereby becoming active. A second protein is ASCT2. LAT1 transports neutral amino acid with long side chains, such as leucine, and simultaneously counter transports intracellular glutamine [38,39]. The intracellular glutamine supply is maintained via the intracellular Na^+-dependent amino acid transporter ASCT2 that helps facilitate LAT1 function. The interplay between LAT1, ASCT2, and 4F2hc is thought to be important in controlling cancer cell metabolism [40]. For example, co-expression of LAT1 with ASCT2 has been shown in lung cancer, and this co-expression, but not the sole expression of LAT1 or ASCT2, is strongly related to prognosis [41].

In our study, [^{14}C]MeAIB accumulation in four types of human carcinoma cells was correlated with total SNAT expression (Fig. 4B). Since SNAT does not require an activating factor, there is a high possibility of a consistent relationship between SNAT expression and cellular uptake. System L, which transports amino acids into cells, has a counter transport mechanism associated with it. In contrast, system A, which also transports amino acids into cells, has no counter transport activity. Amino acid transporter function can therefore be evaluated directly using MeAIB (system A substrate), allowing differentiation from system L [42,43]. The correlation between the uptake of radiotracers and the gene expression of amino acid transporter in tumor cells has been evaluated in a number of recent nuclear medicine studies. A correlation between 4-borono-2-^{18}F-fluoro-phenylalanine ([^{18}F]FBPA) and L-3-[^{18}F]fluoro-α-methyl tyrosine ([^{18}F]FAMT) uptake and LAT1 expression [15,16,26], a correlation between [*S*-methyl-^{3}H]-D-MET uptake and LAT + ASCT expression [22], and a correlation between *Trans*-1-amino-3-[^{18}F]fluorocyclobutanecarboxylic acid ([^{18}F]FACBC) uptake and ASCT expression [25,44] have all been recently reported. However, it should be borne in mind that gene expression levels may not directly correlate with protein levels. In addition, cell-surface localization and

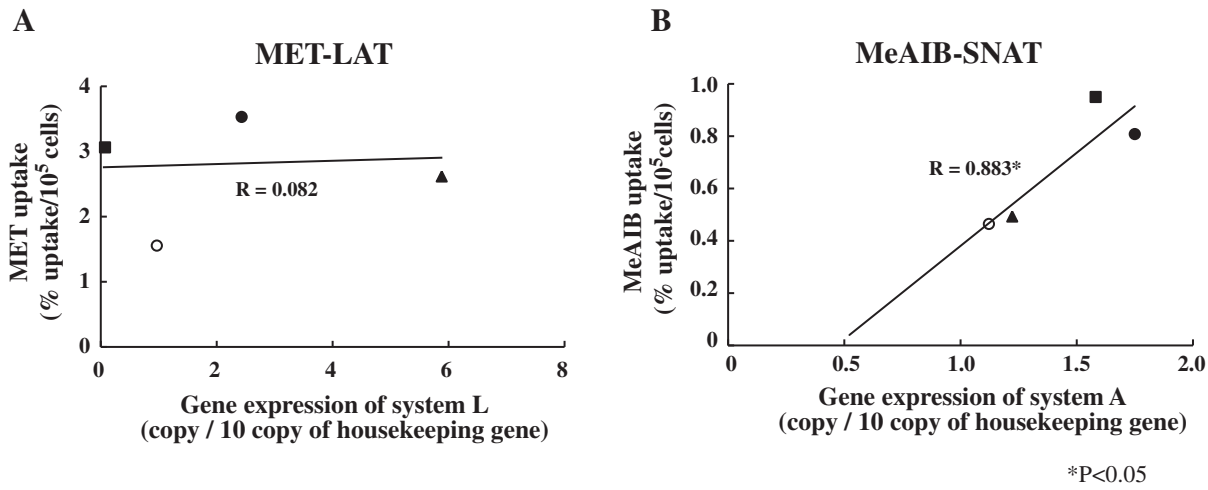

Fig. 4. Correlation between gene expression levels of system A and system L amino acid transporters and uptake of [^{3}H]MET and [^{14}C]MeAIB in human carcinomas. The figures show the relationship between [^{3}H]MET uptake and the gene expression levels of total amino acid transporter system L (total LAT) (A) and between [^{14}C]MeAIB uptake and gene expression levels of total amino acid transporter system A (total SNAT) (B). A431 (●), H441 (▲), PC14 (■) and LS180 (○). Tumor cell uptake of [^{14}C]MeAIB showed a high correlation with the levels of gene expression of amino acid transport system A. *P < 0.05.

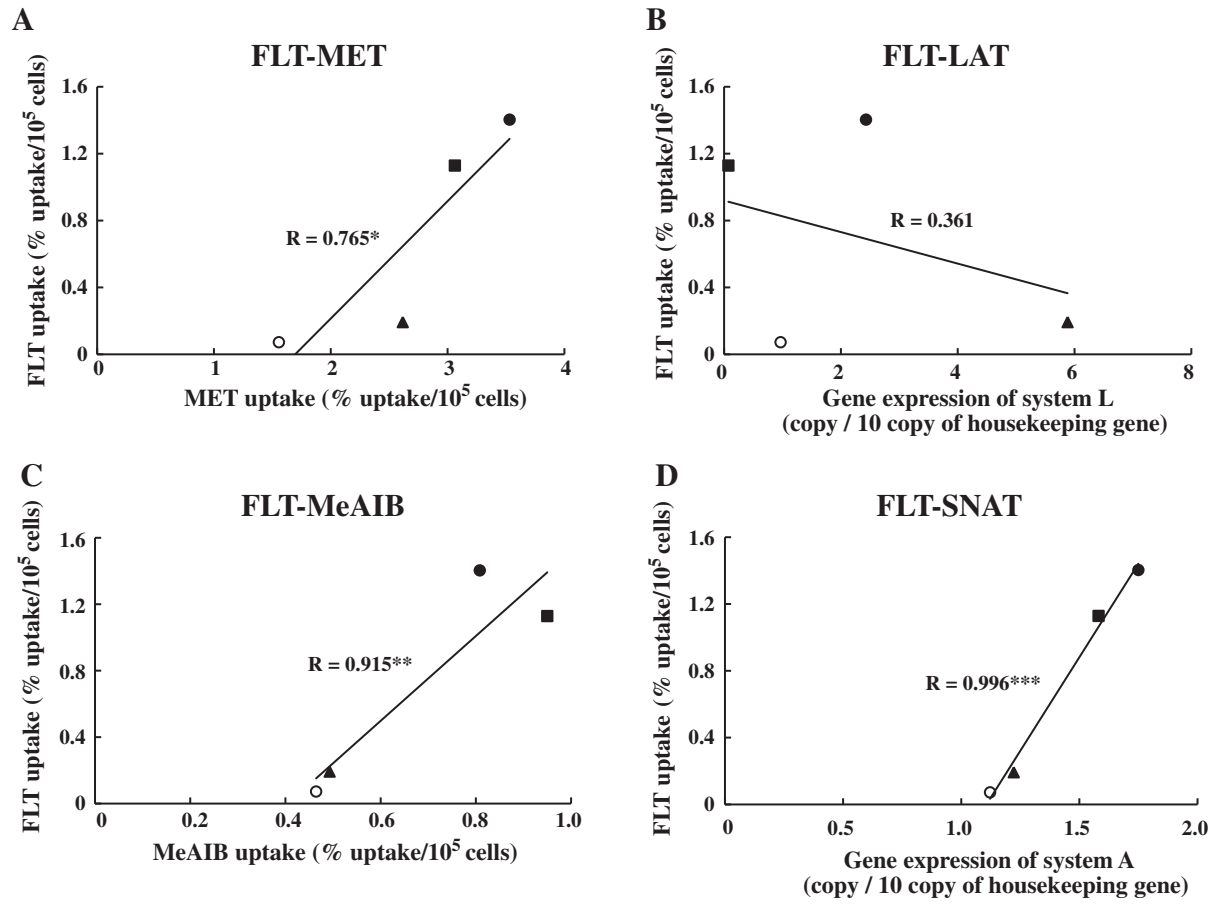

Fig. 5. Correlation of [^{3}H]FLT uptake with either [^{3}H]MET uptake, the gene expression levels of amino acid transporter system L, either [^{14}C]MeAIB uptake or the gene expression levels of amino acid transporter system A in human carcinomas. The figures show the relationship between [^{3}H]FLT uptake and [^{3}H]MET uptake (A), between [^{3}H]FLT uptake and the gene expression levels of total amino acid transporter system L (total LAT) (B), between [^{3}H]FLT uptake and [^{14}C]MeAIB uptake (C) and between [^{3}H]FLT uptake and the gene expression levels of total amino acid transporter system A (total SNAT) (D). A431 (●), H441 (▲), PC14 (■) and LS180 (○). Tumor cell uptake of [^{3}H]FLT showed a high correlation with [^{14}C]MeAIB uptake and with the total gene expression levels of amino acid transport system A (total SNAT) and no correlation with the total gene expression levels of amino acid transport system L (total LAT). *P < 0.05, **P < 0.025, ***P < 0.001.

activity of amino acid transporter proteins may not necessarily be predicted by gene expression levels.

Our data also showed that [^{3}H]FLT accumulation was strongly correlated with SNAT expression and [^{14}C]MeAIB accumulation (Fig. 5C and D). Uptake of 3′-deoxy-3′-[^{18}F]fluorothymidine ([^{18}F]FLT), followed by phosphorylation by Thymidine Kinase 1 (TK1) leads to accumulation of the phosphorylated form of [^{18}F]FLT in cells. The TK1 activity of cells has been reported to be related to cellular proliferative activity, which can be easily evaluated based on [^{18}F]FLT accumulation [45–47]. Based on these data, since [^{3}H]FLT accumulation is correlated with [^{14}C]MeAIB accumulation, it is reasonable to conclude that [^{11}C]MeAIB accumulation is correlated not only with SNAT expression but also with cellular proliferative activity. For [^{3}H]MET, the R^2 value between accumulation of [^{3}H]FLT and accumulation of [^{3}H]MET was lower comparison with [^{14}C]MeAIB (Fig. 5A and C). From the perspective of statistics, the correlation of [^{3}H]FLT uptake needs to be examined with either [^{3}H]MET uptake or [^{14}C]MeAIB uptake in many human carcinoma cells. Studies using Ki-67 in many human carcinoma cells are underway to confirm the uptake results observed correlating [^{14}C]MeAIB and [^{3}H]MET to [^{3}H]FLT uptake.

Glucose metabolism, as well as amino acid metabolism, increases as a requirement to supply an energy source for cell proliferation in cancer cells [48]. Moreover, amino acids and amino acid transporters play important roles other than in energy metabolism, such as in macromolecular synthesis, mTOR activation, and ROS homeostasis beyond energy metabolism [49]. There are approximately fifty different types of amino acid transporters, but only LAT1 [50], LAT3 [51], ASCT2 [52], $ATB^{0,+}$ [53] and xCT [54] have been reported to be expressed at high levels on the surface of cancer cells. Recently, there have been numerous reports in cancer cells related to glutamine transport via ASCT2 and LAT1 [55–57]. Moreover, the expression levels of the SNAT amino acid transporter, which belongs to the SLC38 family and like ASCT2 and LAT1 is related to glutamine transport, have been examined in a range of different cancers. Overexpression of SNAT was observed in gastric cancer [58], human hepatocellular carcinoma [59], breast cancer [60,61], hilar cholangiocarcinoma [62], C6 glioma [63], prostate cancer [25], HeLa epithelial cervical cancer cells, and 143B osteosarcoma cells [64]; and SNAT expression in stomach and breast cancers is also related to Ki-67 [61] and PCNA (proliferating cell nuclear antigen) expression [58] as indicated by proliferative activity.

Reportedly, the accumulation of [^{11}C]AIB, an unnatural amino acid and amino acid transporter system A substrate, accumulation in cancer cells post-radiation therapy is correlated with changes in SLC38A1 expression [65]. Data from the Oncomine analysis (a gene expression database of 947 human cancer cell lines, http://www.broadinstitute.org/ccle) revealed that ASCT2, SNAT1 and SNAT2 were overexpressed, which could lead to a new treatment for preventing proliferation of cancer cells [64]. The studies reported here highlight the potential importance of SNAT in cancer. As described earlier, there have been an increasing number of studies highlighting the role of SNAT in cancer cells. Since [^{11}C]MeAIB accumulation is correlated with SNAT expression and SNAT expression is correlated with [^{18}F]FLT accumulation in cancer cells, this suggests that there is a relationship with tumor proliferative activity.

Our results along with previous investigations support the concept that SNAT mediates the uptake of [^{11}C]MeAIB by tumor cells. We expect that accurate PCR studies examining the relationship between amino acid transporter gene expression and amino acid transporter activity in human carcinoma cells will be performed in the future.

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Appendix A. Supplementary data

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