Analysis of L-type amino acid transporter in canine hepatocellular carcinoma

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(Received 1 August 2014/Accepted 23 December 2014/Published online in J-STAGE 29 January 2015)

ABSTRACT. Analysis of L-type amino acid transport expression of hepatocellular carcinoma cells (HCCs) of the dog was performed. The leucine transport activity of canine HCCs was 0.628 ± 0.018 nmol/mg protein/min. The inhibitor of LAT 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH) reduced 90% of the activity at 1 mM. The deduced amino acid sequences of canine LAT2, LAT3 and LAT4 were well conserved in mammalians, exhibiting 89, 88 and 77% homology, respectively. RT-PCR revealed distinct LAT1 expression compared with normal hepatocytes. Western blotting analysis confirmed the potent LAT1 expression in canine HCCs but not hepatocytes, and real-time RT-PCR analysis indicated that canine HCCs possessed 28 times higher LAT1 expression than hepatocytes. These results indicated that the leucine transport activity of canine HCCs was due to LAT1.

KEY WORDS: canine, hepatoma, LAT, transporter

doi: 10.1292/jvms.14-0392; J. Vet. Med. Sci. 77(5): 527-534, 2015

Many amino acid transport systems have been distinguished based on differences in their substrate-selectivity, ion-dependence, pH sensitivity, kinetics and regulatory properties by using membrane vesicle preparations or cultured cells [5, 6]. Among them, system L is an amino acid transport system that mediates sodium-independent transport of neutral amino acids. It was first described in Ehrlich ascites tumor cells as a sodium-independent transport system for neutral amino acids that is specifically inhibited by a bicyclic amino acid, 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH) [28, 29, 37]. Later, 2 subtypes for system L with distinct characteristics in substrate affinity, selectivity, and transport properties were reported and named systems L1 and L2 [37]. To date, 4 isoforms of system L have been identified at the molecular level. The first cloned transporters with system L activity were LAT1 and LAT2, which are members of the SLC (solute carrier) 7 family of transporters [17, 21, 34, 36]. They mediate sodium-independent amino acid exchange and recognize a wide range of large neutral amino acids as substrates [35], expanding to small neutral amino acids in the case of LAT2 [30, 31, 33]. These proteins form heteromeric complexes via a disulfide bond with the heavy chain of 4F2 antigen (4F2hc, SLC3A2), a single transmembrane domain protein essential for the functional expression of LAT1 and LAT2.

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Later, other transporters were identified by expression cloning [1, 3], and it was reported that they exhibited Naindependent, BCH-sensitive neutral amino acid transport activity. In spite of possessing the properties of system L, these transporters (LAT3 and LAT4) were structurally different from system L1 (LAT1 and LAT2), and additionally, they did not require co-expression with 4F2hc to elicit transport activity at the plasma membrane. LAT3 and LAT4 transport activities show specific properties: (i) preferential substrate specificity [6], (ii) complex kinetics [6], which are compatible with 2 simultaneous apparent affinities and (iii) inactivation by the thiol reagent N-ethylmaleimide [25]. Northern blot analysis indicated the tissue distribution of system L2 was different between LAT3 and LAT4. Potent expression of LAT3 was found in the placenta and kidney, and that of LAT4 was found in the pancreas and skeletal muscle [1, 3]. Previously, we reported the cDNA sequence and distribution of canine LAT1 in the healthy dog and in several tumor cells [27]. The accumulating data indicated the close relationship between tumor malignancy and LAT1 expressions in human tumor tissues [15, 16]. Notably, the expression of LAT1 has been described to be a significant factor indicating a poor outcome in various human cancers [2, 12, 14, 22, 32]. Hepatocellular carcinoma is the most common primary hepatic tumor in dogs and arises from the uncontrolled proliferation of hepatocytes. Viral infections have been associated with hepatocellular carcinoma in humans, but no causal link with hepatocellular carcinoma in dogs has yet been established. While Fukumoto et al. reported the LAT1 expression in the canine mammary gland tumor and melanoma in the veterinary clinic [9–11], little information is available concerning LAT expression in hepatocellular carcinoma cells (HCCs) of dog. In this study, we examined functional analysis of LAT

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r	Sequence (5'-3')	Product (bp)		
Oligonucleotides for RT-PCR				
Sense	TGTACGGCTCGCTGCCCGCCTTCCT	468		
Antisense	GATGGCCAGGGGCAGGTTTCTGTAGGG			
Sense	GGTGGGCCACCCGGGTTCAAGACATTT	609		
Antisense	TGTCGCTGGTGACCAGCATCAGCAGGG			
Sense	GGTCGGGACTGGCCTGTCTCATCTTTC	624		
Antisense	AGCGTGGTCTGGCAGACTTGGTGGCCG			
Sense	GCCATCACCCTGCCTCTAGGCATCGTC	578		
Antisense	TTATGGCCCTCCTGCAGTGCCGCTTGC			
Sense	ATC ACC ATC TTC CAG GAG CGA GA	192		
Antisense	GTC TTC TGG GTG GCA GTG ATG G			
al-time RT-PCR				
sense	CCTGGTGTACGTGCTGACGAA	106		
Antisense	TCCCAGGTGATAGGTCCCAAAG			
sense	CCTTCCTCAAAAAGTCTGGG	95		
Antisense	CTTCTCATCGTAGGGACGAAG			
	r T-PCR Sense Antisense Sense Antisense Sense Antisense Sense Antisense Sense Antisense sense Antisense sense Antisense sense Antisense sense Antisense Sense Antisense	r Sequence (5'-3') T-PCR Sense TGTACGGCTCGCTGCCGGCCTTCCT Antisense GATGGCCAGGGGCAGGTTTCTGTAGGG Sense GGTGGGCCACCCGGGTTCAAGACATTT Antisense TGTCGCTGGTGACCAGCATCAGCAGGG Sense GGTCGGGACTGGCCTGTCTCATCTTTC Antisense AGCGTGGTCTGGCAGACTTGGTGGCCG Sense GCCATCACCCTGCCTCTAGGCATCGTC Antisense TTATGGCCCTCCTGCAGTGCCGCTTGC Sense ATC ACC ATC TTC CAG GAG CGA GA Antisense GTC TTC TGG GTG GCA GTG ATG G ial-time RT-PCR sense CCTGGTGTACGTGCTGACGAA Antisense TCCCAGGTGATAGGTCCCAAAG sense CCTTCCTCAAAAAGTCTGGG Antisense CTTCCTCATCGTAGGACGAAG		

 Table 1.
 Sequences of oligonucleotides used in this study

Accession numbers are indicated in parentheses.



Fig. 1. Histologic section from the specimen from the liver of the dog. HE stain (A, B). Hepatocellular carcinoma (*) and cholangiocellular carcinoma (arrow) (A). In part, hepatic cells with a normal pattern were observed (arrow). The tumor cells contained various-sized cord-like structures and conspicuous nucleoli (*). Bar=20 μm. (B). Histologic section of the nude mouse with a transplanted tumor. HE stain (C, D). Bar=100 μm (C). The cells of 2 nuclei were observed in various-sized and inequality (arrow). Bar=20 μm (D).

activity, determined the molecular structure of canine LAT3 and LAT4 and investigated the expression of the 4 isotypes of LATs by RT-PCR and Western blot analysis.

MATERIALS AND METHODS

Animal and cells samples: All experiments were performed according to the guidelines of the Laboratory Animal Care Committee of Azabu University, and were in compliance with the Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions. A tumor was removed from an 11-year female Yorkshire Terrier with hepatocellular carcinoma and diagnosed histologically. Tissues samples were fixed in 20% buffered formalin, embedded in paraffin, sectioned at 4 μ m and stained with hematoxylin and eosin (HE). A cell line was established after transplantation to a nude mouse, cultured through >100 passages and maintained in Eagle's MEM (Gibco, Carlsbad, CA, U.S.A.) supplemented with 10% fetal bovine serum, 6.6 mg/l spectinomycin and 2 mM glutamine in a 60 mm dish (BioCoat Collagen I 60 mm Dish, Corning, Tokyo, Japan) 37°C in a 5% CO₂ humidified incubator. Tissue samples were also obtained from a healthy 5-year-old male Shiba dog.

Determination of the cDNA sequence of canine LATs: Determination of the cDNA sequence was carried out as described previously [26]. Total RNA was isolated from tissues of the healthy dog using an RNA extraction solution (Isogen. Nippon Gene, Tokyo, Japan). The primers used in this study were prepared according to the conserved sequence region between the human and mouse. The DDBJ accession numbers of the human and mouse LATs used in this study were as follows: human LAT2, AF171669; mouse LAT2, AF171668; human LAT3, AB103033; mouse LAT3, AB103034; human LAT4, AB120364; mouse LAT4, AB120363). RT-PCR amplification was performed by employing a SuperScript III first-Strand Synthesis System kit (Invitrogen, Carlsbad, CA, U.S.A.) with Hot start Ex Taq DNA polymerase (Takara Bio, Kyoto, Japan). The band was excised from the agarose gel and purified using a Wizard SV gel clean-up system (Promega, Tokyo, Japan). The extracted and purified DNA was



Fig. 2. Leucine transport activity of canine HCCs in the presence or absence of 1 mM BCH. The values represent the means and SD of 4 individual experiments.

cloned into a pCR II-TOPO cloning vector (Invitrogen) and sequenced with a BigDye Terminator kit ver.3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, U.S.A.). The nucleotide sequence obtained exhibited high similarities (>85%) to human LATs cDNA sequences. In order to determine the 3' and 5' regions of cDNA, RACE methods were carried out using a SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA, U.S.A.) and a set of canine LATs sequences obtained.

dLAT2 aa	1 : MEKGARHRNNAEKNHPGGSE LEASQETGSTGGOVALKKEI GLVSACGI I VON I IGSGI FVSPKOVLENAGSVG LALI VWI VTGLI TAVGALCYAELGVTI	100
hLAT2 aa	1 : MEEGARHRNN TEKKHPGGGESDASPEAGSGGGVALKKEI GLVSACG I IVGN I IGSGI FVS PKOVLENAGSVG LAL IVW IVTGFI TVVGALCYAELGVTI	100
mLAT2 aa	1 : MEKGARORINITAKIN HP - GSDTS PEAE AS SGGGOVALKKE I GIVSACG I IVON I IGSGI FVS PKOVLENAGSVG LALIVI IVTGI I TAVGALCYAE LGVTI	99
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di 172	ו בינו אים בידי התאמד בשמערצ בישאמוריון זים בביו מים מסמי מידי קימו ואיז איז מידי או איז איז מיני ביב בינו ביב ב	20.0
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LLAI2_da		200
mLAT2_aa	100 : PRSGGDY S YVRDI F GG LAGF LR LWI AVLVI Y PTNQAVI ALT FSNYVLQP LFPT CFP PPESGLR LLAAI CLLL LTWANCSSVRWATRVQD I FTAGRL LA LAL	199

dLAT2_aa	201:11 IMGVVQ ICKGEYFWLEPKNAFENFQEPDIGLIALAFLQGSFAYGGWNFLNYVTEELVDPYKNLPRAIFISIPLVTFVYVFANVAYVTAMSPQELLASN	300
hLAT2_aa	201 : I I IMG IVQ ICKGEYFW LEPKNAFENFQEPDIG IVA LAFLQGSFAYGGWNFLNYVTEELVDPYKN LPRAIFISIP IVTFVYVFANVAYVTAMSPQELLASM	300
mLAT2 aa	200 : I I IMG IVQ ICKGEFFW LEPKNAFENFQEPDI GIVA LAF LQGSFAYGGWNFLNYVTEELVDPYKN LPRA IF I SI PIVTEVYVFANI AYVTAMS PQELLASM	299
-	***** ******* *************************	
dLAT2 aa	301 : AVXVT FGEKLLGVMAW I MP I SVALST FGGVNGSLFTS SRLFFAGAREGHLPSVLAM I HVKRCTP I PALLFTCI STLLMLVTSDMYTLI NYVGFI NYLFYG	400
hLAT2 aa	301 : AVAVT FGEKLLOVMAN I MP I SVALST FGOVINGSLFTS SRLFFAGAREGHLPSVLAM I HVKRCTP I PALLFTCI STLLMLVTSDMYTLI NYVGFI NYLFYG	400
mLAT2 an	300 - AVANTEGERILIANMANINDISVAISTEGANGSLETSSELEFA GARECHLESVIAMINNEDCTEIDALIETCISTIIMINTSDMYTLINNAGENVLEVG	30.0
u		222
413 72		500
ALAIZ_AA	TUL VI ROUIVERWERPDI PE I ELEVITI PI I TELEVELLI I SUSEVOCO IGLAI NEI GOVVILLOVINGE PE I LOSQUECANO PENDA	500
RLAIZ_aa	401: VIVAGUIVERWARPDIPEPIALALLEPIITELEWARLEVESEWSEVVCGIGLAIALIGVEVILGVIMURAPACESDEIELEIDSORACVVVIPEVER	500
mLAT2_aa	400 : VTVAGQI VLRWKKPDI PRPI KVS LLFPI I YLLFWAFLLIFS LWSEPVVCG IGLAIMLTGVPVYFLGVYWQHKPKCFNDFI KS LTLVSQKMCVVVYPQ-EG	498
	.**********************************	
dLAT2_aa	501 : GSRTEETNED TEEQRQP IYH PTASKDQE QPQP	532
hLAT2_aa	501 : GS GT E EANEDMEE QQQ PMY Q PT P TKD KD VAGQ PQ P	535
mLAT2 aa	499: NSGAEETTDD LEEQHKP IF KPT PVKD PD SEE QP	531
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Fig. 3. Amino acid sequences of canine LAT4 were compared with those of the human and mouse. Multiple sequence alignments were performed using the GENETYX program (ver. 10). Asterisks and dots indicate identical residues and conservative substitutions, respectively. The cDNA sequences data of canine LAT2 were deposited in the DNA Data Bank of Japan (accession numbers AB923978).

dLAT3	1: MAPT LOOAYRRRWMACTAVLENLF FSAVL LOWGSLL IMLKEEGFYSSLCPA-NTTNATRDEORKWLSCDROEEMLNLGFT I GSFVLSATT LPLGI LMDR	99
hLAT3	1: MAPT LOOAY RRRWMACTAVLENLF FSAVL LGWGSLL II LKNEGFYSST CPAESS TNT TO DEORRW PGC DOO DEMLNLGFT I GSFVL SATT LP LGI LMDR	100
mLAT3	1 · MAPTIKOAYR REWINACTAWENT FESAVLICINASLI INLEKE GEYSSLCPAENE TO TO DECHOW TSC DOOD KMINIGET I GSFLLSATTIPLGILMDR	100
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dLAT3	100: FGPR FTR LVGSAS FAASCT LMALAS RDT RVLSPLIF LALS LNG FGG ICLT FSSLT LPNMF GNLRST FMALMI GSYASSA IT FPG I KLIYDAGVS FVVIMF	199
hLAT3	101: FGPR FVR IVGSACFTASCT LMALAS ROVEALSPLIFLALS LNGFGG ICLTFTSLT LPNMF GNLRST LMALMI GSYASSA ITFPG I KLIYDAGVAFVVINF	200
mLAT3	101: FGPR PLR INGSACFAASCT LMA LAS RDTEVLSPL IF LALS LNGFAG IC LTFTSLT LPNNFGNLRSTFNALMIGSYASSA ITFPG I KLIYDAGVPFTVINF	200
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dLAT3	200: TWSGLACLI FLNCALNWPSEAF PAPEEVNYTKK I KLSGLALDH KVTGDR FYTHVT IVGOR LSOKAPSLEEGADVF I SSODVRGASGS PPEKSI PFROSLC	299
hLAT3	201: TWSGLACLI FLNCTLNWP I EAF PAPEEVNYTKK I KLSGLALDH KVTGDLFYTHVTTMGORLSOKAPS LEDGSDAFMSPODVRGTSEN LPERSVPLRKSLC	300
mLAT3	201: TWSGLACLI FLNCALNWPAEAF PAPEEVDYTKK I KLIGLALDHKVTGDRFYTHVT IVGORLSOKSPSLEEGADAF I SSPDI PGTSEETPEKSVPFRKSLC	300

dLAT3	300: SPIFLWSLLTMGMTQLEVIFYMAAMNKMLEYLVTGGQEHETDILKQAAETVGFYSSIFGAMQLLCLLTCPLIGYIMDWRIKDCVDAPSEGTALG	394
hLAT3	301: SPTF LWS LLTMGMTQ LRI I FYMAAVNKMLEY LVTGGQEHETNEQQQXVAETVGFYSSVFGAMQ LLC LLTCPL I GY I MDWRI KDCVDA PTQGTVLG	395
mLAT3	301: SPIFLWS LVTMGMTQ LRVI FYMGAMNKI LEF IVTGGKERE TNEQRQ RVEETVEFYSS I FGVMQ LLC LLT CPL I GY I MDWRI KDCVDAPTEGTLNENASFG	400
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dLAT3	395: DAREGAATKSAR PRYRKIQKLTNA I NAFTLTNEL LVGFG I TCL INS LALQEVTEVLATMVRGEYHSACGS LYARVE PSNHEGTLTGLQSL I SAVEAL LQQ	494
hLAT3	396: DARDGVATKS IR PRYCKI QKLTNA I SAFTLTNLLIVGFG I TCLINNLHLQFVTFVLHT IVRGFFHSACGS LYAAVFPSNHFGTLTGLQSLI SAVFALLQQ	495
mLAT3	401: DARDGASTKFTR PRYRKVQKLTNA I NAFTLTNI LUVGFG I ACLIKNLALQLLAFVLHTIVRGFFHSACGGLYARVFPSNHFGTLTGLQSLISAVFALLQQ	500
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dLAT3	495: PLFMANVGPLKGEPFWVNLGLLLFSLLGFLLPSYLFYYRARLQREYVAYWEGPKKVLGSS	558
hLAT3	496: PLFMANVGPLKGEPFWVNLGLLLFSLLGFLLPSYLFYYRARLQQEYAANGMGPLKVLSGSEVTA	559
mLAT3	501: LLEMANVGPLNGDPFWVNLGLLLLSFLGFLLPSYLYYYRSRLQREYATNLVDPQKVLNTSKVATTPEAKRLGGQAVKA	578

Fig. 4. Amino acid sequences of canine LAT3 were compared with those of the human and mouse. Multiple sequence alignments were performed using the GENETYX program (ver. 10). Asterisks and dots indicate identical residues and conservative substitutions, respectively. The cDNA sequences data of canine LAT3 were deposited in the DNA Data Bank of Japan (accession numbers AB924118).

dLAT4 mLAT4 hLAT4	1: MAPT LATAH RERWIMACTAVLENLLFSAVLLGW GSLLIMLKSE GFYSYLCTE SEMVSN GTVGGTAE PEHEEMSIMN GWLSCKAQDEMLNLAFTVGSFLLS 1: MAPT LATAH RERWIMACTAVLENLLFSAVLLGW GSLLIMLKSE GFYSYLCTK PENVTN STVGGSAE PEPEELS LVN GWLSCKAQDEI LNLAFTVGSFLLS 1: MAPT LATAH RERWIMACTAVLENLLFSAVLLGW GSLLIMLKSE GFYSYLCTE PENVTN GTVGGTAE PGHEEVSWMN GWLSCGAQDEMLNLAFTVGSFLLS **********************************	100 100 100
dLAT4	101: A IT LPLG IVMDKYGPRKLRLLGSACFAVSCLLI AYGASN PNS LSVL IFVSLALNGFGGMCMTFTSLTLPNMFGDLRSTF IALMI GSYASSAVTFPG I KVI	200
mLAT4	101: A IT LPLG I I MDKYGPRKLR LLGSAC FAVSC LLI AYGASN PDS LSVL IF I ALA LNGFGGMCMTFTSLT LPNMFGDLRSTFI ALMI GSYASSAVTFPGI KLI	200
hLAT4	101: A IT LPLG IVMDKYGPRKLR LLGSACFAVSC LLI AYGASKPNALSVLIF I ALALNGFGGMCMTFTSLT LPNNFGDLRSTF I ALMI GSYASSAVTFPGI KLI	200

dLAT4	201: YDF GVS F I I I LIVVWAS CS GLVF LINC FFNWP LEP F PG PEDMDY SVK I KF SWLGFDHKI T GKQFY KQVT TVGRR LSVGSSMRSAKEQAALQEGHKLCLS T I D	300
mLAT4	201: YDAGASFIGI LVVWAGCSGLVFFNCFFNWPLEPFPGSEDMDYSVKIKFSWLGFDHKITGKQFYKQVTTVGRRLSVGSSMRTAKEQAALQEGHKLCLSTVD	300
hLAT4	201: YDAGVSF IVVIVVIAGCSGLVF LINCFFNWP LEPF PGPEDMDYSVK I KESWLGFDHKI TGKOFYKOVT TVGRR LSVGSSMRSAKEOVALOEGHKLCLSTVD	300
	.*.* .*****.***********************	
dLAT4	301: LEVKCKPDAAAAPSPMHSVFSPILLS LVTMCITQLRLIFYMGAMNNILKE LVSGDQDVVGLYTSIFGVLQLLCLLTAPVIGYIMDWRLKECEDAS	396
mLAT4	301: LEVKCOPDAAAAPSFMHSVFSPLLVLSLVTMCVTQLRLI FYMGAMNSI LEFLVRGDQKTVALYTSI FGALQLLCLLTAPVI GYIMDWKLKECEDTS	396
hLAT4	301: LEVKCOPDAAVAPSEMISVESPILLISLVTMOVTOLRLIFYMGAMINILKELVSGDOKTVMATVGLYTSIFGVLOLLCLITAPVIGYIMDWRLKECEDAS	400
	******.********************************	
dLAT4	397: EEPEEKDANGGE-KKKKRDRGIQKVTNAMRAFAFTNLLLVGFGVTCLIPNLPLGILSFILHTIVRGFIHSAVGGLYAAVYPSTQFGSLTGLQSLVSALFA	495
mLAT4	397: EEPEEEEGTQGE-KKQKRDRQIQKVTNAMRAFAFTNVLLVGFGVTCLIPNLPLQIFSFVLHTIVRGFIHSRVGGLYAAVYPSTQFGSLTGLQSLVSALFA	495
hLAT4	401: EEPEEKDANOGEKKKKKRDROIOKITNAMRAFAFTNLLLVGFGVTCLIPNLPLOILSFILHTIVRGFINSAVGGLYAAVYPSTOFGSLTGLOSLISALFA	500
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dLAT4	496: LLQQPLFLAMMGPLQGDPLWVNVGLLGLSMLGFCLPLYLICYRRQLERQLQQKREDDKLFLKLNGSSNREAFV	568
mLAT4	496: LLQQPLY LAMMGPLGGDPLWWWGLLAMSMLGFCLPLYLICYRRQLERQLQQKREDSKLFLKINGSSNREAFV	568
hLAT4	501: LLQQPLFLAMMGPLQGDPLWWWGLLLLSLLGFCLPLYLICYRRQLERQLQQRQEDDKLFLKINGSSNQEAFV	573

Fig. 5. Amino acid sequences of canine LAT4 were compared with those of the human and mouse. Multiple sequence alignments were performed using the GENETYX program (ver. 10). Asterisks and dots indicate identical residues and conservative substitutions, respectively. The cDNA sequences data of canine LAT4 were deposited in the DNA Data Bank of Japan (accession numbers AB924119).

Measurement of leucine transport activity: Radioactive (³H-) L-leucine was purchased from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). Na-independent leucine uptake was measured as described elsewhere [20]. Simply, all buffers containing Na were replaced with N-methyl-D-glucamine. The cells were plated 5×10^5 cells/6-well plate

24 hr before the experiment. After washing the cells, the medium containing 10 μ M leucine with a radioisotope was added and incubated at 37°C. To study the effect of BCH, the inhibitor of LAT, was included in an incubation medium containing radiolabeled leucine. Uptake was terminated by washing with ice-cold phosphate-buffered saline. After

LEUCINE TRANSPORT IN HEPATOCELLULAR CARCINOMA

dLAT1	0:	0
dLA12	0:	U
dLAT3 dLAT4	1: NAPT LQQAY RERWINACTAVLENLFFS AVLLGWGSLLINLKE EGFYSS LCPANTINATRDEQRKWLSCD RQEEMLMLGFT I GSFVLS 1: MAPT LATAH RERWINACTAVLENLLFS AVLLGWGSLLINLKS EGFYSY LCTE SEMVS NGTVGGT AE PEHEEMS LMNGWLSCK AQDEMLNLAFTVGSFLLS	87 100
dlat1	0:	0
dLAT2	0:	0
dLAT3	88: ATT LPLG I LMDRF GPR PTR LVGSAS FAASCT LMA LAS RD TRVLSP L IF LALS LNGFGG IC LTFS SLT LPNMF GNLRSTFMALMI GSYASSA IT FPG I KL I	187
dlat4	101: A IT LPLG IVMDKYGPRKLR LLGSACFAVSC LLI AYGASN PNS LSVL IFVSLALNGFGGMCMTFT SLT LPNNFGDLR STFIALMI GSYASSAVTFPG I KVI	200
dLAT1	0:	0
dLAT2	0:	0
dLAT3	188: YDAGVS FVVI MET WSGLACLI FLNCALNWPS EAF PAPEEVNYTKKI KLSGLALDH KVTGDR FYTHVT IVGORLSOKAPS LEE GADVFI SSOD	279
dlat4	201: YDF GVSF III I LWWASCSG LWF LNCFFNWP LEPF PGPEDMDY SVK I KF SWLGFDHKI TGKQFY KQVTTVGRR LSVGSSMRSAKEQAALQEGHKLCLSTID	300
dLAT1	1:MLAGKRADGGDGGDGGDGAEGVTLQRS I TLFNGVAI IVGT I I GSGI FVT FFGVLKEAGSPGLALVVWAVCGVFSI VGALCYAELG	85
dLAT2	1:MERGARHERNAEKNHPGGSELEASQETGSTGGGVALKKEIGLVSACGIIVGNIIGSGIIVSPRGVLENAGSVGLALIVWIVTGLITAVGALCYAELG	97
dLAT3	280: VRGASGSPPEKSIPFRQSLCSPIFLWSLLTMCHTQLRVIFYMAAMNEMLEYLVTGGQEHETDILEKQAAETVGFYSSIFGAMQLLCLLTCPLIGYIMDWR	379
dlat4	301: LEVKCKPDAAAAPSFMHSVFSPILLLSLVTMCITQLRLIFYMGAMINILKFLVSGDQDVVGLYTSIFGVLQLLCLLTAPVIGYIMDWR	388
	* *	
dLAT1	8 6: TTI TKS GCDY AYMLEVYGS LPAFLR W I ELLI I RPS SQY IVA LVFATYLLKP LFPTC PVPE SAAKLVAC LOVLLLTAVNCY SVKAATEVQDAF AAAKLLA	185
dLAT2	98: VTI PKS GGDY SYVKD I FGG LAGFLR W I AVLVI YPTNQAVIA LTF SNYVLOP LFPTCF PPESG LRL LAA I CLL LLTW/NCASVRWATEVQD IF TAGKLLA	197
dLAT3	380: I KDCVDAPSEGTALGDAREGAATKSARPRYRKI QKLTNA I NAFTLTNFLLVGFG I TCLINSLHLQFVTFVLHTNVRGFYHSACGSLYAAVFPSNHFGTLT	479
dLAT4	389: LKECEDASEE PEEKDANQGEKKKKRDRQIQKVTNAMBAFAFTNLLLVGFGVTCLIPNLPLQILSFILAT IVRGFIHSAVGGLYAAVYPSTOFGSLT	484
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41 2 71	186. I או דו ובמינט במהגבוו הסער לדמ. דעו האדע או על אין על האדער איז ארע האדער או גער איז ארע אין איז איז איז איז איז איז איז איז איז איז איז איז איז	284
dLAT2	198-141 HIMGAND CREEVENEED AN FENTLED AN AUGUST ALS AN AGENT COMPANY AND	201
41 3 72	190. BALTINGYYICABIT WERANTERYET GUTALLEUGETA GUTALEN BERNING TATAT TITTT TITTTANA TALAPAS	559
AL ATA	100: OLQS LI SAVERE LQCE LI RARVOF EMBERI WARDOL LI SLUGELES I LI TERRE LQET VETEDORAVI. OSSEVIA SAVER E LA SAVERA LA SA	568
GLAIT		200
dLAT1	285: TSEAVAVDFGTYH LGVMSWII PVFVGLSCFGSVNGSLFTSSRLFFVGSREGHLPSILSMIHPQLLTPMPSLVFTCIMTLLYAFSRDIFSVINFFSFFNWL	384
dLAT2	298: A SNAVAVTE GEKL LOVMAN IMP I SVALS TE GOVNGS LETS SR LEFF AGAREGH LP SVLAMI HVKRCT P I PALLETC I ST LLMLVT SDMYTL I NYVGE I NYL	397
dLAT3	558:	558
dLAT4	568:	568
dLAT1	385: CVALAI AQMLWLRYKKPE LERP IKVNLALPVFF I LACLFL IAVSFWKTPVECGI GFT I ILSGLPVYFLGVWWKNKPKWLLQG IFSTTVLCQKLMQVVPQE	484
dLAT2	398: FYGVTI AQQ IVLRWKKPD I PRPIKI NLLFPI IYLLFWAFLLI FSLWSEPWCGTGLA I MLTGVPVYFLGVYWQHKPKCFNNFI ES LTLVSQKMCVAVYPE	497
dLAT3	558:	558
dlat4	568:	568
dLAT1	465:T	485
dLAT2	498: MUAGSKTEETNEDTEEQKQPIYHPTASKDQEQPQP	532
dLAT3	>>8:	558
aLAT4	300:	208

Fig. 6. Amino acid sequences of the four isotypes of canine LATs were compared. Multiple sequence alignments were performed using the GENETYX program (ver. 10). Asterisks and dots indicate identical residues and conservative substitutions, respectively.

solubilizing the cells with 1% SDS, the radioactivity was measured with a liquid scintillation counter, and the protein content was determined by the Micro BCA method.

RT-PCR analysis of the 4 isotypes of LAT expression in hepatocarcinoma and normal hepatocytes: In order to examine the expression of LATs, we performed RT-PCR using the primers specific to each LAT (Table 1). RT-PCR conditions were as follows: 25 cycles of three steps, 94°C for 15 sec, 68°C for 15 sec and 72°C for 20 sec.

Real-time RT-PCR evaluation of LAT1: The housekeeping gene RP19 was used as a reference gene. The primer set for LAT1 used for real-time PCR was designed and purchased from Takara (Table 1). Real-time quantitative PCR was performed using a Thermal Cycler Dice[®] Real Time System II (Takara). Samples (final volume of 25 μ l) were run in duplicate and contained the following: X1 SYBR[®] Premix Ex TaqTM II (Takara), 1 μ l 10 μ M of each primer and 2 μ l cDNA template. Amplification was conducted using the following protocol: initial denaturation phase at 95°C for 10 sec and then 40 cycles at 95°C for 5 sec for denaturation and 60°C for 20 sec for annealing and extension. The qRT-PCR results are presented as the gene expression of the target gene (LAT1) relative to that of the housekeeping gene (RP19), and LAT1 gene expression levels were obtained using the standard method of quantification.

Western blot analysis of LAT: The anti-canine LAT1 serum was prepared as described previously [26]. The cell membrane of the tissues for Western blot analysis was prepared as reported by Denker *et al.* [7]. In brief, the cells were homogenized at 4°C in the buffer containing 0.1 M KCl, 5 mM Na₂HPO₄, pH 7.5, 0.75 mM Na-EGTA, pH 7.5, 1 mM DDT, 5 mM MgCl₂, 200 μ g/ml phenylmethylsulfonyl fluoride and 4 μ g/ml leupeptin. Homogenates were centrifuged for 10 min to remove debris. The 1 ml supernatant was laid

over a 5 ml sucrose solution containing 0.8 mM sucrose and 2 mM Na-EGTA, and was centrifuged at 32,000 g for 40 min. Protein concentration of the pellet was determined by the BCA method and was used for Western blot analysis. The membranes were simply solubilized, electrophoresed into 12% polyacrylamide gels and immunoblotted with a chemiluminescence autoradiograph. The membrane was then treated with a primary antibody against the C-terminus of canine LAT1, followed by a secondary antibody (anti-rabbit IgG (H+L) goat IgG Fab' HRP, ×100,000, Seikagaku Corp., Tokyo, Japan). The LAT1 protein was detected with an ECL Plus chemiluminescence detection system (GE Healthcare Bioscience, Chalfont, UK) and exposed to x-ray film.

RESULTS

Histologic sections from the specimen of hepatocellular carcinoma from the dog are shown in Fig. 1 (A, B). The tumor was composed of 2 dissimilar forms, hepatocellular carcinoma and cholangiocellular carcinoma. The tumor cells contained various-sized cord-like structures and conspicuous nucleoli. Histologic sections of a nude mouse with a transplanted tumor are also shown in Fig. 1 (C, D). Mitosis was observed, but cord-like structures disappeared. The tumor cytoplasm was abundant and eosinophilic.

The leucine transport activity of canine HCCs is indicated in Fig. 2. The transport activity was 0.628 ± 0.018 nmol/ mg protein/min, while in the presence of 1 mM BCH, the specific inhibitor of carnitine transporter, the transport activity was reduced by 90%. Figures 2-4 show the deduced amino acid sequences of canine LAT2-LAT4 and a comparison with those of other mammalians. Canine LAT2 was 1 amino acid longer than that of the mouse, and 3 amino acids shorter than that of the human (Fig. 8). Canine LAT3 was 1 amino acid shorter than that of the human. Mouse LAT3 possessed 5 and 14 amino acid insertions in the same location compared with those of the dog and human (Fig. 4). Canine LAT4 consisted of the same number of amino acid resides as human LAT4, while mouse LAT4 possessed 4 amino acid insertions (Fig. 5). Canine LAT2, LAT3 and LAT4 exhibited 89, 88% and 77% homology among mammalians, respectively. The homology of canine LAT1 and LAT2 was 54%, and LAT3 and LAT4 showed 58% identity (data not shown). On the other hand, significant homology was not observed among LAT1-LAT4 (Fig. 6). Figure 7 shows the RT-PCR analysis of LAT expression in HCCs and hepatocytes from the healthy dog. A potent signal was observed only in LAT1 in the hepatocarcinoma, while LAT1 was not observed in hepatocytes. Signals of LAT3 (weakly) and LAT4 were detected in hepatocytes, while LAT3 and LAT4 were not observed in HCCs. As a control experiment, the expressions of LAT2 and LAT4 were confirmed in the kidney, and that of LAT3 was confirmed in the pancreas (Fig. 7B). Detailed evaluation of LAT1 expression in the hepatocarcinoma and healthy liver was investigated by real-time RT-PCR analysis. Expression of LAT1 in the hepatocarcinoma was 28 times higher than that in the healthy liver (Fig. 8A). Western blotting analysis using anti-LAT1 serum confirmed the certain



Fig. 7. RT-PCR analysis of the four isotypes of LATs in canine HCCs and normal hepatocytes. Integrity of RNA was examined by using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (A). Control experiments of LAT2-LAT4 were also examined.



Fig. 8. Real-time RT-PCR analysis of mRNA of canine LAT1 in canine HCCs and normal hepatocytes (A) and Western blot analysis of LAT1 expression using antiserum against the C-terminus of canine LAT1 peptides (B).

signal in the hepatocellular carcinoma (Fig. 8B). RT-PCR analysis indicated no LAT3 and LAT4 expression in canine HCCs, while hepatocytes possessed weak expression.

DISCUSSION

In this study, we investigated the leucine transport activity and confirmed potent BCH-sensitive, Na-independent transport activity in canine HCCs. To evaluate the contribution of 4 LAT isoforms, we determined the cDNA sequences of LAT2, LAT3 and LAT4, and the expression of the 4 isotypes of LATs was examined by RT-PCR analysis. Distinct LAT1 expression in canine HCCs was observed, while other signals were either weak or not detected.

Real-time RT-PCR evaluation of LAT1 indicated that the expression of LAT1 was 28 times higher in HCCs than that in hepatocytes. Western blotting analysis using anti-LAT1 serum supported these results. Therefore, potent BCH-sensitive, Na-independent leucine transport activity occurred due to LAT1. LAT1 has been closely associated with cancerous or proliferative cells, and previous studies have shown it to be highly expressed in proliferating tissues, many tumor cell lines and primary human tumors [18, 24, 38]. The expression of LAT1 has been described to be a significant factor indicating a poor outcome in various human cancers [12, 16, 22, 33]. Cancer cells require considerable nourishment to meet their demands for rapid growth and cell division. Therefore, upregulation of amino acid transporters, especially the system L amino acid transporters that supply large neutral amino acids including many essential amino acids, has been reported [8]. Notably, downregulation of LAT1 by silencing LAT1 shRNA introduction or BCH treatment suppressed invasion and migration of cholangiocarcinoma [13]. Hepatocellular carcinoma is the most common primary hepatic tumor in dogs. Canine hepatocellular carcinoma arises from the uncontrolled proliferation of hepatocytes. The pathogenesis of hepatocellular carcinoma is a multistep process involving sequential events, such as chronic inflammation, hyperplasia, dysplasia, and ultimately, malignant transformation [4]. Several epigenetic and genetic alterations are involved in hepatocellular carcinoma, which ultimately lead to alterations of molecular pathways. Upregulation of LAT1 was reported to be associated with a poor prognosis in malignant tumors [19, 23]. Potent expression of LAT1 may involve the alternation of amino acid metabolism in hepatocellular carcinoma. Therefore, alteration of LAT1 function could possibly be a therapeutic target for canine hepatocellular carcinoma. Although we confirmed that LAT1 played an important role in leucine transport in canine HCC in this study, only one cell line was investigated. We will next investigate more cell lines of canine hepatocellular carcinoma or other tumor cell lines. The data clarified in this paper may facilitate the study on understanding of the pathophysiology of canine hepatocellular carcinoma.

ACKNOWLEDGMENT. This study was in part supported by a research project grant awarded by the Azabu University Research Services Division.

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