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Oncogenicity of L-type amino-acid transporter 1 (LAT1) revealed by targeted gene disruption in chicken DT40 cells: LAT1 is a promising molecular target for human cancer therapy

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ABSTRACT

L-type amino-acid transporter 1 (LAT1) is the first identified light chain of CD98 molecule, disulfide-linked to a heavy chain of CD98. Following cDNA cloning of chicken full-length *LAT1*, we have constructed targeting vectors for the disruption of chicken *LAT1* gene from genomic DNA of chicken *LAT1* consisting of 5.4 kb. We established five homozygous *LAT1*-disrupted ($LAT1^{-/-}$) cell clones, derived from a heterozygous $LAT1^{+/-}$ clone of DT40 chicken B cell line. Reactivity of anti-chicken CD98hc monoclonal antibody (mAb) with $LAT1^{-/-}$ DT40 cells was markedly decreased compared with that of wild-type DT40 cells. All $LAT1^{-/-}$ cells were deficient in L-type amino-acid transporting activity, although alternative-splice variant but not full-length mRNA of *LAT1* was detected in these cells. $LAT1^{-/-}$ DT40 clones showed outstandingly slow growth in liquid culture and decreased colony-formation capacity in soft agar compared with wild-type DT40 cells. Cell-cycle analyses indicated that $LAT1^{-/-}$ DT40 clones have prolonged cell-cycle phases compared with wild-type or $LAT1^{+/-}$ DT40 cells. Knockdown of human *LAT1* by small interfering RNAs resulted in marked *in vitro* cell-growth inhibition of human cancer cells, and *in vivo* tumor growth of HeLa cells in athymic mice was significantly inhibited by anti-human LAT1 mAb. All these results indicate essential roles of LAT1 in the cell proliferation and occurrence of malignant phenotypes and that LAT1 is a promising candidate as a molecular target of human cancer therapy.

1. Introduction

L-type amino-acid transporter 1 (LAT1), also referred to as solute carrier (SLC) 7A5, is the first identified light chain (lc) of CD98 [1,2], disulfide-linked to a heavy chain (hc; SLC3A2) of CD98.

We have reported higher expression of CD98 in various cancer cells using specific anti-CD98hc monoclonal antibodies (mAb) [3,4], growth inhibition of cancer cells by anti-CD98hc mAb [5–7] or by liposomes containing anti-cancer drug and coated with anti-CD98hc mAb [8], malignant transformation of mouse fibroblasts by DNA transfection of human and rat cDNAs of *CD98hc* [9–11], and cooperation between CD98hc and CD98lc in the malignant transformation [10].

Six CD98lcs, LAT1, LAT2 (SLC7A8), y+LAT1 (SLC7A7), y+LAT2 (SLC7A6), ASC1 (SLC7A10) and xCT (SLC7A11) exist [1,2,12], however, CD98lc responsible for CD98hc-mediated malignant transformation has not been identified.

Here, we demonstrate the essential roles of LAT1 in cell growth and malignant transformation using experimental genetics, namely, we established chicken *LAT1*-disrupted ($cLAT1^{-/-}$) DT40 cell lines, and compared various biological characteristics among wild-type, $cLAT1^{+/-}$ and $cLAT1^{-/-}$ DT40 cell lines. Furthermore, we characterized human LAT1 (hLAT1) as a promising target for the therapy of human cancers using experiments showing in *vitro* and *in vivo* growth inhibition of human cancer cells with *hLAT1* small interfering RNAs (siRNAs) and anti-hLAT1 mAb.

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2. Materials and methods

2.1. Construction of cLAT1-targeting vector

On the basis of polymerase chain reaction (PCR)-amplified partial cDNA of *cLAT1* corresponding to human *LAT1* cDNA (145–1223 bp), a full-length cDNA coding *cLAT1* was obtained by 5′ and 3′ rapid amplification of cDNA ends (RACE) using chicken intestine and testis cDNAs as a template. A partial genomic DNA segment corresponding to the C-terminal half of a cLAT1 region with six transmembrane domains was amplified by long-range PCR using DT40 genomic DNA as a template. Gene-targeting constructs for chicken *LAT1*, namely, *cLAT1-His* and *cLAT1-Bsr*, were made by replacing the 4th cytoplasmic domain with 7th and 8th transmembrane domains of *cLAT1* with *histidinol (His)*- or *blasticidin (Bsr)*-selection marker cassettes. Nucleotide sequences of the inserted fragments were confirmed by a 373A DNA sequencer (Applied Biosystems, Foster City, CA, USA).

2.2. Cell culture, DNA or siRNA transfection and reverse transcription (RT)-PCR

DT40 cells [13] were cultured at 39 °C in RPMI1640 medium (RPMI) supplemented with heat-inactivated 10% fetal bovine serum (FBS; ICN Biomedicals, Aurora, OH) and 1% chicken serum (Sigma–Aldrich, Tokyo, Japan) in a humidified CO_2 incubator. For gene targeting, cells (1×10^7) were electroporated with 25 μg of linearized *cLAT1*-targeting constructs using a Gene Pulser apparatus (BioRad, Hercules, CA, USA) at 550 V and 25 mF as described elsewhere [13]. Drug-resistant colonies were selected in 96-well plates with culture media containing 1 mg/ml His and 20 μg/ml Bsr. Genomic DNA or cDNA was isolated from drug-resistant clones and gene disruption was confirmed by PCR or RT-PCR. Hybridoma cells secreting rat mAb against hLAT1 [14,15] and mouse CD44v [15] were cultured at 37 °C in 10% FBS-RPMI. HCT116 colon, HeLa cervix and T24 bladder cancer cells (American Type Culture Collection) were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich) supplemented with 10% FBS. Human *LAT1* siRNAs (50 nM, No. 1-*hLAT1*: GUGAACUGCUACAGCGUGA, and No. 2-*hLAT1*: GGAAGGGUGAUGU GUCCAA Dharmacon, Lafayette, Colorado, USA) or control siRNA (50 nM) were transfected to human cancer cells with Lipofectamine RNAi Max reagent (Invitrogen). Following 48 h-culture of cells, the expression of *hLAT1* mRNA was evaluated by RT-PCR and compared to the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA.

2.3. Anti-chicken CD98hc and anti-human LAT1 mAb

N71 (IgG, κ) mouse mAb recognizing chicken CD98hc was produced by the cell fusion of spleen cells of a mouse immunized against recombinant chicken CD98hc proteins produced in *Escherichia coli* with NS-1 mouse myeloma. Anti-hLAT1 or anti-mouse CD44v rat mAb was produced respectively by the cell fusion of spleen cells of a rat immunized against RH7777 rat hepatoma cells expressing hLAT1 or mouse CD44v fused to green fluorescent protein (GFP) with X63 mouse myeloma [14,15].

2.4. Western blot

Cells (3×10^7) were extracted by 1-ml lysis solution containing 1% SDS, 1% Triton X-100, 1 mM dithiothreitol and inhibitors (1 mM PMSF, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM NaF, 0.1 mM Na3VO4 and 10 mM glycerophosphate) in Dulbecco's phosphate-buffered saline (PBS; pH7.4). The solution was incubated on ice for 20 min and then ultracentrifuged at 45,000 rpm for 45 min at 4 °C. The cleared cell lysate (1×10^6 cells-equivalent in each lane) was separated on SDS–PAGE and transferred to Fluorotrans membranes (Pall BioSupport, Port Washington, NY) using a semidry transfer apparatus. Membranes were treated with Block Ace (Dainihon Seiyaku, Osaka, Japan) 1:2 diluted with PBS, and incubated sequentially with N71 mAb, rabbit anti-mouse immunoglobulins (Dako) 1:300 diluted in PBS with 1% bovine serum albumin (ICN Biomedicals), and HRP-conjugated protein A (Zymed Laboratories Inc., South San Francisco, CA, USA) diluted 1:10,000 in PBS containing 0.05% Tween 20 (T-PBS). Between each step, the membranes were washed extensively with T-PBS. HRP activity was detected using 0.05% 3,3′-diaminobenzidine (Dojindo, Kumamoto, Japan) and 0.01% hydrogen peroxide in 0.1 M Tris buffer, pH 7.5.

2.5. Evaluation of the activity of amino-acid transport

Uptake of ^{3}H-leucine in wild-type, $cLAT1^{+/-}$ and $cLAT1^{-/-}$ DT40 cell clones in the Na^+-independent or Na^+-dependent condition was measured by a previously reported method [16] with a cell-harvester (Skatron-Molecular Devices, Tokyo, Japan) and a liquid-scintillation counter (Beckman Coulter, Tokyo, Japan).

2.6. Cell-growth evaluation of DT40 clones and siRNA-treated human cancer cells

DT40 cells (5×10^3) were seeded into each well of 96-well plates and cultured in 200 μl of 10% FBS-RPMI. Ten microliters of WST-8 (Dojindo) was periodically added to each well, and absorbance at 450 nm was measured 3 h after the addition of WST-8. Human cancer cell lines treated with control or *hLAT1* siRNAs (50 nM) for 24 h were seeded into each well of 96-well plates (1×10^4/well) and cultured in 200 μl of 10% FBS-DMEM for additional 48 h. WST-8 (10 μl) was added to each well and absorbance at 450 nm was measured 4 h after the addition of WST-8.

WST-8 is a monosodium salt of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, which produces a water-soluble formazan dye upon bioreduction in the presence of an electron carrier.

2.7. Cell-cycle analysis

Cells were prepared with Cycle TEST plus DNA Reagent Kit (BD; Beckton Dickinson, Sunnyvale, CA). Subsequent flow cytometry (FCM) was performed with a BD LSR flow cytometer and data were analyzed using CellFit software (BD). Cells labeled with FITC-BrdU (BD) were cultured at 39 °C and subjected to FCM after the staining of cells with 4,6′-diamidino-2-phenylindole (DAPI, 1 μg/ml).

2.8. Cell-death analysis

Mitochondrial membrane potential was assessed with 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$, 10 μg/ml) and DAPI (1 μg/ml) staining. Cells were suspended in PBS containing $DiOC_6$ and DAPI, incubated for 30 min at 37 °C and were analyzed by FCM.

2.9. Soft-agar assay

Anchorage-independent growth of DT40 cell clones was examined as described previously [9–11]; briefly, wells of 35-mm dishes (BD) were coated with 2 ml of bottom agar mixture (RPMI with 10% FBS and 0.53% agar). After the bottom layer had solidified, 2 ml of the top agar mixture (RPMI with 10% FBS and 0.3% agar) containing the cells (1×10^3) was added. On day 15 after plating, colonies were counted under a microscope.