Expression of system L amino acid transporters in colorectal cancer cell (HT-29)

การแสดงออกของตัวขนส่งกรดอะมิโนระบบแอลในเซลล์มะเร็งลำไส้ (HT-29)

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ABSTRACT

Treatment of colorectal cancer (CRC) is at present ineffective, therefore new molecular targets for the treatment are required. System L amino acid transporter is upregulated in several types of tumors. Therefore, we aim to investigate the expression of system L1 (LAT1, LAT2) and system L2 (LAT3, LAT4) amino acid transporters in colorectal cancer cells (HT-29). By quantitative real time PCR, we found that HT-29 expressed system L1 (LAT1 and its associating protein, 4F2hc), system L2 amino acid transporter (LAT3) and ASCT2 at high levels. LAT1, 4F2hc and LAT3 proteins were localized predominantly at plasma membrane of HT-29 cells. Leucine transport in HT-29 cells was inhibited by BCH, a specific system L inhibitor and *N*-ethylmaleimaide (NEM) suggesting the presence of system L1 and L2 in HT-29 cells. Collectively, it is proposed that beside LAT1, LAT3 could be a novel therapeutic target for colorectal cancer.

บทคัดย่อ

เนื่องจากการรักษาโรคมะเร็งลำไส้ใหญ่ในปัจจุบันยังไม่มีประสิทธิภาพเพียงพอ ทำให้ต้องมีการค้นคว้าหาเป้าหมายใหม่ในการรักษา ที่ผ่านมาพบว่ามีการเพิ่มการแสดงออกของตัวขนส่งกรดอะมิโนในเซลล์มะเร็งหลายชนิด ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาการแสดงออกของตัวขนส่งกรดอะมิโนระบบแอล 1 (LAT1, LAT2) และระบบแอล 2 (LAT3, LAT4) ในเซลล์มะเร็งลำไส้ใหญ่ (HT-29) ซึ่งพบว่าเซลล์นี้มีการแสดงออกของ LAT1, 4F2hc, LAT3 และ ASCT2 ยีนสูง และยังพบการแสดงออกของ LAT1 4F2hc และ LAT3 โปรตีนเด่นที่บริเวณเยื่อหุ้มเซลล์ การขนส่งกรดอะมิโนลิวซีนเข้าสู่เซลล์ถูกยับยั้งด้วยสาร BCH ซึ่งเป็นตัวยับยั้งที่จำเพาะต่อตัวขนส่งกรดอะมิโนระบบแอล และ *N*-ethylmaleimaide (NEM) จึงยืนยันได้ว่ามีการแสดงออกของตัวขนส่งกรดอะมิโนทั้ง 2 ระบบ ดังนั้นนอกจาก LAT1 แล้ว LAT3 จึงเป็นโปรตีนที่สำคัญที่จะสามารถพัฒนาไปเป็นเป้าหมายใหม่ในการรักษาโรคมะเร็งลำไส้ได้

Key Words: LAT3, System L2 amino acid transporter, Colorectal cancer
คำสำคัญ: LAT3 โปรตีนตัวขนส่งกรดอะมิโนระบบแอล 2 มะเร็งลำไส้ใหญ่

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Introduction

Colorectal cancer (CRC), an adenomatous polyps-cluster, arising from abnormal cells in the glands covering the inner wall of the colon is the fourth most common cancer in men and the third most common in women worldwide. It accounts for an estimated 1.2 million new cancer cases and over 630,000 cancer deaths per year, almost 8% of all cancer deaths (Kamangar et al., 2006). Although the incidence rate of CRC in Thailand is low, CRC case has rapidly increased since 2001-2003 in both male and female with a male/female ratio of 1.4:1 (Sriamporn et al., 2007). The highest incidence rate of CRC for both sexes has been found in Bangkok (Sriamporn et al., 2007). The risk factors for colorectal cancer development appear to be associated with smoking, physical inactivity, obesity, and alcohol consumption (Ferrari et al., 2007). The treatments of CRC depend on the stage of disease. Primary tumors without metastasis are usually treated by surgical resection whereas the combination with chemotherapeutic drugs or radiation therapy may be employed for metastasis cases. However, up to 30% of CRC cases treated with chemotherapy usually suffer from liver injury (Pawlik et al., 2007). Thus, new therapeutic targets for CRC are urgently needed.

System L amino acid transporters have been proposed to be crucial to supply the essential amino acids for tumor cell growth and proliferation. L-type amino acid transporter 1 (LAT1), the first isoform of system L1 amino acid transporter was isolated from C6 rat glioma cells by expression cloning (Kanai et al., 1998). LAT1 is a 12-membrane-spaning protein, which mediates a Na^+-independent amino acid exchange. LAT1 forms a heterodimeric complex with the heavy chain of 4F2 antigen (4F2hc) for proper recruitment to the plasma membrane (Verrey et al., 2004). The second isoform, L-type amino acid transporter 2 (LAT2) was identified and showed amino acid sequence similarity to LAT1 (50% identity) (Pineda et al., 1999; Segawa et al., 1999). Similar to LAT1, LAT2 also forms a heterodimeric amino acid transporter with 4F2hc and the transport activity is inhibited by a system L-specific inhibitor BCH (2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid). In addition to system L1, system L2 amino acid transporters have been identified, which are composed of L-type amino acid transporter 3 (LAT3) and L-type amino acid transporter 4 (LAT4) (Babu et al., 2003; Bodoy et al., 2005). Unlike system L1, system L2 do not require 4F2hc for functional expression. LAT3 and LAT4 mediate a Na^+-independent amino acid transport and are inhibited by BCH, consistent with the properties of system L, and sensitive to inhibition of *N*-ethylmaleimide (NEM) (Babu et al., 2003; Bodoy et al., 2005). The upregulation of LAT1 and CD98 has been observed in several solid tumors such as brain, colon, lung, liver and skin to support continuous growth and proliferation (Fuchs and Bode, 2005). In contrast, LAT2 plays a significant role in growth of normal cells. However, the role of system L2 amino acid transporters in cancer biology is still unknown. Recent study has demonstrated that LAT3 played role in prostate cancer cell growth (Wang et al., 2011). However, the information regarding the expression of system L2 amino acid transporters in colorectal cancer is limited. In this present study, we investigated the expression of system L1 and system L2 amino acid transporters in human colorectal adenocarcinoma cell line (HT-29). We found that LAT1 and its associated protein 4F2hc highly

expressed in HT-29 cells. Interestingly, LAT3, a system L2 amino acid transporter was also detected at high level. The leucine transport was inhibited by BCH (system L specific inhibitor) and sentitive to inhibition of NEM pretreatment. Collectively, this present study suggests that beside LAT1, LAT3, a system L2 amino acid transporter could be a new molecular target for colorectal cancer treatment.

Objectives of the study

To investigate the expression of system L1 and L2 amino acid transporters in human colorectal cancer cells.

Methodology

Cell culture, antibodies and reagents

Human colon adenocarcinoma cell (HT-29) was obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM/F-12 medium containing 100 U/ml penicillin, 100 µg/ml streptomycin and 10 % fetal bovine serum (Invitrogen, Carlsbad, CA). Cells were incubated at 37 °C with 5% CO_2 incubator. The following antibodies were used: anti-SLC7A5 from Abcam; anti-CD98 (H-300) from Santa Cruz Biotechnology Inc.; anti-SLC43A1/LAT3 from MBL; HRP goat anti-rabbit IgG (H+L) antibody from Jackson ImmunoResearch Laboratories, Inc.; goat anti-rabbit IgG (H+L) Alexa Fluor 488, and TO-PRO3 from Invitrogen. The following reagents were used: TRIzol reagent from Invitrogen; cDNA kit from Bio-Rad; SYBR kit from Biosystem; complete Mini EDTA-free from Roche; SupperSignal West Pico chemiluminescent from ThermoScientific; and [^{14}C]L-leucine from Perkin Elmer.

Western blot analysis

Cells were cultured in a 60 mm dish at density 5 x 10^5 cells/dish and incubated at 37 °C with 5% CO_2 incubator for 24 h. Cell were lysed with modified RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and protease inhibitor cocktail). After 20 min incubation on ice, cells were centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was collected and the protein concentrations were measured. Equal amount of protein samples were used for Western blot analysis. Proteins were mixed with Laemmli's sample buffer with or without DTT and resolved by SDS-PAGE, subsequently transferred onto a nitrocellulose membrane by electro-blotting. Membranes were incubated with anti-SLC43A1/LAT3 antibody overnight at 4°C before incubating with HRP-conjugated goat anti-rabbit IgG for 1 h at room temperature. The signals were detected using the enhanced SuperSignal West Pico Chemiluminescent reagent.

Total RNA extraction and RT-PCR quantification

HT-29 cells were cultured in a 60 mm dish at density 5 x 10^5 cells/dish and incubated at 37 °C with 5% CO_2 incubator for 24 h. Total RNAs were extracted from cells by using Trizol reagent according to the recommendation of the manufacturer with a slight modication. cDNA synthesis was conducted by using iScript™ cDNA synthesis kit according to the manufacturer's protocols. Quantitative real time PCRs of LAT1, LAT2, LAT3, LAT4, 4F2hc, and ASCT2 were analyzed using SYBR Green I dye with ABI PRISM 7500 Sequence Detection System and analysis software (Applied Biosystem). Primers were

designed using NCBI/Primer-Blast and 7 IDT Scitools Olioanalyzer 3.1. The primers used are summarized in table 1. The mRNA level of each gene was normalized to mRNA level of a housekeeping gene, β-actin.

Table 1 The primers for quantitative real time PCR

LAT1	5'-TTCGTCCAGATCGGGAAGGGTGA-3'
	5'-TCCATAGGCAAAGAGGCCGCT-3'
LAT2	5'-GGGAGCCCTCTGCTATGCTGAAC-3'
	5'-GAACCCAGCCAGTCCTCCGAAGA-3'
LAT3	5'-GCTGGTTGGCAGTGCCTGCT-3'
	5'-GCCATTCAGGGACAGCGCCA-3'
LAT4	5'-AACCAAACGCTCTCTCCGTGCT-3'
	5'-ACCGAAGGTCGCCGAACATGT-3'
4F2hc	5'-GGCGCAGAAGTGGTGGCACA-3'
	5'-CCCCTTCAGACCCGCCAG GT-3'
ASCT2	5'-TGGTACGAAAATGTGGGCA-3'
	5'-GTGCCCCAGCAGGCAGCACA-3'
β-actin	5'-TACCCTGGCATTGCCGACAGGA-3'
	5'-TACCCTGGCATTGCCGACAGGA-3'

Immunofluorescence microscopy

HT-29 cells were grown on glass coverslips in a 24-well culture plate. At 50-60% confluence, cells were washed with cold PBS containing Ca^{2+}/Mg^{2+} (PBS^{++}) and fixed with cold methanol for 5 min. To permeabilize the cells and block non-specific protein-protein interaction, cells were incubated in buffer containing 0.3% Triton X-100, 0.3% BSA and 10% normal goat serum for 30 min. The samples were then incubated overnight at 4°C with anti-SLC7A5, anti-SLC43A1/LAT3 and anti-CD98 (H-300) diluted in antibody dilution buffer (0.3% Triton X-100, 0.3% BSA). Cells were washed with PBS^{++} for 5 times, and then incubated with Alexa Fluor 488 goat anti-rabbit IgG for 1 h at room temperature. Cells were washed with PBS^{++} for 5 times, stained with TO-PRO 3 (1:2000) for 10 min at room temperature, and then washed again with PBS^{++} for 3 times. The stained coverslips were mounted and visualized at room temperature with a confocal laser microscope (FV10i/w, Olympus).

Functional characterization

HT-29 cells were seeded in 48-well plates at cells density of 1 x 10^5 cells/well and incubated in 37°C under humidified 5% CO_2 incubator for 48 h. Cells were washed twice with D-PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, 0.5 mM $MgCl_2$, 1mM $CaCl_2$ 5.6 mM D-Glucose, and adjusted pH to 7.4) and then incubated for 10 min in D-PBS at 37°C. Cells were incubated with D-PBS containing [^{14}C]L-leucine (1μM) with or without 10 mM BCH for 2 min. For NEM study, cells were preincubated with 0.1 mM NEM for 10 min before uptake experiment. Uptake was stopped by removing the uptake solution and washed with 500 μl of ice-cold D-PBS 3 times. Cells were solubilized with 10% SDS in 0.1 M NaOH and neutralized with 0.1 M HCl. The radio-activity was counted by scintillation spectrometry.

Statistical analysis

All data were expressed as mean and standard error of mean (mean ± S.E.M). The statistical analysis was performed by using the statistical software package, GraphPad Prism version 5.0. The statistically significant differences among groups were compared using one-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc test. Statistical significance was considered when $p < 0.05$.

Results

mRNA expression of system L1 and L2 amino acid transporters in HT-29 cells

Previous study has demonstrated the expression of LAT1 and 4F2hc in human colon cancer cells, however, the expression of LAT3 and LAT4, system L2 amino acid transporters in these cells have not been reported. In this study, the mRNA expressions of system L1 and system L2 amino acid transporters in HT 29 cells were examined by quantitative real time-

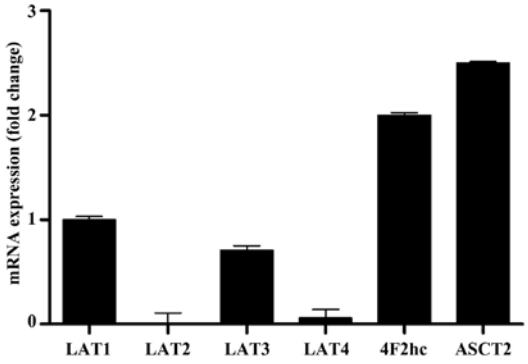

Figure 1 mRNA expression of system L1 and L2 amino acid transporters in HT-29 cells. Total RNA was isolated from HT-29 cells and used for quantitative real time PCR by specific primer pairs for system L1 amino acid transporters (LAT1 and LAT2), system L2 amino acid transporters (LAT3 and LAT4), 4F2hc and ASCT2. The mRNA expression level of each gene was normalized to mRNA level of a housekeeping gene, β-actin. Data is expressed as fold change compare to the expression level of LAT1

PCR. As shown in Fig. 1, HT 29 cells revealed high level expression of LAT1 and its associated protein 4F2hc. Interestingly, system L2 amino acid transporter, L-type amino acid transporter 3 (LAT3) was also detected at almost the similar level to LAT1. In contrast, the expressions of LAT2 and LAT4, the second isoform of system L1 and system L2 amino acid transporters, respectively were also detected. However, the expression level was very low compared to that of LAT1 and LAT3. Interestingly, the expression of a Na^+- coupled amino acid transporter, ASCT2 was found to be higher than LAT1 and LAT3 suggesting an important role of ASCT2 in colon cancer in addition to system L amino acid transporter.

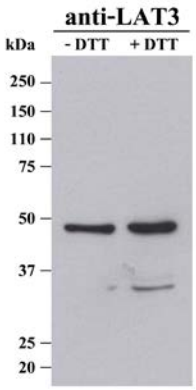

Figure 2 Protein expression of LAT3 in HT-29 cells. HT-29 cells were lysed with modified RIPA lysis buffer and equal amount of protein samples were used for western blot analysis in the presence [DTT (+)] or absence [DTT (-)] of DTT using anti-SLC43A1/LAT3 antibody. The signals were detected using the enhanced SuperSignal West Pico Chemiluminescent

Plasma membrane expression of system L1 and system L2 amino acid transporters

The expression of system L2 amino acid transporter protein was investigated using western blotting. 4F2hc/CD98 is not required for functional expression of LAT3, that is why LAT3 protein was detected at 48 kDa band under both reducing and

nonreducing conditions (Fig. 2). The cellular localization of system L1 and system L2 amino acid transporter proteins was further investigated using confocal immunofluorescence. LAT1 and its associated protein 4F2hc were predominantly localized at the plasma membrane of HT-29 cells (Fig. 3). Interestingly, LAT3, system L2 amino acid transporter was also detected at the plasma membrane (Fig. 3). Collectively, these results indicate that not only system L1 heterodimeric amino acid transporter (LAT1-4F2hc) but also system L2 amino acid transporter (LAT3) is expressed at high level at the plasma membrane of HT-29 cells and is the major amino acid transporters that provides the essential amino acids necessary for colon cancer cell growth.

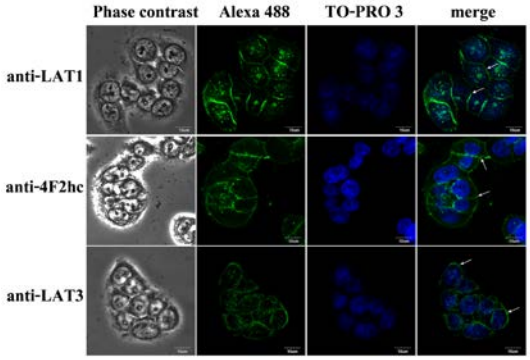

Figure 3 Plasma membrane localization of system L1 and L2 amino acid transporters in HT-29 cells. HT-29 cells were fixed and stained with anti-SLC7A5, anti-SLC43A1/ LAT3 and anti-CD98 (H-300) as primary antibodies followed by Alexa Fluor 488 (green). TO-PRO3 was used as nuclear marker. Arrow indicates plasma membrane protein localization. Bar, 10 μM

Inhibition of leucine transport in HT-29 cells

System L-mediated amino acid transport was determined by measuring [^{14}C]L-leucine uptake in HT-29 cells in the presence and absence of specific inhibitors. It was noted that system L1 and system L2 amino acid transporters are sensitive to inhibition by BCH (2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid), whereas only system L2 is affected by NEM (*N*-ethylmaleimide) (Yanagida et al., 2001; Babu et al., 2003). As shown in Fig. 4, the uptake of [^{14}C]L-leucine was significantly inhibited by 10 mM BCH suggesting the presence of amino acid transport system L in colon cancer cells. Interestingly, preincubation with 0.1 mM NEM significantly reduced system L2-mediated [^{14}C]L-leucine uptake. However the inhibition was less than that of BCH. In addition, co-treatment with BCH and NEM significantly inhibited [^{14}C]L-leucine uptake compared with BCH alone. Collectively, these results indicate that both system L1 and system L2 amino acid transporters are present in HT-29 cells and inhibition of both systems could provide a rationale for combination treatment of colon cancer.

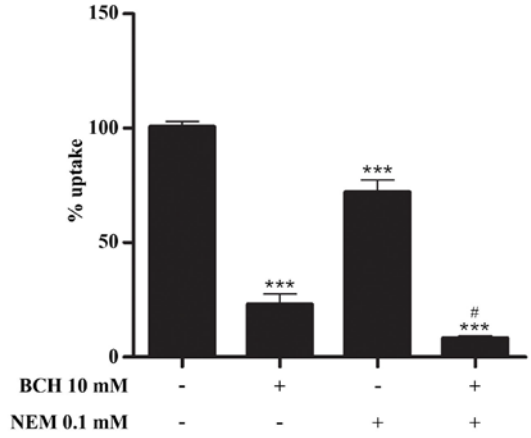

Figure 4 System L1 and L2 amino acid transporters mediate leucine transport in HT-29 cells. Cells were incubated in 1 μM [^{14}C]L-leucine in D-PBS for 2 min in the presence or absence of 10 mM BCH. To study the effect of NEM, cells were preincubated with 0.1 mM NEM for 10 min before measuring [^{14}C]L-leucine uptake in the presence or absence of 10 mM BCH. Data shown are % inhibition and represented as mean ± S.E.M (n=3). ***, $p < 0.001$ compared with control (without inhibitor); $^{\#}$, $p < 0.05$ compared with BCH treatment group (ANOVA)

Discussion and Conclusions

Earlier study has reported the expression of system L1, LAT1 and 4F2hc in human colorectal adenocarcinoma cells (HT-29) however mRNA and protein expression of system L2 amino acid transporters in this cancer type is not well studied. In this study, we investigated the mRNA and protein expressions of system L1 and system L2 amino acid transporters in HT-29 cells. We found that cancer specific system L1 amino acid transporter, LAT1 and its associating protein, 4F2hc was highly expressed. Interestingly, the recently identified system L2 amino acid transporter, LAT3 was also detected. System L amino acid transporters mediated the essential amino acid uptake in HT-29 cells and were inhibited by system L specific inhibitor, BCH. Therefore, it is proposed that system L amino acid transporters could be new molecular targets for treatment of colorectal cancer.

Amino acids are necessary for growth and proliferation of cancer cells. We found that HT-29 cells highly expressed LAT1/4F2hc, a heterodimeric amino acid transporter, which transports essential amino acids into the cell. Consistent with an earlier study (Oda et al., 2010), we demonstrated that LAT1 and 4F2hc were expressed at the plasma membrane of HT-29 cells. Indeed, the upregulation of LAT1 and 4F2hc has been observed in several solid tumors such as brain, colon, lung, liver and skin (Fuchs and Bode, 2005), and in several cancer cell lines including leukemia cells, lung small cell carcinoma cells (RERF-LC-MA), uterine cervical carcinoma cells (HeLa), and bladder carcinoma cells (T24) (Oda et al., 2010; Yanagida et al., 2001). Recent studies have demonstrated that high-level expression of LAT1 and 4F2hc was associated with poor prognosis in various human cancers, such as pancreatic cancer (Kaira et al., 2012), stage 1 sequamous cell carcinoma of the lung (Kaira et al., 2009), primary astrocyte tumor (Nawashiro et al., 2006), and triple negative breast cancer (Furuya et al., 2012). These significant correlations of LAT1 and 4F2hc expression with clinicopathological features and patient survival suggest the prognostic value of LAT1 and 4F2hc in tumors. However, prognostic significance of LAT1 and 4F2hc expression in colorectal cancer patient is currently unknown. LAT1 is obligatory amino acid exchanger (Kanai et al., 1998) and by this definition, it cannot mediate net influx of essential amino acids into cells, however, the hetero-exchange mechanism does.

Therefore, it has been proposed that transporters that have overlapping substrate selectivity provided efflux substrates necessary for LAT1. Among them, ASCT2, a sodium-coupled amino acid transporter has the overlapping substrate selectivity with LAT1 and appears to be upregulated in cancer (Fuchs and Bode, 2005). A recent study reported that indeed, ASCT2 provided net uptake of glutamine into cells that is necessary for LAT1 in exchange with the essential amino acids (Nicklin et al., 2009). Interestingly, we detected high expression levels of ASCT2 in HT-29 cells. It has been reported that glutamine uptake in colon cancer cell lines was mediated by system ASC and that inhibition of glutamine uptake suppressed human colon carcinoma cell (WiDr) proliferation (Pawlik et al., 2000). Our results suggest that ASCT2 provides the sufficient intracellular concentration of glutamine, while LAT1 uses glutamine to adjust the essential amino acids uptake to meet the metabolic demands for growth and proliferation of colorectal cancer cells.

Recently, system L2 amino acid transporters have been identified and named LAT3 (Babu et al., 2003), and LAT4 (Bodoy et al., 2005). By quantitative real time PCR, we found that HT-29 expressed high level of LAT3 mRNA and detectable level of LAT4 mRNA. In contrast, Haase et al. detected overexpression of LAT4 but not LAT3 in HT-29 and FaDu cells (Haase et al., 2007). This discrepancy might be due to the differences in passage number and culturing condition. Indeed, it has been shown that passage number and culturing time affected P-glycoprotein efflux transporter (P-gp) expression in Caco-2 cells (Anderle et al., 1998; Siissalo et al., 2007). Therefore, western blotting and immunofluorescence were used to confirm the LAT3 protein expression in HT-29 cells. LAT3 localized clearly at the plasma membrane of colorectal cancer cells. The upregulation of LAT3 in HT-29 cells is not due to the compensatory response from down regulation of system L1 amino acid transporter because HT-29 cells exhibited high expression levels of LAT1 and its associating protein, 4F2hc. System L2 amino acid transporters-mediated amino acid uptake was inhibited by the pretreatment with *N*-ethylmaleimaide (NEM) (Babu et al., 2003; Bodoy et al., 2005). NEM, a thiol reagent, inhibited LAT3 transport activity by reacting with exposed sulfhydryl groups rather than competing with substrates at the binding site (Babu et al., 2003). Consistent with previous study, we found that leucine uptake in HT-29 cells was reduced after pretreatment with NEM. This result further confirms the expression of amino acid transport system L2 in colorectal cancer cells. The uptake by system L1 and system L2 is inhibited by BCH. Therefore, BCH exhibited a greater inhibitory effect on leucine uptake in HT-29 cells than NEM. However, the role of system L2 amino acid transporters in cancer progression is unclear. LAT3 was identified from human hepatocarcinoma-derived cell line FLC4 (Babu et al., 2003). Knockdown of LAT3 in prostate cancer cell lines (LNCaP and PC-3) by shRNA resulted in a significant decrease in cell growth (Wang et al., 2011) suggesting the significant role of system L2 amino acid transporters in cancer biology. Wang et al. have proposed the relationship between roles of LAT1 and LAT3 in prostate cancer. They found that androgen receptor signaling activated LAT3 expression in primary prostate cancer and induced cancer cell growth through activation of mTORC1 signaling pathway (Wang et al., 2011). LAT3 expression was decreased after hormone ablation therapy and resulted in reduction of intracellular leucine concentration. Low level of leucine concentration activated LAT1 transcription and restored mTORC1 activity (Wang et al., 2011). Although, the association between male sex hormone and the incidence

rate of colorectal cancer is not known, however, the prostate cancer patients who received hormone ablation therapy had 30-40% increased risk of colorectal cancer (Gillessen et al., 2010).

We have shown, for the first time, that colorectal cancer cells expressed high level of system L2 (LAT3) amino acid transporter. The inhibition of LAT3 is expected to suppress cancer cell growth. Therefore, it is propose that system L2 amino acid transporters might be a novel molecular target for treatment of colorectal cancer. Moreover, we also detected the expression of LAT1 and its associating protein, 4F2hc together with ASCT2, an amino acid transporter required for LAT1-mediated uptake of essential amino acids. Targeting of LAT3 together with these transporters might be improving the treatment outcome for colorectal cancer.

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