L-type Amino-Acid Transporter 1 Expression Predicts the Response to Preoperative Hyperthermo-Chemoradiotherapy for Advanced Rectal Cancer

TAKESHI EBARA[1], KYOICHI KAIRA[2], JUN-ICHI SAITO[1], MARIKO SHIOYA[1],
TAKAYUKI ASAO[3], TAKEO TAKAHASHI[1], HIDEYUKI SAKURAI[4],
YOSHIKATSU KANAI[5], HIROYUKI KUWANO[3] and TAKASHI NAKANO[1]

[1]Department of Radiation Oncology, Gunma University Graduate School of Medicine,
3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan;
[2]Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine,
Showa-machi, Maebashi, Gunma 371-8511, Japan;
[3]Department of General Surgical Science, Gunma University Graduate School of Medicine,
3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan;
[4]Department of Radiation Oncology and Proton Medical Research Center,
Tsukuba University Graduate School of Medicine. 1-1-1 Tennodai, Ibaraki 305-7102, Japan;
[5]Division of Bio-system Pharmacology, Department of Pharmacology,
Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

Abstract. *Aim: To evaluate whether expression of L-type amino acid transporter 1 (LAT1) in pretreatment rectal cancer biopsies is predictive of tumour response to neoadjuvant hyperthermo-chemoradiotherapy (HCRT). Patients and Methods: Forty-four patients with rectal adenocarcinoma who received neoadjuvant HCRT were investigated. LAT1 expression was immunohistochemically evaluated using pretreatment biopsies. The operation was performed after 2-3 months following HCRT and each resected specimen was graded by the histological criteria of the Japanese Classification of Colorectal Carcinoma. Results: A positive LAT1 expression was recognized in 50.0% (22/44) of patients. Resected specimens were divided into 2 groups according to the histological grading criteria: good response (n=29) and poor response (n=15). LAT1-negative tumours had an 81.8% probability of good response and 18.2% probability of poor response. LAT1 expression showed marginally significant association with response to HCRT (p=0.05). Conclusion: LAT1 may be a useful predictive marker of response to HCRT in rectal cancer.*

Recently, preoperative chemoradiotherapy for locally advanced rectal cancer (LARC) has been increasingly used as a neoadjuvant treatment (1-6). The pathologic downstaging after neoadjuvant chemoradiotherapy is correlated with improved survival, decreased local recurrence, and a higher rate of sphincter-preserving surgeries (7-9). Heat is cytotoxic for cells in hypoxic, poor perfusion and low pH environments that are specifically found in tumour tissues. It has been clearly shown that hyperthermia in combination with radiotherapy increases the cytotoxic effects (10). Therefore, in this study local hyperthermia was added to chemoradiotherapy (HCRT) in order to enhance of the local control.

Prospective identification of patients who have a higher likelihood of responding to preoperative neoadjuvant therapy could be important in deceasing treatment morbidity. In addition, patients who are unlikely to respond could be offered alternative approaches to therapy. Although recent studies have evaluated the potential of genetic biomarkers to predict the outcome in LARC treated with neoadjuvant chemoradiotherapy, there is no effective method of predicting which patients will respond to neoadjuvant therapy (11).

Amino acid transporters are essential for the growth and proliferation of normal and transformed cells (12, 13). Among various types of amino acid transports, system L is a

Correspondence to: Takeshi Ebara, MD, Department of Radiation Oncology, Gunma University Graduate School of Medicine. 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan. Tel: +81 272208383, Fax: +81 272208397, e-mail: tebara@med.gunma-u.ac.jp

Key Words: LAT1, rectal cancer, preoperative hyperthermo-chemoradiotherapy, tumour response.

Na$^+$-independent, large and neutral amino acid transport agent (12, 14). L-Type amino acid transporter 1 (LAT1) transports large neutral amino acids such as leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, methionine and histidine (15, 16). Previous studies showed that LAT1 is highly expressed in proliferating tissues including tumour cell lines and primary human tumours and played an essential role in the growth of tumour and the survival of patients (16-18). Kaira *et al*. described that the positive incidence of LAT1 expression was approximately 30% in colon cancer (19). Moreover, LAT1 expression was higher in the metastatic lesions of colon cancer than in the primary lesions. However, it is unclear whether LAT1 expression could predict treatment response in human neoplasms.

The aim of the present study was to determine whether the expression of LAT1 in pretreatment rectal cancer biopsies is predictive of tumour response to HCRT.

Patients and Methods

Patients. Between 2003 and 2006, 44 patients with proven rectal adenocarcinoma who received HCRT followed by surgery were investigated. For diagnostic work-up, all patients underwent staging for distal metastases with computed tomography of the abdomen and thorax. T stage was determined by magnetic resonance imaging, especially T$_2$-weighted imaging. The extent and location of the tumour was classified according to the Japanese Classification of Colorectal Carcinoma (20). Patient characteristics are shown in Table I. The median follow-up was 44.7 months (range: 3.5-77.0 months) for all patients and that for the 35 patients who were still alive at the last follow-up was 50.0 months (range: 29.7-77.0 months).

Treatments. The treatment protocol for LARC has been described in previous reports (21). Radiotherapy was delivered by 10-MV X-rays through an anteroposterior or three- or four-field box technique. The clinical target volume encompassed the primary tumour and the entire mesorectal tissue. The total radiation dose was 40-50 Gy, with daily fractions of 2 Gy on 5 consecutive days per week. Chemotherapy consisted of 5-FU (250 mg/m^2 per day) and leucovorin (25 mg/m^2 per day) administered by continuous infusion at night for 5 days a week in the 1st, 3rd and 5th weeks of radiation. Hyperthermia was performed for 2-5 sessions once a week with an 8 MHz radiofrequency capacitive heating equipment (Thermotron-RF 8; Yamamoto Vinita Co. Ltd., Japan). The operation was performed after 2-3 months following HCRT.

LAT1 expression analysis. LAT1 expression was evaluated using pretreatment biopsies by immunohistochemical staining with an affinity-purified polyclonal rabbit anti-human LAT1 antibody (16). An oligopeptide corresponding to amino acid residues 497 to 507 of human LAT1 (CQKLMQVVPQET) was synthesised. The NH$_2$-terminal cysteine residue was introduced for conjugation with keyhole limpet hemocyanin. Antipeptide antibody was produced as described elsewhere (22). For immunohistochemical analysis, antiserum was affinity purified as previously described (22).

Table I. *Patient characteristics.*

Age (years)	37-77 (median, 59)
Gender	male/female=32/12
Tumour location*	Rs/Ra/Rb/P=4/10/29/1
T stage	T2/3/4=6/33/5

*If more than one division was involved, the division where the bulk of the tumor was located was designated as the tumour location. Rs: Rectosigmoid, the portion of the large intestine located between the promontory and the inferior border of the second sacral vertebra; Ra: upper rectum, the portion of the large intestine located between the inferior border of the second sacral vertebra and the level of the peritoneal reflection; Rb: lower rectum, the portion of the large intestine located between the level of the peritoneal reflection and the superior border of the puborectal muscle; P: proctos (anal canal), the tubular portion extending from the superior border of the puborectal muscle to the anal verge.

The detailed protocol for LAT1 immunostaining has been published previously (19). Immunohistochemical staining was performed on paraffin sections using a polymer peroxidase method (Envision+ horseradish peroxidase; Dako Cytomation, Denmark). Briefly, deparaffinised, rehydrated sections were treated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. To expose antigens, sections were microwaved in 10 mmol/l sodium citrate buffer (pH 6.0) for 5 min and cooled for 30 min. After rinsing in 0.05 mol/l TBS containing 0.1% Tween 20, the sections were incubated with affinity-purified anti-LAT1 antibody (1.2 mg/ml; 1:3,200) overnight at 4°C. Thereafter, they were incubated with Envision+ rabbit peroxidase (DAKO, Carpinteria, CA) for 30 min. The peroxidase reaction was performed using 0.02% 3,3'-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 0.05 mol/l Tris-HCl buffer (pH 7.4). Finally, nuclear counterstaining was done with Mayer's haematoxylin. For negative control, the incubation step with the primary antibody was omitted. The specificity of immunoreactions using the anti-LAT1 antibody was established in previous studies (18, 23).

LAT1 expression was considered positive only if distinct membrane staining was present. Staining intensity was scored as follows: 1, <10% of tumour area stained; 2, 10% to 25% stained; 3, 26% to 50% stained; and 4, >51% stained. The tumours in which stained tumour cells made up more than 10 % of the tumour were graded as positive. According to this scoring protocol, two investigators among from the author team, who had no prior knowledge of the clinical data, independently graded the staining intensity in all cases. To test the intraobserver variability, each section was reassessed by the same investigator after the first assessment had been completed. The time interval between the first and second assessments was at least 4 weeks. The interobserver variability was also determined by comparing the values of the first measurements of two investigators.

Histopathological response for HCRT. Each resected specimen was examined for histological changes after HCRT according to the histological criteria of the Japanese Classification of Colorectal Carcinoma (20). Grades were assigned according to the amount of necrosis, degeneration and/or lytic change of the tumour in the estimated total amount of the lesion (Table II). Grading of

Table II. *Histological criteria for the assessment of response to neoadjuvant therapy.*

Grade 0	No effect
	No tumour cell necrosis or degeneration in response to treatment is seen.
Grade 1	Mild effect
	(a) Minimal effect: Tumour cell necrosis or degeneration is present in less than 1/3 of the entire lesion.
	(b) Mild effect: Tumour cell necrosis, degeneration and/or lytic change is present in more than 1/3 but less 2/3 of the entire lesion.
Grade 2	Moderate effect
	Prominent tumour cell necrosis, degeneration, lytic change, and/or disappearance is present in more than 2/3 of the entire lesion but viable tumour cells remain.
Grade 3	Marked effect
	Necrosis and/or lytic change is present throughout the entire lesion and it is replaced by fibrosis with or without granulomatous changes. No viable tumour cells are observed.

histopathological response was performed by a pathologist blinded to the immunohistochemistry results.

Statistical analysis. The association between LAT1 expression and histological response for HCRT was analyzed using the Fisher's exact test. Statistical analysis was performed using StatView J-5.0 Japanese version (HULINKS, Inc. Japan).

Results

LAT1 expression. LAT1 immunostaining was detected in carcinoma cells in tumour tissues and was localised predominantly on their plasma membrane. All positive cells revealed strong membranous LAT1 immunostaining. A positive LAT1 expression was recognised in 50.0% (22/44) of tissues.

Correlation between LAT1 expression and pathological evaluation. The numbers of resected specimens given grades 1a, 1b, 2 and 3 were 5, 10, 20 and 9, respectively. Patients were divided into two groups; those with grade 2 and 3 were considered to have a good response to HCRT, while those with grade 1a and 1b were considered to have a poor response to HCRT. LAT1-negative tumours had an 81.8% probability of good response and 18.2% probability of poor response, while LAT1-positive tumours had a 50.0% probability of good response and 50.0% probability of poor response. LAT1 expression showed marginally significant association with response to HCRT (p=0.05) (Table III).

Discussion

Recently, neoadjuvant chemoradiotherapy has been increasingly used for LARC, and approximately 45% of LARC patients respond to neoadjuvant chemoradiotherapy in terms of downstaging by at least one T stage (24). However, there is no useful molecular biomarker to predict the outcome after neoadjuvant chemoradiotherapy. Thymidylate synthase and epidermal growth factor receptor (*EGFR*) polymorphisms, along with the quantitative

Table III. *Relationship between LAT1 expression and pathological evaluation.*

Pathological evaluation	LAT1 expression		Total
	Positive	Negative	
Good responder	11 (50.0%)	18 (81.8%)	29
Poor responder	11 (50.0%)	4 (18.2%)	15
Total	22	22	

assessment of EGFR and p21, have been shown potential to predict response to treatment in rectal cancer (11). However, it is unclear whether LAT1 expression could predict response and outcome after treatment in patients with rectal cancer. In the present study, the patients with LAT1-positive expression tended to have a poor response for HRCT. The up-regulation of LAT1 has been reported in gliomas, oesophageal carcinomas, non-small cell lung cancer (NSCLC), urothelial carcinomas of the upper urinary tract and prostate cancer (25-30). However, there is no description of the relationship between treatment response and LAT1 expression in these human neoplasms. Further studies should investigate whether LAT1 expression is a biomarker of resistance to treatment in human neoplasms.

LAT1 expression has been associated with the Ki-67 labelling index, indicating an up-regulation of metabolic activity (31). The tumours with LAT1 expression have a tendency for rapid proliferation. In addition, highly proliferative activity can lead to hypoxic components in tumour tissue because of insufficient blood supply. Hoskin *et al*. showed that there is a correlation between hypoxia markers (glucose transporter-1 protein and carbonic anhydrase IX) and Ki-67 in bladder cancer (31).

The present study had some limitations, which are discussed briefly. The use of biopsies rather than whole tumours to assess LAT1 should be interpreted with caution.

Biopsy samples are only parts of the tumour and are not representative of the whole tumour tissue. Another limitation of the study was due to the fact that it was performed retrospectively on a small number of patients. Further investigations with an increased number of cases are required.

References

1 Sauer R, Becker H, Hohenberger W, Rödel C, Wittekind C, Fietkau R, Martus P, Tschmelitsch J, Hager E, Hess CF, Karstens JH, Liersch T, Schmidberger H and Raab R: German Rectal Cancer Study Group: Preoperative *versus* postoperative chemoradiotherapy for rectal cancer. N Engl J Med 351: 1731–1740, 2004.

2 Nelson H and Sargent DJ: Refining multimodal therapy for rectal cancer. N Engl J Med 345: 690–692, 2001.

3 Kapiteijn E, Marijnen CA, Nagtegaal ID, Putter H, Steup WH, Wiggers T, Rutten HJ, Pahlman L, Glimelius B, van Krieken JH, Leer JW and van de Velde CJ: Dutch Colorectal Cancer Group: Preoperative radiotherapy combined with total mesorectal excision for respectable rectal cancer. N Engl J Med *345*: 638-646, 2001.

4 Janjan NA, Khoo VS, Abbruzzese J, Pazdur R, Dubrow R, Cleary KR, Allen PK, Lynch PM, Glober G, Wolff R, Rich TA and Skibber J: Tumor downstaging and sphincter preservation with preoperative chemoradiation in locally advanced rectal cancer: The M.D. Anderson Cancer Center experience. Int J Radiat Oncol Biol Phys *44*: 1027-1038, 1999.

5 Kim JS, Kim JS, Cho MJ, Song KS and Yoon WH: Preoperative chemoradiation using oral capecitabine in locally advanced rectal cancer. Int J Radiat Oncol Biol Phys *54*: 403-408, 2002.

6 Glynne-Jones R and Harrison M: Locally advanced rectal cancer: What is the evidence for induction chemoradiation? Oncologist *12*: 1309-1318, 2007.

7 Crane CH, Skibber JM, Feig BW, Vauthey JN, Thames HD, Curley SA, Rodriguez-Bigas MA, Wolff RA, Ellis LM, Delclos ME, Lin EH and Janjan NA: Response to preoperative chemoradiation increases the use of sphincter-preserving surgery in patients with locally advanced low rectal carcinoma. Cancer *97*: 517-524, 2003.

8 Rödel C, Martus P, Papadoupolos T, Füzesi L, Klimpfinger M, Fietkau R, Liersch T, Hohenberger W, Raab R, Sauer R and Wittekind C: Prognostic significance of tumor regression after preoperative chemoradiotherapy for rectal cancer. J Clin Oncol *23*: 8688-8696, 2005.

9 Valentini V, Coco C, Picciocchi A, Morganti AG, Trodella L, Ciabattoni A, Cellini F, Barbaro B, Cogliandolo S, Nuzzo G, Doglietto GB, Ambesi-Impiombato F and Cosimelli M: Does downstaging predict improved outcome after preoperative chemoradiation for extraperitoneal locally advanced rectal cancer? A long-term analysis of 165 patients. Int J Radiat Oncol Biol Phys *53*: 664-674, 2002.

10 van der Zee J, González González D, van Rhoon GC, van Dijk JD, van Putten WL and Hart AA: Comparison of radiotherapy alone with radiotherapy plus hyperthermia in locally advanced pelvic tumours: a prospective, randomised, multicentre trial. Lancet *355*: 1119-1125, 2000.

11 Kuremsky JG, Tepper JE and McLeod HL: Biomarkers for response to neoadjuvant chemoradiation for rectal cancer. Int J Radiat Oncol Biol Phys *74*: 673-688, 2009.

12 Christensen HN: Role of amino acid transport and countertransport in nutrition and metabolism. Physiol Rev *70*: 43-77, 1990.

13 McGivan JD and Pastor-Anglada M: Regulatory and molecular aspects of mammalian amino acid transport. Biochem J *299*: 321-334, 1994.

14 Oxender DL and Christensen HN: Evidence for two types of mediation of neutral amino acid transport in Ehrlich cells. Nature *197*: 765-767, 1963.

15 Kanai Y, Segawa H, Miyamoto K, Uchino H, Takeda E and Endou H: Expression cloning and characterization of a transporter for large neutral amino acids activated by the heavy chain of 4F2 antigen (CD98). J Biol Chem *273*: 23629-2332, 1998.

16 Yanagida O, Kanai Y, Chairoungdua A, Kim DK, Segawa H, Nii T, Cha SH, Matsuo H, Fukushima J, Fukasawa Y, Tani Y, Taketani Y, Uchino H, Kim JY, Inatomi J, Okayasu I, Miyamoto K, Takeda E, Goya T and Endou H: Human L-type amino acid transporter 1 (LAT1): characterization of function and expression in tumor cell lines. Biochim Biophys Acta *1514*: 291-302, 2001.

17 Fuchs BC and Bode BP: Amino acid transporters ASCT2 and LAT1 in cancer: partners in crime? Semin Cancer Biol *15*: 254-266, 2006.

18 Nawashiro H, Otani N, Shinomiya N, Fukui S, Ooigawa H, Shima K, Matsuo H, Kanai Y and Endou H: L-Type amino acid transporter 1 as a potential molecular target in human astrocytic tumors. Int J Cancer *119*: 484-492, 2006.

19 Kaira K, Oriuchi N, Imai H, Shimizu K, Yanagitani N, Sunaga N, Hisada T, Tanaka S, Ishizuka T, Kanai Y, Endou H, Nakajima T and Mori M: L-Type amino acid transporter 1 and CD98 expression in primary and metastatic sites of human neoplasms. Cancer Sci *99*: 2380-2386, 2008.

20 Japanese Society for Cancer of the Colon and Rectum: Japanese Classification of Colorectal Carcinoma. Japanese Society for Cancer of the Colon and Rectum. Second English Edition. Tokyo, Kanehara & Co., Ltd., 2009.

21 Asao T, Sakurai H, Harashima K, Yamaguchi S, Tsutsumi S, Nonaka T, Shioya M, Nakano T and Kuwano H: The synchronization of chemotherapy to circadian rhythms and irradiation in pre-operative chemoradiation therapy with hyperthermia for local advanced rectal cancer. Int J Hyperther *22*: 399-406, 2006.

22 Chairoungdua A, Segawa H, Kim JY, Miyamoto K, Haga H, Fukui Y, Mizoguchi K, Ito H, Takeda E, Endou H and Kanai Y: Identification of an amino acid transporter associated with the cystinuria-related type II membrane glycoprotein. J Biol Chem *274*: 28845-28848, 1999.

23 Matsuo H, Tsukada S, Nakata T, Chairoungdua A, Kim DK, Cha SH, Inatomi J, Yorifuji H, Fukuda J, Endou H and Kanai Y: Expression of a system L neutral amino acid transporter at the blood–brain barrier. Neuroreport *11*: 3507-3511, 2000.

24 Read TE, McNevin MS, Gross EK, Whiteford HM, Lewis JL, Ratkin G, Picus J, Birnbaum EH, Fleshman JW, Kodner IJ and Myerson RJ: Neoadjuvant therapy for adenocarcinoma of the rectum: Tumor response and acute toxicity. Dis Colon Rectum *44*: 513-522, 2001.

25 Kobayashi K, Ohnishi A, Promsuk J, Shimizu S, Kanai Y, Shiokawa Y and Nagane M: Enhanced tumor growth elicited by L-type amino acid transporter 1 in human malignant glioma Cells. Neurosurgery *62*: 493-504, 2008.

26 Kobayashi H, Ishii Y and Takayama T: Expression of L-type amino acid transporter 1 (LAT1) in esophageal carcinoma. J Surg Oncol *90*: 233-238, 2005.

27 Kaira K, Oriuchi N, Imai H, Shimizu K, Yanagitani N, Sunaga N, Hisada T, Kawashima O, Kamide Y, Ishizuka T, Kanai Y, Nakajima T and Mori M: CD98 expression is associated with poor prognosis in resected non-small-cell lung cancer with lymph node metastases. Ann Surg Oncol *16*: 3473-3481, 2009.

28 Imai H, Kaira K, Oriuchi N, Yanagitani N, Sunaga N, Ishizuka T, Kanai Y, Endou H, Nakajima T and Mori M: L-type amino acid transporter 1 expression is a prognostic marker in patients with surgically resected stage I non-small cell lung cancer. Histopathology *54*: 804-813, 2009.

29 Nakanishi K, Ogata S, Matsuo H, Kanai Y, Endou H, Hiroi S, Tominaga S, Aida S, Kasamatsu H and Kawai T: Expression of LAT1 predicts risk of progression of transitional cell carcinoma of the upper urinary tract. Virchows Arch *451*: 681-690, 2007.

30 Sakata T, Ferdous G, Tsuruta T, Satoh T, Baba S, Muto T, Ueno A, Kanai Y, Endou H and Okayasu I: L-Type amino-acid transporter 1 as a novel biomarker for high-grade malignancy in prostate cancer. Pathol Int *59*: 7-18, 2009.

31 Hoskin PJ, Sibtain A, Daley FM and Wilson GD: GLUT1 and CAIX as intrinsic markers of hypoxia in bladder cancer: relationship with vascularity and proliferation as predictors of outcome of ARCON. Brit J Cancer *89*: 1290-1297, 2003.

32 Takeuchi K, Ogata S, Nakanishi K, Ozeki Y, Hiroi S, Tominaga S, Aida S, Matsuo H, Sakata T and Kawai T: LAT1 expression in non-small cell lung carcinomas: Analyses by semiquantitative reverse transcription-PCR (237 cases) and immunohistochemistry (295 cases). Lung Cancer *68*: 58-65, 2010.