Neoplasia • Vol. 6, No. 1, January/February 2004, pp. 74–84 74

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# L-Type Amino Acid Transporter-1 Overexpression and Melphalan Sensitivity in Barrett's Adenocarcinoma[1]

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## Abstract

**The L-type amino acid transporter-1 (LAT-1) has been associated with tumor growth. Using cDNA microarrays, overexpression of LAT-1 was found in 87.5% (7/8) of esophageal adenocarcinomas relative to 12 Barrett's samples (33% metaplasia and 66% dysplasia) and was confirmed in 100% (28/28) of Barrett's adenocarcinomas by quantitative reverse transcription polymerase chain reaction. Immunohistochemistry revealed LAT-1 staining in 37.5% (24/64) of esophageal adenocarcinomas on tissue microarray. LAT-1 also transports the amino acid–related chemotherapeutic agent, melphalan. Two esophageal adenocarcinoma and one esophageal squamous cell line, expressing LAT-1 on Western blot analysis, were sensitive to therapeutic doses of melphalan ($P < .001$). Simultaneous treatment with the competitive inhibitor, BCH [2-aminobicyclo-(2,1,1)-heptane-2-carboxylic acid], decreased sensitivity to melphalan ($P < .05$). In addition, confluent esophageal squamous cultures were less sensitive to melphalan ($P < .001$) and had a decrease in LAT-1 protein expression. Tumors from two esophageal adenocarcinoma cell lines grown in nude mice retained LAT-1 mRNA expression. These results demonstrate that LAT-1 is highly expressed in a subset of esophageal adenocarcinomas and that Barrett's adenocarcinoma cell lines expressing LAT-1 are sensitive to melphalan. LAT-1 expression is also retained in cell lines grown in nude mice providing a model to evaluate melphalan as a chemotherapeutic agent against esophageal adenocarcinomas expressing LAT-1.**

*Neoplasia* (2004) **6**, 74–84

**Keywords:** Esophageal adenocarcinoma, L-type amino acid transporter-1, amino acid transporters, melphalan, chemotherapy.

## Introduction

The uptake of amino acids is essential for a number of cellular functions including protein synthesis, metabolism, and cell growth. The L-type amino acid transporter-1 (LAT-1) transports large, branched-chain and aromatic, neutral amino acids, including several essential amino acids, and is a member of the sodium-independent amino acid transport system L [1]. Unlike the system L isoform LAT-2, which is more widely expressed, LAT-1 is normally expressed in fetal liver, bone marrow, brain, placenta, and testes [2]. LAT-1 has also been reported to be highly expressed in cultured cells and malignant tumors, and is important in allowing continuous growth [2–5]. All tumor cell lines examined by Yanagida et al. [5] expressed LAT-1 including bladder, small cell lung, cervical, and teratocarcinoma cells. In addition, Ohkame et al. [6] showed a significant relationship between the expression of LAT-1 and the size of hepatic nodules in a model for metastatic rat colon carcinoma.

A remarkable characteristic of the LAT-1 transporter is its broad substrate selectivity that allows it to transport amino acid–related compounds including L-DOPA, thyroid hormones, gabapentin, and the chemotherapeutic agent, melphalan [1,7–11]. In a *Xenopus* oocyte expression system, 100 μM melphalan was able to inhibit 50% of the LAT-1–mediated uptake of [$^{14}$C]phenylalanine [12]. LAT-1 upregulation in tumor cells has been proposed as one of the major routes in the uptake of melphalan into the cell [5]. In addition, the level of system L amino acid transporter activity in tumor cells has been reported to be correlated with sensitivity to melphalan [13,14]. To our knowledge, melphalan has not been previously evaluated as a chemotherapeutic agent in the treatment of esophageal adenocarcinoma.

Over the past two decades, the incidence of esophageal adenocarcinoma has increased greatly, whereas the 5-year survival remains low at <10% [15,16]. Although esophagectomy remains the primary means of treatment, there is an urgent need for both novel therapies and early detection methods. Based on initial cDNA microarray studies suggesting that LAT-1 is overexpressed in Barrett's adenocarcinoma, the present study was undertaken to characterize the expression

Abbreviations: 4F2hc, 4F2 heavy chain; BCH, 2-aminobicyclo-(2,1,1)-heptane-2-carboxylic acid; DAB, diaminobenzadine; DMEM, Dulbecco's modified Eagle's medium; GUS-B, glucuronidase-; LAT-1, L-type amino acid transporter-1; TMA, tissue microarray

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[1]This work was supported by Pharmacia Corporation, National Cancer Institute grant CA71606, and NIH T32 Surgical Oncology Research Training Program CA009672-13.

Received 18 September 2003; Revised 11 November 2003; Accepted 19 November 2003.

of LAT-1 in esophageal adenocarcinomas and in the precursor Barrett's mucosa. Due to its ability to transport melphalan, we also hypothesized that Barrett's adenocarcinoma cell lines expressing LAT-1 would be sensitive to therapeutic doses of melphalan and that this sensitivity would be decreased by the competitive inhibitor 2-aminobicyclo-(2,1,1,)-heptane-2-carboxylic acid (BCH).

## Methods

### *Patients and Tissues*

Written consent and approval of the Institutional Review Board were obtained to collect specimens from patients undergoing esophagectomy at the University of Michigan Medical Center (Ann Arbor, MI). Patients in this study had no preoperative radiation or chemotherapy. Specimens were transported to the laboratory in Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc., Carlsbad, CA) on ice. A portion of each sample was frozen in ornithine carbamoyltransferase (OCT) compound (Miles Inc., Elkhart, IN) for cryostat sectioning. The remainder was frozen in liquid nitrogen and stored at −80°C.

### *Cell Lines*

Nine esophageal cell lines were used in this study. OE33 [17], Seg-1, Bic-1, and Flo-1 were derived from esophageal adenocarcinomas and have been described previously [18]. H00-T, L65-T, and BA1 also originated from esophageal adenocarcinomas, whereas S23-B was derived from Barrett's metaplasia following immortalization with E6/E7 retroviral infection. BA1 was kindly provided by Dr. Rutten (Oregon Health and Science University, Portland, OR). Het-1A is an esophageal squamous cell line immortalized by SV40 infection [19]. All cell lines were grown in DMEM (Life Technologies Inc.) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA) and 1% penicillin/streptomycin/fungizone (Life Technologies Inc.) at 37°C in 5% carbon dioxide/95% air.

### *Tumor Cell Injection in Nude Mice*

Bic-1 and Seg-1 cells ($5 \times 10^6$ cells) were injected subcutaneously into the flank of athymic nude mice at two separate sites. The tumors were excised 4 weeks after injection and frozen at −80°C for later analysis.

### *RNA Extraction and cDNA Microarray*

RNA samples were stored at −80°C. Total RNA was extracted from tissue samples (four samples each for Barrett's, low-grade and high-grade dysplasia, and stage I and II tumors). Prior to RNA isolation, the tissues were either homogenized using a precooled mortar and pestle in a dry ice ethanol bath, or disrupted using a Mixer Mill MM 300 (Retsch GmbH and Co. KG, Haan, Germany). Total RNA was isolated from tumor and Barrett's esophagus samples using the Totally RNA kit for isolation of total cellular RNA (Ambion, Austin, TX) according to the manufacturer's instructions. Poly $A^+$ RNA was purified using the Oligotex mRNA midi kit (Qiagen Inc., Valencia, CA). Each polyA$^+$ RNA sample was linearly amplified using the protocol detailed by Van Gelder et al. [20] with minor modifications. The amplified RNA was reverse-transcribed using MMLV reverse transcriptase, 0.05 pg/μl oligo-dT primer (21mer), 1 × first strand buffer, 0.03 U/μl RNase inhibitor, 500 μM dATP, 500 μM dGTP, 500 μM dTTP, 40 μM dCTP, and 40 μM dCTP-Cy3 (BDS) or dCTP-Cy5 (Amersham Biosciences Inc., Piscataway, NJ). Specific yeast control polyA$^+$ RNA were included as quantitative controls. After incubation at 37°C for 2 hours, each reaction sample (one with Cy3 and another with Cy5 labeling) was treated with 2.5 ml of 0.5 M sodium hydroxide and incubated for 20 minutes at 85°C to stop the reaction and degrade the RNA. Probes were purified using two successive CHROMA SPIN 30 gel filtration spin columns (Clontech, Palo Alto, CA). After combining, both reaction samples were ethanol-precipitated using 1 ml of glycogen (1 mg/ml), 60 ml sodium acetate, and 300 ml of 100% ethanol. The probe was then dried to completion using a SpeedVAC (Thermo Savant, Holbrook, NY) and resuspended in 14 μl of 5 × SSC/0.2% sodium dodecyl sulfate (SDS).

The hybridization of the labeled probes to the cDNA arrays was performed at Incyte Genomics (St. Louis, MO). Briefly, the probe mixture was heated to 65°C for 5 minutes, aliquoted onto the cDNA microarray, and covered with a 1.8-$cm^2$ coverslip. The arrays were incubated for 6.5 hours at 60°C and washed for 10 minutes at 45°C in high-stringency wash buffer (1 × SSC, 0.1% SDS) and three times for 10 minutes each at 45°C in low-stringency wash buffer (0.1 × SSC) before drying. Differential gene expression was detected using a microscope equipped with an Innova 70 mixed gas 10-W laser (Coherent Lasers, Santa Clara, CA) capable of generating spectral lines at 488 nm for excitation of Cy3 and 632 nm for excitation of Cy5. The differential expression values were quality-controlled internally and reported as percent fold increase over a pool of Barrett's esophagus samples. A filtering algorithm was used to select genes that were overexpressed in adenocarcinomas or dysplastic Barrett's mucosa when compared to Barrett's metaplasia samples. Only those genes with overexpression at least twice that of Barrett's metaplasia and in a minimum of two samples were considered significant.

### *Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)*

The level of LAT-1 gene expression was determined by real-time quantitative PCR on an ABI 7900 HT sequence detection system (Applied Biosystems, Foster City, CA). Briefly, the primer sets were designed using Primer 3 (Whitehead Institute/MIT Center for Genome Research, Cambridge, MA) and synthesized by Operon Inc. (Houston, TX). The LAT-1 forward and reverse primers were 5′-CAACCCAGCCCAGTGTAAC-3′ and 5′-GACACCC-TCTGCCGAGTAAT-3′. The glucuronidase-β (GUS-B) forward and reverse primers were 5′-CAGTGCCCATTCC-TATGCCATCGTG-3′ and 5′-ACCTGGACCAGGTTGCT-GAT-3′. cDNA was generated using the ABI Taqman

Reverse Transcription Reagents kit (Applied Biosystems) with RNA extracted from 12 Barrett's metaplasia, 4 low-grade and 10 high-grade dysplasia, and 15 stage I and 13 stage II esophageal adenocarcinoma samples. Gene expression was analyzed using the SYBR green real-time quantitative PCR assay (Applied Biosystems). The standard reaction mix included 350 nM of each primer and 9.1 μl of a 1/10 dilution of the cDNA. GUS-B was used to normalize input RNA concentrations. SYBR green dye detects PCR products by measuring the increase in fluorescence caused by the binding of SYBR green dye to the minor groove of double-stranded DNA. Significant differences of relative quantification were determined using the 2(-delta delta C(T)) method [21]. These data are represented as the fold increases in LAT-1 gene expression normalized to the endogenous reference gene (GUS-B) relative to the Barrett's esophagus control pool.

*RT-PCR*

RNA samples were treated with DNase I (Promega, Madison, WI), and 2 μg of total RNA was reverse transcribed using reverse transcriptase (Life Technologies Inc.) with $(dT)_{18}$ and random hexamers in a 20-μl solution of 10 mM of each dNTP, 5 × reaction buffer, 0.1 M DTT, and RNasin (Promega). PCR amplification of LAT-1 was performed using 1.5 μl of the cDNA products with the forward and reverse primers 5′-TCCCGCGGTGCTGACT-GAG-3′ and 5′-GTTGTGGTGGGTTGTGCTTGAAAA-3′. GAPDH PCR amplification was performed using the primers 5′-AGTCCATGCCATCACTGCCA-3′ and 5′-GGTGTCGCTGTTGAAGTCAG-3′. The following cycling parameters were used: initial denaturing for 2.5 minutes at 95°C and then 40 seconds at 95°C, 1 minute at 59°C, and 1 minute at 72°C for 35 cycles. The PCR products were resolved on a 1% agarose gel with ethidium bromide (0.25 μg/ml), and the products were visualized using UV transillumination.

*Immunohistochemistry and Tissue Microarray (TMA)*

A TMA was constructed, as previously described [22], with formalin-fixed, paraffin-embedded tissues from 70 patients including 64 tumor, 8 lymph node metastases, 8 dysplastic Barrett's mucosa, and 11 nondysplastic Barrett's metaplasia samples. Normal esophagus was also included.

Immunohistochemical staining was performed on the DAKO Autostainer (DAKO, Carpinteria, CA) using DAKO $LSAB^+$ and diaminobenzadine (DAB) as the chromagen. All samples were included on a single TMA and were stained for the same amount of time. Dewaxed and rehydrated sections of the TMA at 4-μm thickness were labeled with LAT-1 antibody (mouse monoclonal antibody, 1:200 dilution; Cosmo Bio Co., Tokyo, Japan) or ASCT-1 antibody (rabbit polyclonal antibody, 1:1000; Chemicon International, Temecula, CA). Microwave citric acid epitope retrieval for 20 minutes was used for both antibodies. Slides were lightly counterstained with hematoxylin. Each sample was then scored using a scale of 0, 1, 2, or 3 corresponding to absent, light, moderate, or intense staining.

*Protein Extraction and Western Blot Analysis*

Protein was extracted from cell pellets and tissue samples using NP-40 detergent lysis buffer and protease inhibitor cocktail (20 μl/1 ml NP-40; Sigma-Aldrich Corp., St. Louis, MO). Fifty micrograms of proteins from each sample was then mixed with one fifth the volume of sample buffer [0.35 M Tris–Cl, pH 6.8, 10% SDS, 30% glycerol, 9.3% dithiothreitol (DTT), and 0.175 mM bromophenol blue] and heat-denatured by boiling for 6 minutes. Extracts were stored at −80°C until use.

The proteins were then electrophoresed in a 10% SDS polyacrylamide gel along with Benchmark Prestained Protein Ladder (Life Technologies Inc.). The separated proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA) overnight at 4°C and then incubated with a blocking solution (0.05% Tween 20, 50 mM Tris, pH 8.0, 150 mM NaCl, and 5% powdered nonfat milk) for 1 hour. The membranes were incubated with either a 1:500 dilution of mouse anti–LAT-1 antibody (Cosmo Bio Co.) or a 1:1000 dilution of rabbit anti–ASCT-1 antibody (Chemicon International) for 1 hour. Secondary antibodies used were 1:5000 dilutions of either a goat anti–mouse (Southern Biotechnology Associates, Birmingham, AL) or anti–rabbit antibody (Vector Laboratories Inc., Burlingame, CA) for an additional hour. Primary antibody binding was visualized using an enhanced chemiluminescence kit (Pierce, Rockford, IL) and Hyperfilm-MP (Amersham International, Buckinghamshire, UK). β-Actin expression was determined with a 1:1000 dilution of an anti–β-actin monoclonal antibody (Abcam, Cambridge, UK) and used as a loading control.

*Treatment of Cell Cultures with Melphalan*

Cell culture treatments were performed as previously described [12,23–25]. Briefly, once cell cultures reached 70% confluence, they were trypsinized and transferred to six-well plates. Each well was plated with $2 \times 10^5$ cells and incubated for 24 hours in DMEM (Life Technologies Inc.) with 10% FBS (Atlanta Biologicals) and 1% penicillin/streptomycin/fungizone (Life Technologies Inc.). For experiments on confluent cultures, $5 \times 10^5$ cells were plated per well and incubated for an average of 3 days until cultures were confluent. Cells were then treated with 5 to 25 μM melphalan (Sigma-Aldrich Corp., St. Louis, MO) dissolved in acidified ethanol (40:1), as previously described [12,23,24]. Control cultures were treated with an equivalent amount of acidified ethanol. After 4 hours, the treatment medium was removed and replaced with fresh media. Cells were allowed to grow for 48 hours before being trypsinized and centrifuged at 6000 rpm. All experiments were repeated in triplicate.

To evaluate responses to the system L competitive inhibitor, BCH (Sigma-Aldrich Corp.), 70% confluent cultures were treated with 0 to 10 mM BCH in 1 N NaOH [2,14]. Five micromolar melphalan was added 10 minutes after BCH, whereas control cultures received an equivalent dose of NaOH and melphalan. Cells were treated with BCH/melphalan for 4 hours after which treatment medium was removed and replaced with fresh media. Cells were then allowed to grow for 48 hours before being trypsinized and

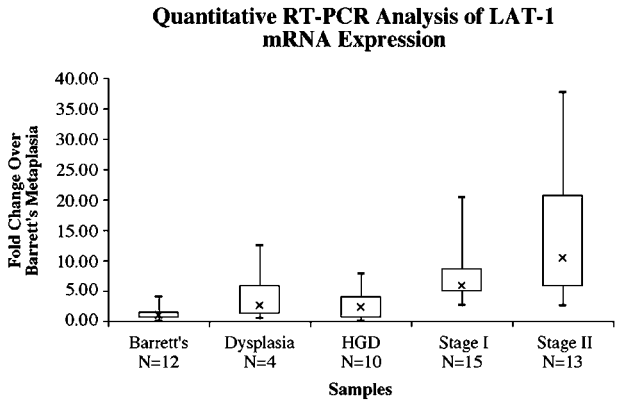


**Figure 1.** *Quantitative RT-PCR confirmed overexpression of LAT-1 in 15 stage I and 13 stage II esophageal adenocarcinomas relative to 12 Barrett's metaplasia samples. The median level of gene expression for each sample type was normalized against the median expression level of the pooled Barrett's metaplasia samples. (Boxed areas represent the first to third quartile. Crossbars indicate maximum and minimum values, and Xs represent median levels.) HGD = high-grade dysplasia.*

centrifuged. Cell pellets were resuspended in phosphate-buffered saline and counted using a hemocytometer. The ratio of live to dead cells was determined using 0.5% trypan blue exclusion and a hemocytometer as previously described [26,27]. All experiments were repeated in triplicate.

### *Statistical Analysis*

Statistical analysis was performed by comparing the experimental group to control cultures using a two-sided Student's *t*-test on Microsoft Excel v. X for the Macintosh.

## Results

### *Expression of LAT-1 mRNA in Barrett's Mucosa and Adenocarcinoma*

When compared to the level of expression in Barrett's metaplasia, cDNA microarray analysis revealed 2- to 16-fold overexpression of LAT-1 mRNA in seven of eight tumor samples including both stage I and stage II tumors. These results were confirmed by quantitative RT-PCR in 100% (28/28) of esophageal adenocarcinomas relative to 12 samples of Barrett's metaplasia with 3- to 38-fold overexpression (Figure 1). There were no differences in expression between Barrett's metaplasia and dysplasia.

### *Analysis of Primary Tissues Using TMA*

Staining of LAT-1 protein on the esophageal TMA is summarized in Table 1. LAT-1 staining was present in 37.5% (24/64) of esophageal adenocarcinomas (Figure 2, *C* and *D*). Light staining was found in Barrett's metaplasia (1/11), dysplasia (1/8), and a normal esophageal sample (Figure 2, *A* and *B*). However, the most intense staining was found in tumor tissues of which 21.9% (14/64) showed moderate or intense staining (Table 2; Figure 2, *C* and *D*). Of the lymph node metastases, 50% (4/8) were positive for LAT-1 staining.

### *Western Blot Analysis of Tumor Tissues and Esophageal Cell Lines*

Western blot analysis confirmed the TMA results showing high LAT-1 protein expression (predicted molecular mass, 55 kDa) in D48-T tumor and moderate amounts in S92-T (Figure 3*A*). Smaller amounts were found in tumors C55-T, S43-T, S36-T, and S68-T, and a sample of Barrett's metaplasia D48-B. In contrast, normal esophagus expressed low levels of LAT-1 protein. Nine esophageal cell lines, including seven lines derived from esophageal adenocarcinoma, one from Barrett's mucosa, and an esophageal squamous cell line, were then evaluated for LAT-1 protein expression by Western blot analysis. As shown in Figure 3*B*, all nine esophageal cell lines showed expression of LAT-1 protein.

### *Analysis of ASCT-1 mRNA and Protein Expression*

During the course of these experiments, the amino acid transporter, ASCT-1, a transporter of small neutral amino acids, was also evaluated. ASCT-1 has been reported by Younes et al. [28] to be overexpressed in esophageal adenocarcinomas. However, on cDNA microarray analysis, no difference was found for ASCT-1 mRNA expression in esophageal adenocarcinoma compared to nondysplastic and dysplastic Barrett's mucosa. On Western blot analysis, ASCT-1 protein was expressed in esophageal adenocarcinoma cell lines Bic-1, Flo-1, Seg-1, and H00-T (Figure 3*C*). However, similar levels of expression were also seen in S23-B derived from Barrett's mucosa. Although staining for ASCT-1 protein was seen in 96.9% (62/64) of esophageal adenocarcinoma samples on TMA immunohistochemistry (Table 2; Figure 2*H*), staining was also seen in all Barrett's metaplasia and dysplasia samples (Table 2; Figure 2, *F* and *G*). In fact, the majority of Barrett's metaplasia (54.5%) and dysplasia (63.6%) samples showed intense staining. Staining was also found in a sample of normal esophagus (Figure 2*E*). Thus, in contrast to LAT-1, ASCT-1 does not appear to be overexpressed in esophageal adenocarcinoma.

**Table 1.** Immunohistochemical Analysis of LAT-1 Expression in Esophageal Tissues Using Tissue Microarray.

| | Light | Moderate | Intense | Total |
|---|---|---|---|---|
| Barrett's metaplasia | 1/11 (9.1%) | 0/11 | 0/11 | 1/11 (9.1%) |
| Dysplasia | 1/8 (12.5%) | 0/8 | 0/8 | 1/8 (12.5%) |
| Esophageal adenocarcinoma | 10/64 (15.6%) | 10/64 (15.6%) | 4/64 (6.3%) | 24/64 (37.5%) |
| Lymph node metastases | 1/8 (12.5%) | 3/8 (37.5%) | 0/8 | 4/8 (50%) |

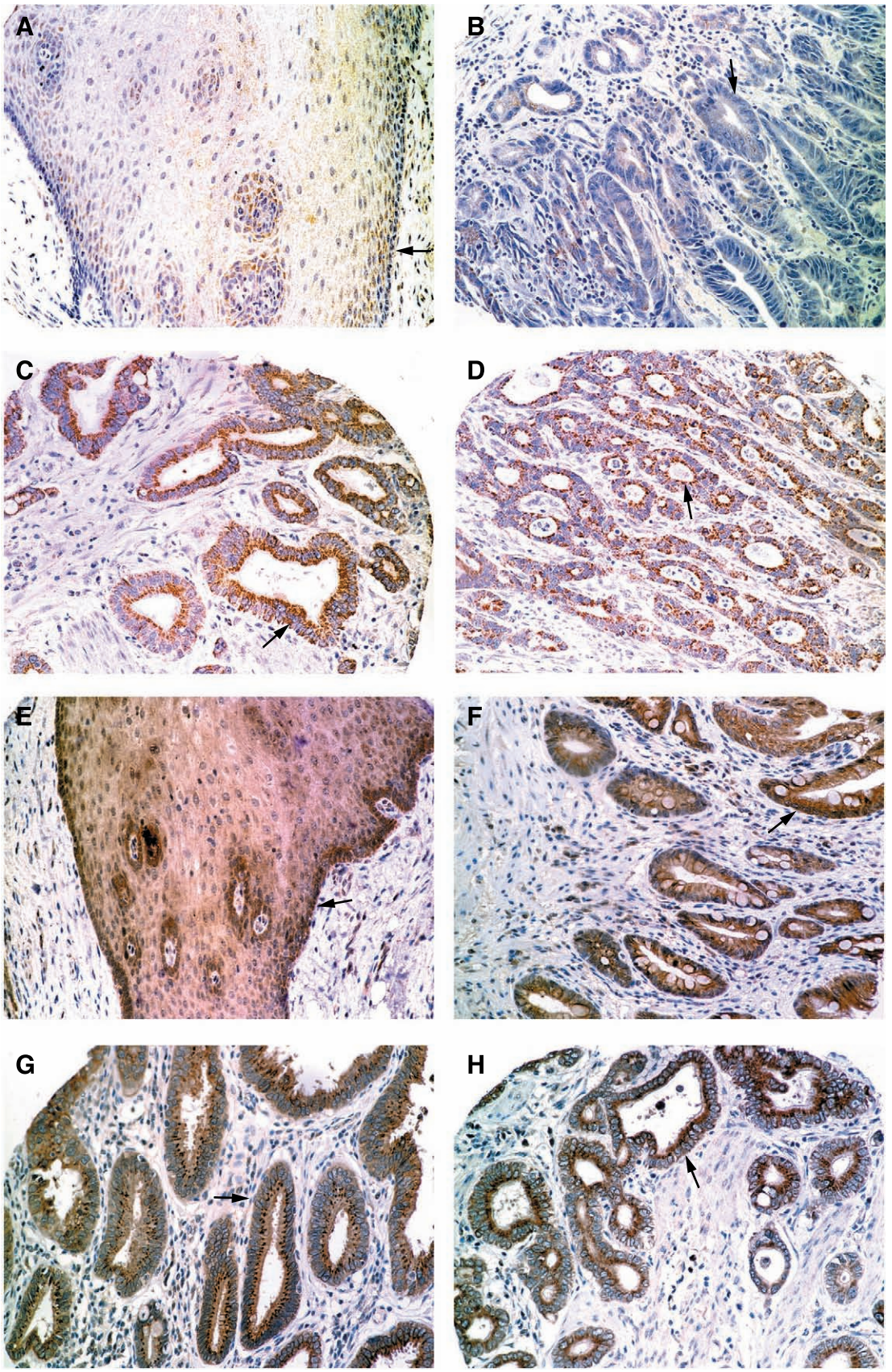


**Figure 2.** *Tissue microarray immunohistochemistry showing (A) light staining of LAT-1 in a sample of normal esophagus; (B) moderate staining in a sample of dysplastic Barrett's mucosa; and (C and D) intense staining in two esophageal adenocarcinoma samples. Intense staining of ASCT-1 was found in (E) normal esophagus, (F) Barrett's metaplasia, (G) Barrett's dysplasia, and (H) esophageal adenocarcinoma. Arrows indicate esophageal mucosa. Original magnifications, ×200. Sections were counterstained with hematoxylin.*

**Table 2.** Immunohistochemical Analysis of ASCT-1 Expression in Esophageal Tissues Using Tissue Microarray.

| | Light | Moderate | Intense | Total |
|---|---|---|---|---|
| Barrett's metaplasia | 2/11 (18.2%) | 3/11 (27.3%) | 6/11 (54.5%) | 11/11 (100%) |
| Dysplasia | 0/11 | 4/11 (36.4%) | 7/11 (63.6%) | 11/11 (100%) |
| Esophageal adenocarcinoma | 27/64 (42.2%) | 25/64 (39%) | 10/64 (15.6%) | 62/64 (96.9%) |
| Lymph node metastases | 1/8 (12.5%) | 3/8 (37.5%) | 2/8 (25%) | 6/8 (75%) |

### *Esophageal Cell Lines Are Sensitive to Therapeutic Doses of Melphalan*

The esophageal adenocarcinoma cell lines, Bic-1 and Seg-1, as well as an SV-40–immortalized esophageal squamous cell line, Het-1A, were sensitive to therapeutic doses of melphalan (5–25 μM) [29] with significant decreases in total cell number and the ratio of live to dead cells ($P < .001$) (Figure 4). Simultaneous treatment with the competitive inhibitor, BCH (5 mM), decreased sensitivity to 5 μM melphalan with total cell numbers and ratio of live to dead cells significantly greater than control cultures ($P < .05$) (Figure 5). Treatment with BCH alone did not result in any significant differences in total cell number or ratio of live to dead cells. In addition, confluent cultures of Seg-1 and Het-1A had a marked decrease in LAT-1 protein expression as determined by Western blot analysis (Figure 6) and were significantly less sensitive to treatment with melphalan ($P < .05$) (Figure 7).

### *LAT-1 Expression Is Maintained in Cell Lines Grown In Vivo*

Two esophageal adenocarcinoma cell lines, Seg-1 and Bic-1, were injected in the flank of athymic nude mice. Immunohistochemical analysis could not be performed because the only commercially available anti–LAT-1 antibody is also produced in mice. However, using a semiquantitative technique with ImageQuant 1.2 normalizing LAT-1 mRNA expression to GAPDH after RT-PCR (Figure 8), LAT-1 mRNA expression was found to be maintained at relatively high levels in tumors derived from Seg-1 (75% of cell culture levels) and Bic-1 (95% of cell culture levels).

## Discussion

LAT-1 is a member of the transport system L and is a sodium-independent, high-affinity transporter of large, branched-chain and aromatic, neutral amino acids [1]. LAT-1 is a hydrophobic protein composed of 507 amino acids with 12 transmembrane domains and a predicted molecular mass of 55 kDa [30]. LAT-1, like other light chain subunits of the heterodimeric amino acid transporters including LAT-2, y+LAT-1, y+LAT-2, xCT, and asc-1, is not able to transport amino acids on its own but must associate with the type II membrane glycoprotein 4F2 heavy chain (4F2hc) to be functional [2]. Although 4F2hc expression is ubiquitous, LAT-1 is normally expressed in fetal liver, bone marrow, brain, placenta, and testes [2,5].

The heterodimeric amino acid transporters have a variety of transport properties. LAT-1 transports large, neutral amino acids such as tryptophan, phenylalanine, leucine,

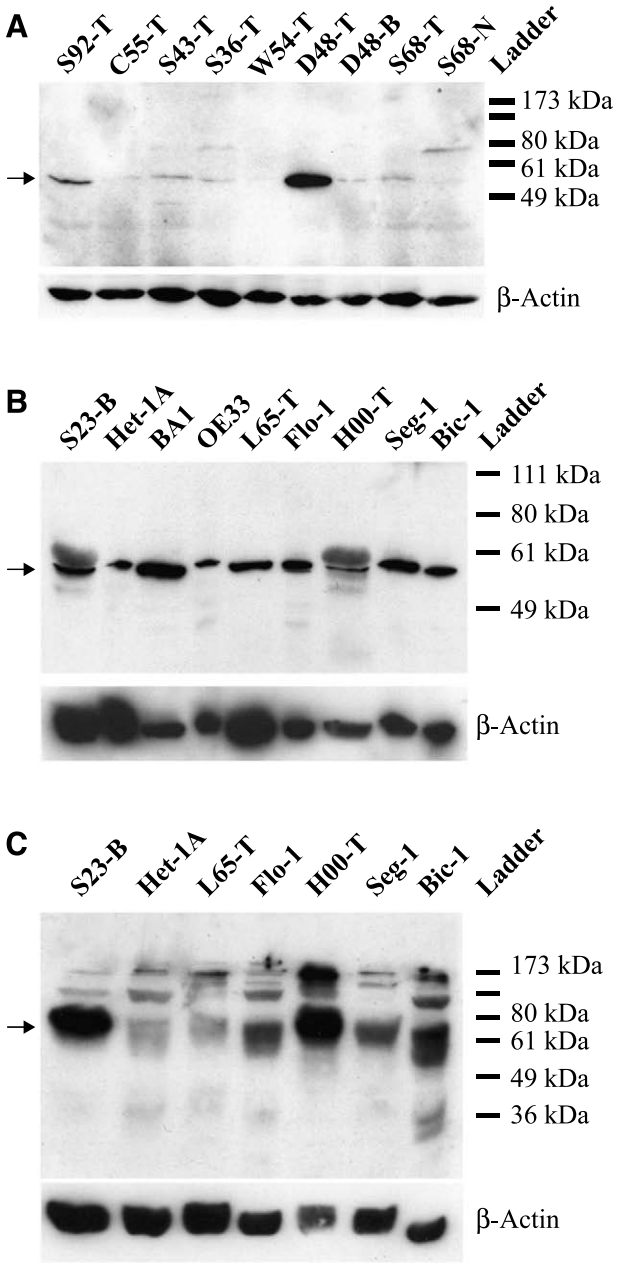


**Figure 3.** *Western blot analysis shows (A) high levels of expression of LAT-1 (arrow, 55 kDa) in esophageal adenocarcinoma sample D48-T and moderate amounts in S92-T. A lower amount of LAT-1 protein is seen in the esophageal adenocarcinoma samples C55-T, S43-T, S36-T, and S68-T, as well as the Barrett's mucosa sample D48-B. (B) Nine esophageal cell lines showing expression of LAT-1 (arrow, 55 kDa) in all cell lines including Het-1A, a squamous cell line, and S23-B, derived from Barrett's mucosa. (C) ASCT-1 (arrow, 55–70 kDa) expression in nine esophageal cell lines including esophageal adenocarcinoma–derived Bic-1, Seg-1, H00-T, and Flo-1 as well as the Barrett's mucosa-derived S23-B. Smaller amounts of ASCT-1 protein are seen in the esophageal adenocarcinoma cell line, L65-T, and the squamous cell line, Het-1A. Higher molecular weight immunoreactive bands were also present in all samples. β-Actin expression was used as a loading control.*

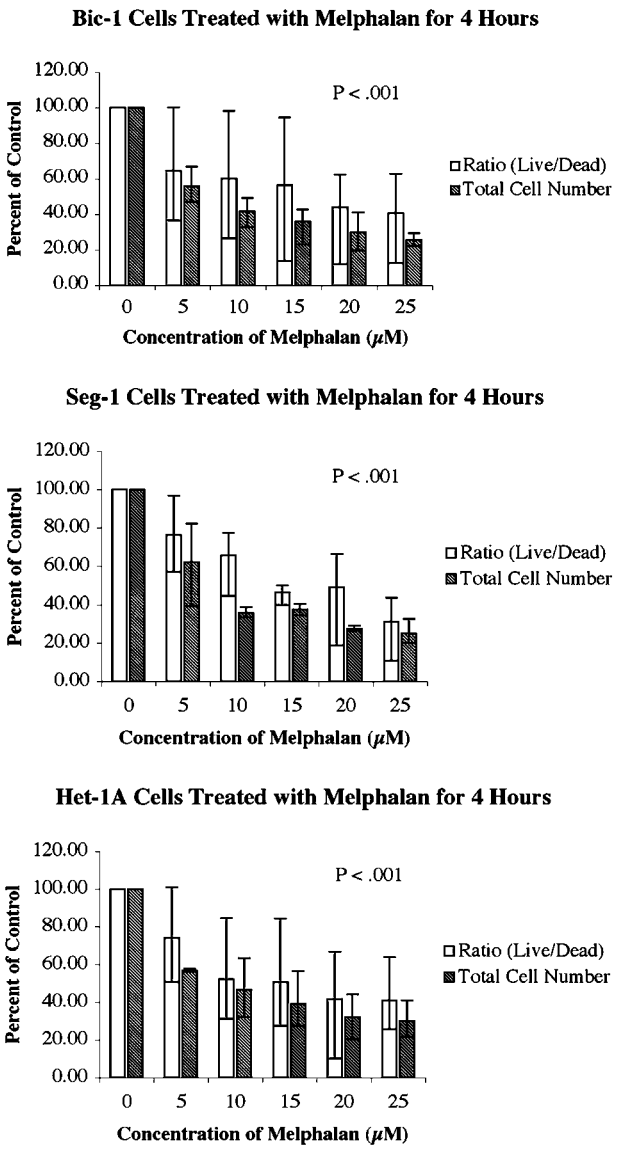


**Figure 4.** *Two esophageal adenocarcinoma–derived cell lines, Bic-1 and Seg-1, as well as an esophageal squamous cell line, Het-1A, were treated with therapeutic doses of melphalan for 4 hours followed by a 48-hour incubation in fresh media. All three cell lines were sensitive to melphalan, with significant decreases in the ratio of live to dead cells and total cell number after treatment. All experiments were repeated in triplicate, with error bars indicating maximum and minimum values.*

and histidine with a high affinity ($K_m$ = ~15–50 μM) and is not affected by pH [2,5,31–33]. LAT-1 has a lower affinity ($K_m$ = ~150–200 μM) for L-glutamine and L-asparagine and is also able to transport D-amino acids such as D-leucine and D-phenylalanine [5]. LAT-1 mediates an exchange of amino acids, and Yanagida et al. [5] have hypothesized that the net influx of amino acids is mediated by an efflux of L-glutamine, which is present at high levels intracellularly. Although LAT-1 transports neutral amino acids, it has been classified with several cationic amino acid transporters in solute carrier family 7 and is officially identified as SLC7A5 [34].

LAT-1 has been associated with growth, proliferation, and tissue development [2–4]. Also identified as CD98, the heterodimeric complex of LAT-1 and 4F2hc is also involved in lymphocyte activation [35,36], and upregulation of CD98 results in tumorigenicity of Balb3T3 fibroblast cells in nude mice [37]. The rat LAT-1 amino acid sequence was found to have 100% identity with the rat integral membrane protein E16 (TA1), which is a highly conserved oncofetal protein involved in carcinogenesis and cell activation [30]. LAT-1 has been found to be highly expressed in cultured cells and malignant tumors, and overexpression of LAT-1 has been proposed as a mechanism allowing continuous cell growth in tumor cells [2–5]. All tumor cell lines examined by Yanagida et al. [5] expressed LAT-1 including bladder, small cell lung, cervical, and teratocarcinoma cells. In addition, Ohkame et al. [6] showed a significant relationship between the

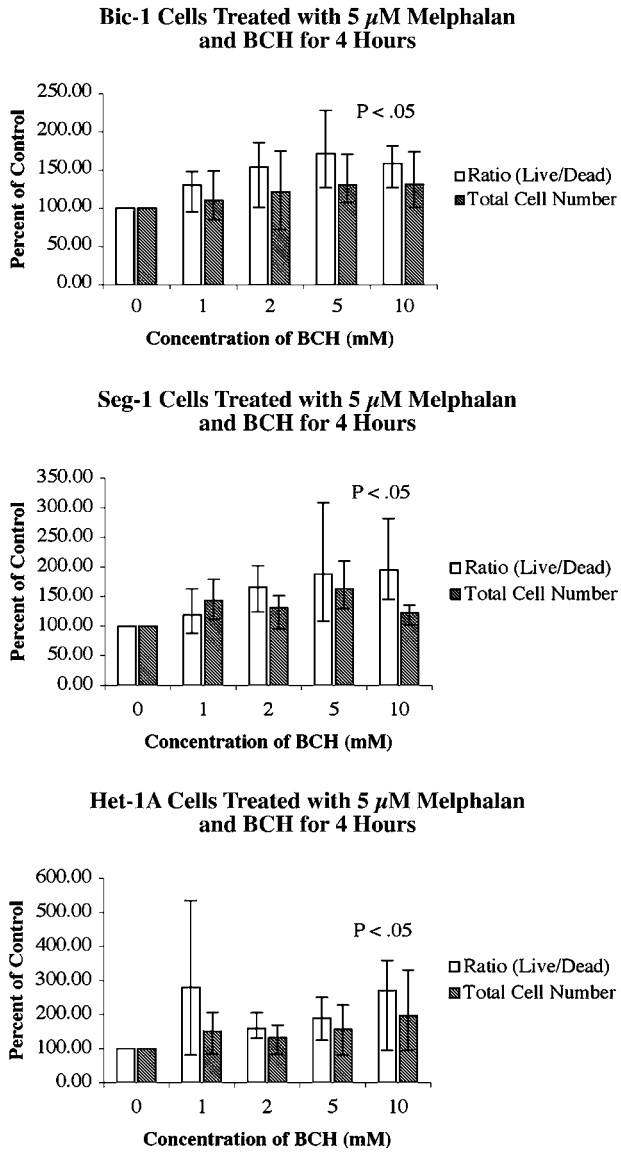


**Figure 5.** *Two esophageal adenocarcinoma–derived cell lines, Bic-1 and Seg-1, and an esophageal squamous cell line, Het-1A, were treated simultaneously with the competitive inhibitor, BCH, and 5 μM melphalan for 4 hours followed by a 48-hour incubation in fresh media. Sensitivity to melphalan was significantly decreased as determined by the ratio of live to dead cells and the total cell number. All experiments were repeated in triplicate, with error bars indicating maximum and minimum values. BCH = 2-aminobicyclo-(2,1,1)-heptane-2-carboxylic acid.*

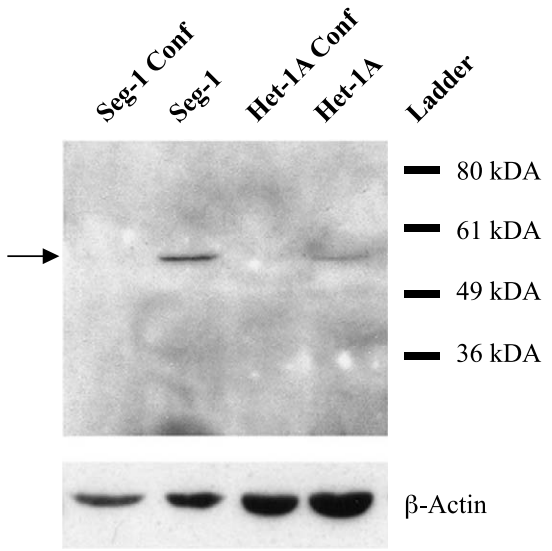


**Figure 6.** *Confluent cultures (Seg-1 Conf and Het-1A Conf) showed markedly decreased LAT-1 (arrow, 55 kDa) protein expression by Western blot analysis of the esophageal adenocarcinoma–derived Seg-1 as well as the esophageal squamous cell line, Het-1A. β-Actin expression was used as a loading control.*

expression of LAT-1 at the protein level and the size of hepatic nodules in a model of rat colon carcinoma.

To our knowledge, LAT-1 expression has not been previously described in esophageal adenocarcinoma. In the current study, LAT-1 mRNA overexpression was found in 87.5% (7/8) of stage I and stage II esophageal adenocarcinomas and was confirmed in 100% (28/28) of esophageal adenocarcinomas between 3- and 38-fold higher than the levels found in Barrett's metaplasia. Meanwhile, LAT-1 protein expression was found in 37.5% (24/64) of esophageal adenocarcinomas (Table 1) using immunohistochemistry of TMA. Differences in expression at the mRNA and protein levels have been reported in the literature for other genes. Our laboratory has previously described the discordance between protein and mRNA expression in lung adenocarcinoma [38]. This difference in expression may be the result of several factors. LAT-1 may be posttranscriptionally regulated through changes in mRNA stability [39], transcript localization [40], or translational efficiency. Differences in expression may also be due to the rate of LAT-1 protein degradation in different samples. In addition, the discordance in mRNA and protein expression may be partially due to differences in sensitivity between mRNA-based and antibody-based assays such as immunohistochemistry.

Although low levels of LAT-1 protein were present in Barrett's metaplasia (1/11), dysplasia (1/8), and a normal esophageal sample on immunohistochemistry, much higher levels were found in tumor tissues, with moderate to intense staining seen in 21.9% (14/64) of esophageal adenocarcinomas (Figure 2, *C* and *D*). All esophageal cell lines in this study including those derived from esophageal adenocarcinoma, Barrett's metaplasia, and normal squamous epithelium expressed LAT-1 protein on Western blot analysis (Figure 3*B*) consistent with previous reports that cultured

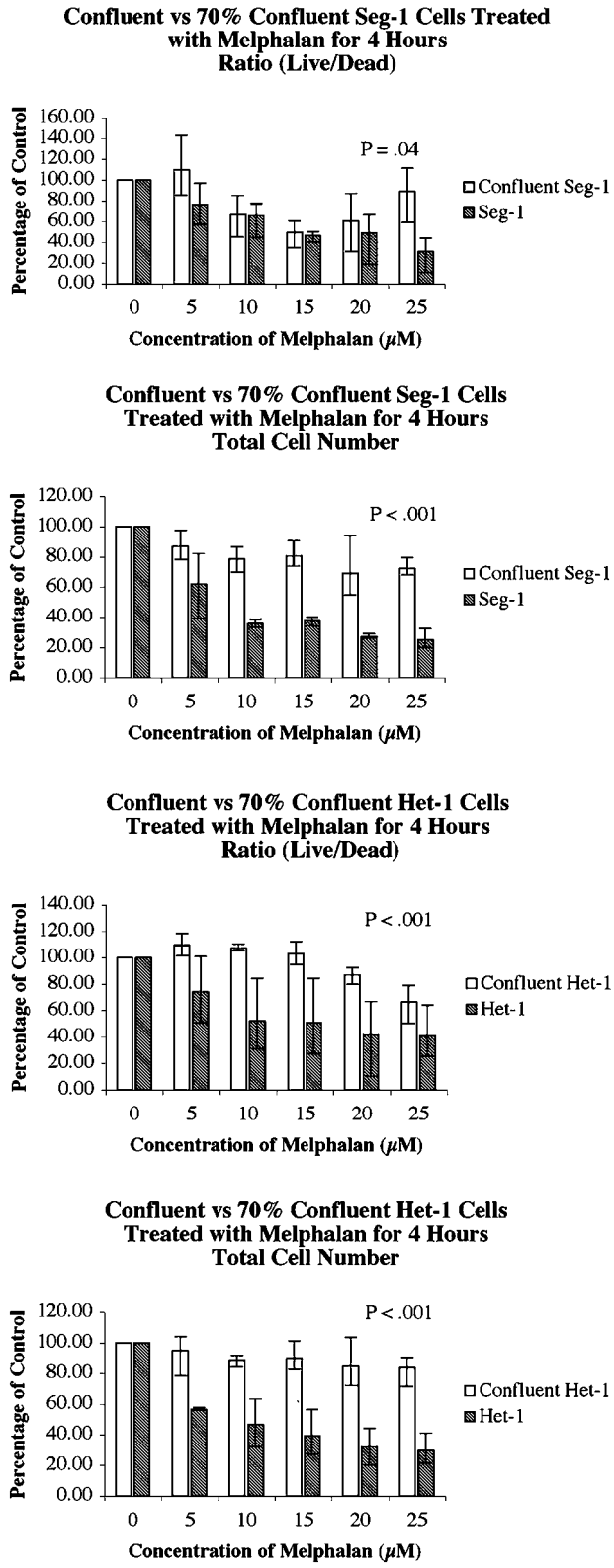


**Figure 7.** *Confluent cultures of the esophageal adenocarcinoma–derived Seg-1 and the esophageal squamous cell line, Het-1A, were treated with therapeutic doses of melphalan for 4 hours followed by a 48-hour incubation in fresh media. Both cell lines were significantly less sensitive to treatment with melphalan as determined by the ratio of live to dead cells and total cell number. All experiments were repeated in triplicate, with error bars indicating maximum and minimum values.*

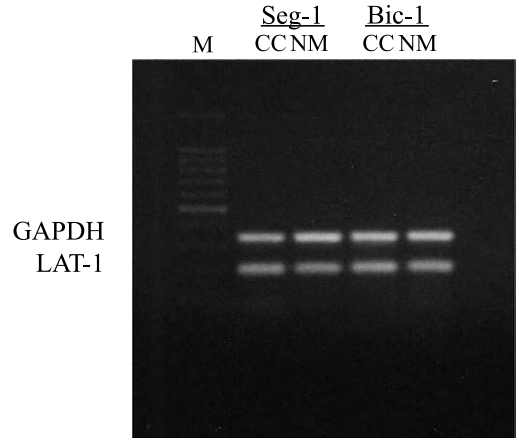


**Figure 8.** *LAT-1 mRNA expression is maintained in tumors derived from two esophageal adenocarcinoma–derived cell lines, Seg-1 and Bic-1, after injection into nude mice as determined by RT-PCR. GAPDH was coamplified as an internal control for each reaction. M=size marker; CC=cell culture; NM=tumors grown in nude mice.*

cells express high levels of LAT-1 [2–5]. On Western blot analysis, LAT-1 was identified as a 55-kDa protein, which is consistent with its predicted molecular weight. Although LAT-1 protein has been described as a 35- to 45-kDa protein on SDS-PAGE [2,5,33], Okamoto et al. [41] also reported a larger 50-kDa band in placental tissues on Western blot analysis, which was confirmed using a blocking peptide. We have also confirmed the size of the LAT-1 band using a second anti–LAT-1 polyclonal antibody (Serotec, Oxford, UK). This size discrepancy may be due to posttranslational modifications such as glycosylation [30].

The expression of another amino acid transporter, ASCT-1, has been described in esophageal adenocarcinoma by Younes et al. [28]. ASCT-1 is a transporter of small neutral amino acids and has been reported to be overexpressed in the majority of esophageal adenocarcinomas. The results of the current study show ASCT-1 protein staining in 96.9% (62/64) of esophageal adenocarcinoma samples (Table 2) and protein expression in several esophageal adenocarcinoma cell lines including Bic-1, Flo-1, Seg-1, and H00-T (Figure 3*C*). However, expression of this transporter was also found in 100% of Barrett's metaplasia (11/11) and dysplasia (11/11) samples (Table 2; Figure 2, *F* and *G*), as well as the Barrett's mucosa-derived cell line, S23-B (Figure 3*C*). In addition, the most intense ASCT-1 staining was seen in Barrett's metaplasia and dysplasia samples. Although ASCT-1 is expressed in esophageal adenocarcinomas, its expression may be reflective of the cell type of origin, rather than a critical role in the growth, proliferation, and development of esophageal adenocarcinoma.

An interesting characteristic of the LAT-1 transporter is its broad substrate selectivity, allowing it to transport amino acid–related compounds including L-DOPA, thyroid hormones, gabapentin, and the chemotherapeutic agent, melphalan [1,7–11]. Melphalan, or L-phenylalanine mustard, is an alkylating agent that was first synthesized in 1953 [42]. Vistica et al. [43] demonstrated that the uptake of melphalan is an active process with an approximately 10-fold difference between intracellular and extracellular levels. Goldenberg et al. [8] identified two amino acid carrier systems involved in the uptake of melphalan. One is a system L sodium-independent transporter inhibited by BCH, whereas the other is sodium-dependent, inhibited by alanine, serine, and cysteine, and appears to be a system ASC-like amino acid transporter.

LAT-1 upregulation in tumor cells has been identified as one of the major routes in the uptake of melphalan [5]. Begleiter et al. [44] have reported a $K_m$ of 80 μM for system L–mediated uptake of melphalan, and in a *Xenopus* oocyte expression system, 100 μM melphalan was able to inhibit 50% of the LAT-1–mediated uptake of [$^{14}$C]phenylalanine [12]. In addition, the level of system L amino acid transporter activity in tumor cells has been reported to correlate with melphalan sensitivity [13,14]. Downregulation of LAT-1 (CD98) has also been found in melphalan-resistant myeloma cells with reduced melphalan uptake [13].

Melphalan has been utilized in the treatment of multiple myeloma, ovarian and breast carcinoma, sarcoma, and metastatic melanoma, and the most common side effect is bone marrow suppression. To our knowledge, melphalan has not been previously described in the treatment of esophageal adenocarcinomas. We hypothesized that esophageal adenocarcinoma cells expressing LAT-1 would be sensitive to melphalan. To evaluate the sensitivity of esophageal cells to melphalan, two esophageal adenocarcinoma cell lines, Bic-1 and Seg-1, and an esophageal squamous cell line, Het-1A, were treated with 5 to 25 μM melphalan. Although doses as high as 50 to 100 μM [12,23] have been used in cell culture studies in the literature, this dosage range was chosen as more clinically relevant. According to Anderson et al. [29], patient plasma levels range from 2.3 to 54 μM, with higher doses being used for bone marrow ablative therapy.

The esophageal adenocarcinoma–derived cell lines, Bic-1 and Seg-1, as well as the squamous cell line, Het-1A, expressed LAT-1 (Figure 3) and were sensitive to therapeutic doses of melphalan. Significant decreases in total cell number and the ratio of live to dead cells were seen after a single treatment with 5 to 25 μM melphalan ($P < .001$) (Figure 4). A dose response was found, with total cell numbers decreased up to 75% and the ratio of live to dead cells up to 69% after treatment with 25 μM melphalan.

Simultaneous treatment with BCH (5 mM), a competitive inhibitor of the system L amino acid transporter, decreased sensitivity to 5 μM melphalan, with total cell numbers and the ratio of live to dead cells significantly greater than control cultures ($P < .05$; Figure 5). By inhibiting uptake through LAT-1, BCH is able to decrease sensitivity to melphalan, confirming the importance of LAT-1 in the uptake of melphalan as previously reported [5,12–14]. Treatment with BCH alone did not result in significant differences in cell number or the ratio of live to dead cells, indicating that either adequate LAT-1 function remains despite inhibition with BCH, or that other amino acid transport systems are able to compensate, providing nutrients to support continuous cell growth and proliferation.

Although proliferating esophageal squamous Het-1A cells express LAT-1 and are sensitive to melphalan, confluent cultures may be a more accurate reflection of the majority of esophageal squamous epithelium *in vivo* and were significantly less sensitive to treatment with melphalan ($P < .001$; Figure 7). Total cell number and the ratio of live to dead cells in confluent cultures were approximately 40% to 50% greater when compared to 70% confluent cultures treated with 5 μM melphalan. Although confluent cells are no longer rapidly proliferating and may be expected to be less sensitive to a variety of chemotherapeutic agents due to their decreased growth rate, melphalan, like most bifunctional alkylating agents, is cell cycle–independent and is active against both resting and rapidly dividing cells [45]. An alternative mechanism for lower melphalan sensitivity in confluent Het-1A cells is a decrease in the uptake of melphalan due to the markedly reduced levels of LAT-1 protein seen on Western blot analysis (Figure 6). These findings agree with results previously reported by Blosmanis et al. [46] evaluating melphalan sensitivity of BALB/c 3T3 fibroblasts. Proliferating cells were found to have a threefold higher uptake of melphalan than nondividing cells with a progressive decrease in uptake the longer cells were kept in the resting phase. They conclude that the lower cytotoxic activity of melphalan in resting cells was due to the decreased level of melphalan uptake.

LAT-1 is highly expressed in a subset of esophageal adenocarcinomas, and Barrett's adenocarcinoma cell lines expressing LAT-1 are sensitive to melphalan. These results suggest that treatment with amino acid nitrogen mustards like melphalan may be beneficial in a subset of patients with Barrett's adenocarcinomas expressing LAT-1. This study also shows that LAT-1 mRNA expression is maintained in tumors derived from two esophageal adenocarcinoma cell lines, Seg-1 and Bic-1, after injection in the flank of athymic nude mice as determined by RT-PCR (Figure 8). These findings provide a potential animal model that could be used to further study treatment with melphalan in hopes of finding an effective therapy against esophageal adenocarcinoma, for which few successful treatments are currently available.

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