SLC7A5 is linked to increased expression of genes related to proliferation and hypoxia in estrogen-receptor-positive breast cancer

RASMUS TÖRNROOS[1], ELISABET TINA[1,2] and ANNA GÖTHLIN EREMO[1,2]

[1]School of Medical Sciences and [2]Department of Clinical Research Laboratory, Faculty of Medicine and Health, Örebro University, SE-70185 Örebro, Sweden

Received June 11, 2021; Accepted October 25, 2021

DOI: 10.3892/or.2021.8228

Abstract. The amino acid transporter named solute carrier family 7 member 5 (SLC7A5) is suggested to play a part in altered cell metabolism and proliferative signaling and has been reported to be overexpressed in various types of cancer, including breast cancer. Estrogen-receptor-positive (ER^+) breast cancers constitute the most common type of breast malignancies and are often treated with anti-estrogenic therapies. In this group of patients, endocrine resistance is a challenging problem that could lead to recurrent disease. To overcome this, additional prognostic biomarkers are needed. The present study aimed therefore to determine whether SLC7A5 may be considered as a possible prognostic marker in ER^+ breast cancer and to investigate its relation with certain cancer-related genes. We used a local breast cancer cohort (n=154) and immunohistochemistry to analyze the expression of SLC7A5 in association with clinicopathological characteristics and patient outcome. In addition, gene expression analysis was performed on 80 of these tumors. Furthermore, the METABRIC dataset was used for correlation analyses between expression of SLC7A5 and several genes related to breast cancer biology. The results demonstrated that overexpression of SLC7A5 was significantly associated with histopathological grade in patients with breast cancer, and that SLC7A5 mRNA expression was positively correlated with the expression of marker of proliferation Ki-67 and hypoxia inducible factor 1 subunit alpha. Overexpression of SLC7A5 may therefore play a role in the biology of endocrinologically-driven disease. However, when further assessing SLC7A5 using the METABRIC dataset, SLC7A5 mRNA expression level was more significantly increased in ER- subgroups compared with ER^+ disease. All breast cancer subtypes included, SLC7A5 mRNA expression was correlated with a higher number of cancer-related genes than in estrogen receptor positive tumors alone. The present study suggested that SLC7A5 expression may be of importance for breast cancer cell proliferation and survival. In order to further establish the biological and clinical role of SLC7A5 in breast cancer, further investigation using different breast cancer subgroups is required.

Correspondence to: Dr Anna Göthlin Eremo, Department of Clinical Research Laboratory, Faculty of Medicine and Health, Örebro University, Campus USÖ Floor 3, Södra Grev Rosengatan 30, SE-70185 Örebro, Sweden
E-mail: anna.gothlin-eremo@regionorebrolan.se

Key words: breast cancer, large neutral amino acid-transporter 1, solute carrier family 7 member 5, hypoxia, proliferation

Introduction

Breast cancer is one of the most prevalent malignancies worldwide, accounting for 11.7% of all new cancer cases in 2020 (1). Breast malignancies constitute a group of diagnoses that each presents with different characteristics. Current practice subdivides tumors according to expression of certain molecular markers, and this subdivision is used to guide the therapeutic approach (2). Of these general subgroups, estrogen-receptor-positive (ER^+) breast cancers represent almost 2/3 of all cases (3). ER^+ patients are eligible for endocrine therapy, including some agents such as tamoxifen, which aims to inhibit tumor growth by interfering with proliferative estrogen signaling (4). In ER^+ patients, disease progression despite anti-estrogenic treatment may be suggestive of either intrinsic or acquired endocrine resistance. The underlying mechanisms of such resistance are not yet fully understood, although alterations in various signaling pathways, including estrogen-signaling, seem to be of importance (5). As not all patients benefit from current treatment regimens, it is crucial to develop novel risk assessment tools capable of early identification of tumor characteristics associated with aggressiveness, including endocrine resistance in hormone-sensitive cases.

One novel protein of interest is named solute carrier family 7 member 5 (SLC7A5), which is a transmembrane amino acid transporter also known as large neutral amino acid-transporter 1 (LAT1). SLC7A5 has been described in several types of cancer, including breast cancer, and functions by facilitating the exchange of essential amino acids over the cell membrane (6,7). The connection between overexpression of SLC7A5 and cancer has been associated with alterations in cell signaling and metabolic homeostasis, which are both

considered as important hallmarks of cancer (8). SLC7A5 has also been reported to be induced by hypoxia in several tissues, including renal cell carcinoma, suggesting an association between SLC7A5 expression and changes in tumor environment and cell demands (9).

Although largely independent of physiological restrictions, readily proliferating tumors are still to some degree constrained by nutrient and oxygen deprivation as they ultimately outgrow their supporting blood vessels (10). As local tissue hypoxia arises, malignant cells are forced to rearrange their genetic programming, and since exogenous amino acids are essential for the synthesis of various proteins and nucleic acids, tumors might start to overexpress amino acid exchangers in order to gain access to a ready supply of building blocks to sustain their proliferation and existence (11,12). In the case of the amino acid exchanger SLC7A5, the protein also appears to be directly involved in proliferative signaling by facilitating the uptake of leucine, which is an essential activator of the mTORC1 signaling complex (13). SLC7A5 is therefore likely to influence tumor cell behavior and may ultimately contribute to disease progression. Thus, SLC7A5 may be considered as a promising biomarker candidate and a novel future therapeutic target.

Based upon the nature of SLC7A5, we hypothesized that upregulation of SLC7A5 may function as a way for breast cancer cells to prolong their lifespan under conditions that are not suitable for proliferation, such as during hypoxia or in conjunction with impaired cell signaling during anti-estrogenic treatment. To confirm this hypothesis, we aimed to study the gene and protein expression of SLC7A5 in ER^+ breast cancer using immunohistochemistry and microarray analysis. The present study discussed the prognostic and biological role of SLC7A5 in ER^+ breast cancer by determining how SLC7A5 protein expression may be associated with various clinicopathological characteristics linked to patient outcome. The present study also explored the correlation between SLC7A5 mRNA expression and the mRNA levels of certain genes linked to breast cancer biology such as estrogen receptor 1 (ESR1), Erb-B2 receptor tyrosine kinase 2 (ERBB2), MYC proto-oncogene, BHLH transcription factor (MYC) and mechanistic target of rapamycin (MTOR), to cell proliferation such as marker of proliferation Ki-67 (MKI67) and cyclin D1 (CCND1), to hypoxia such as hypoxia inducible factor 1 subunit alpha (HIF1A), endothelial PAS domain-containing protein 1 (EPAS1 or HIF2A) and vascular endothelial growth factor A (VEGFA), and to metabolism such as lactate dehydrogenase A (LDHA), solute carrier family 2, facilitated glucose transporter member 1 (SLC2A1 or GLUT1), carbonic anhydrase 9 (CA9) and NDUFA4 mitochondrial complex associated like 2 (NDUFA4L2).

Materials and methods

Patients and breast cancer specimens. The present study included female patients diagnosed with breast cancer at the Department of Oncology, Örebro University Hospital, Sweden, between January 2000 and December 2010. Patients were identified from the registry of the Regional Cancer Centre Uppsala Örebro, Sweden. Information regarding the patients' primary tumor characteristics was retrieved from the registry, along with clinical data. This study was approved by the regional ethics committee in Uppsala (2011/070) and written consent was obtained from participants who were still alive. All procedures were performed in accordance with the ethical standards of the regional ethics committee and with the 1964 Declaration of Helsinki and its later amendments.

The patients included in the present study (n=154) were all diagnosed with an ER^+ tumor and treated with endocrine therapy, and had no distant metastasis at the time of diagnosis. Records from registry were first retrieved in 2013, when a total of 116 eligible patients were included (patients with recurrence, n=27; recurrence-free patients, n=89). The selection procedure for these patients has been previously described in detail (14). To minimize the risk of including patients with undetected metastasis at the time of primary diagnosis, a cut-off of 24 months was used to distinguish recurrence. Updated records were retrieved from the registry in 2017, and additional patients (patients with recurrence, n=12; recurrence-free patients, n=26) were selected and included in the study based on the same inclusion criteria. The 154 patients were subsequently referred to as the Örebro cohort.

Immunohistochemistry (IHC). Formalin-fixed paraffin-embedded primary breast tumor tissues were available from 152 out of the 154 patients. The protein expression of SLC7A5 was evaluated using IHC on 4-μm sections mounted on glass slides. These tissues were stained as previously described (15). Briefly, tissues were deparaffinized and rehydrated using Tissue-Clear® (Sakura Finetek Europe) followed by a series of ethanol (100, 95 and 70%) and deionized water. Heat-induced epitope retrieval was performed in DIVA Decloaker buffer (Biocare Medical) in a decloaking chamber (Biocare Medical) for 10 min at 110˚C. The staining procedure was then performed using the automated slide stainer IntelliPATH FLX™ (Biocare Medical). For protein detection, a rabbit monoclonal anti-SLC7A5 antibody (cat. no. ab208776; 1:500; 1 h incubation at room temperature; Abcam) was used together with the MACH 1 Universal HRP-Polymer Detection system (Biocare Medical) and Betazoid DAB chromogen according to the manufacturer's instructions. The slides were washed with TBS Automation Wash Buffer (Biocare Medical) in between each reaction. Following subsequent counterstaining with Mayer's haematoxylin, the tissues were dehydrated in ethanol (70, 95 and 100%) and xylene and mounted with coverslips using Pertex® mounting medium. For each run of the staining process, a tissue sample (testis) stained for SLC7A5 protein expression was included as a positive control.

Evaluation of IHC staining. The slides were digitally scanned using the Pannoramic 250 Flash II scanner (3DHistech Ltd.) and the resulting images were analyzed using the CaseViewer software package version 2.4 (3DHistech Ltd.). SLC7A5 protein staining was graded according to HercepTest™ (Dako) scoring guidelines, originally intended for assessment of human epidermal growth factor receptor 2 (HER2)-positivity (Table I). Staining was graded as 0, 1+, 2+ or 3+ according to membrane staining intensity, whether the staining was complete or incomplete, and the amount of positively stained tumor cells in the tissue specimen. Scores of 0 or 1+ were considered as negative while scores of 2+ and 3+ were considered as positive. Grading was performed by two separate observers and final grades were based on consensus scores.

Table I. Immunohistochemistry SLC7A5 protein expression scoring guidelines adapted from the HercepTest™ (Dako) guidelines for human epidermal growth factor receptor 2 protein expression assessment.

Score to report	Protein overexpression assessment	Staining Pattern
0	Negative	No staining is observed, or membrane staining is observed in < 10% of the tumor cells.
1+	Negative	A faint/barely perceptible incomplete membrane staining is detected in > 10% of tumor cells.
2+	Weakly positive	A weak to moderate complete membrane staining is observed in > 10% tumor cells
3+	Strongly positive	A strong complete membrane staining is observed in > 10% of the tumor cells

Microarray. For 80 patients, mRNA gene expression data were obtained through microarray analysis performed on a previous occasion (data not shown). Of these, 78 specimens were also available for IHC staining of SLC7A5. Briefly, freshly frozen primary tumor tissues (stored at -80˚C) with confirmed tumor cell content were homogenized using a TissueLyser II (Qiagen AB) with 5 mm steel beads (Qiagen AB) for 2x2 min at 30 Hz. Lysing and RNA extraction was performed using the Allprep DNA/RNA/Protein mini kit (Qiagen AB). RNA concentrations were evaluated and purity determined according to the absorbance ratio at A260, A280 and A230 nm using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). Values of A260/A280 between 1.8 and 2.1 and A260/A230>2.0 were considered as acceptable purity for microarray. RNA integrity number (RIN) values were determined using the Agilent RNA 6000 Nano Kit for the 2100 Bioanalyzer Instrument (Agilent Technologies, Inc.), with RIN>8 considered as an acceptable quality for subsequent microarray analysis. The RNA was stored at -80˚C.

Microarray data acquisition. High-quality RNA (100 ng) was used to prepare Cy3-labeled and amplified cRNA with the One Color Low Input Quick Amp Labeling Kit (Agilent Technologies, Inc.) according to the manufacturer's instructions. The cRNA concentration and specific activity (pmol Cy3/μg cRNA) was determined using NanoDrop. Following the Gene Expression Hybridization Kit (Agilent Technologies, Inc.) protocol, the samples were hybridized to SurePrint G3 Human Gene Expression 8x60k v2 Microarrays (Agilent Technologies, Inc.) for 17 h at 65˚C in a Hybridization Oven (Agilent Technologies, Inc.). The microarrays were scanned with a G2565CA Microarray Scanner (Agilent Technologies, Inc.) and image analysis and data extraction were assessed with version 10.7.3.1 of Agilent's Feature Extraction Software package (Agilent Technologies, Inc.). All microarrays were verified and passed quality check parameters included in the software package prior to data analysis. Before extracting gene expression data for SLC7A5, ESR1, ERBB2, CCND1, MYC, HIF1A, MKI67, VEGFA, MTOR, EPAS1, LDHA, SLC2A1, CA9 and NDUFA4L2, the arrays underwent quantile normalization and ComBat correction to account for batch variations dependent of sample distributions on different slides (SVA R package) prior to log2-transformation. The normalized fluorescence intensity values for each gene were further transformed into z-values prior to statistical analysis.

METABRIC data. The METABRIC dataset, with both clinical and gene expression data, was downloaded from cBioPortal (16,17). Collection of the METABRIC study specimens was ethically approved by the institutional review board and the METABRIC study protocol was approved by ethics committees in Vancouver and Cambridge. The study was previously described in detail (18). Briefly, tumor RNA from >2,000 breast cancer patients was analyzed using the Illumina HT-12 v3 platform (Illumina_Human_WG-v3). Gene expression data (mRNA expression z-values relative to diploid samples) were available from 1,904 breast cancer patients in total, although one patient with breast sarcoma was excluded, leaving a remaining pool of 1,903 patients.

Statistical analysis. For examining the associations between SLC7A5 protein expression and the clinicopathological characteristics in the Örebro patient cohort, the χ^2 or Fisher's exact tests were used. The Mann-Whitney U test was used for continuous variables. Survival curves were generated by Kaplan-Meier estimation using the log-rank test with distant recurrence set as primary patient outcome. The hazard ratio with 95% confidence interval (CI) was calculated using Cox regression.

Prior to gene correlation analysis, the mRNA microarray data (fluorescence intensity values) were z-transformed, with a z-value of 0 representing the mean and scores of ±1 equaling 1 standard deviation from the mean. Normal distribution of SLC7A5 expression was evaluated using Shapiro-Wilk test, arguing for the use of non-parametric tests for analysis of SLC7A5 correlation to other assessed genes as well as clinical data from both the Örebro cohort and METABRIC data. The correlation between z-values (gene expressions) was estimated using Spearman's rank correlation coefficient (ϱ). The Mann Whitney U-test was used to compare SLC7A5 gene expression with the corresponding protein expression and various clinical variables. One-way ANOVA and Kruskal-Wallis test were used to compare multiple categorical clinical variables. Data analysis was performed using SPSS software version 7.03 (SPSS, Inc.) and GraphPad Prism version 7.03 (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characterization of SLC7A5 expression in ER$^+$ breast cancer tissues. Immunohistochemical staining of SLC7A5 was evaluated in all available cases (n=152). The overall protein expression was found to be heterogeneous, and staining varied

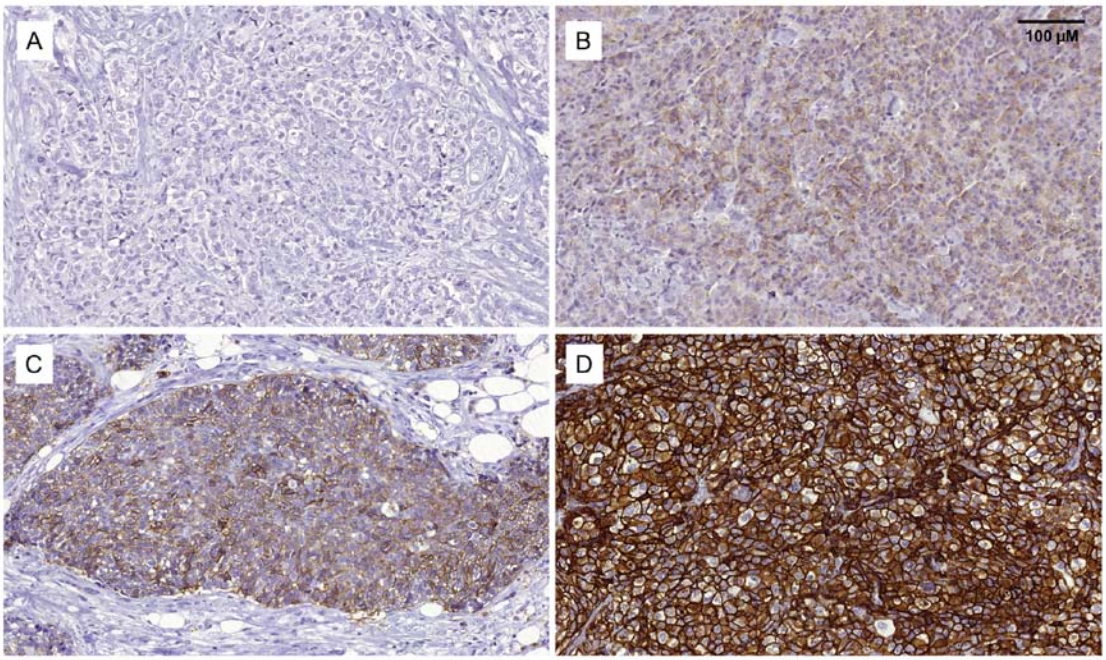

Figure 1. Representative images of SLC7A5 protein staining (magnification, x20). (A) Tumor tissue showing no observable staining (=0). (B) Tumor cells showing weak but incomplete membrane staining (=1+). (C) Tumor tissue showing complete but moderate staining (=2+). (D) Tumor cells showing strong complete membrane staining throughout the tissue specimen (=3+). Scale bar, 100 μm.

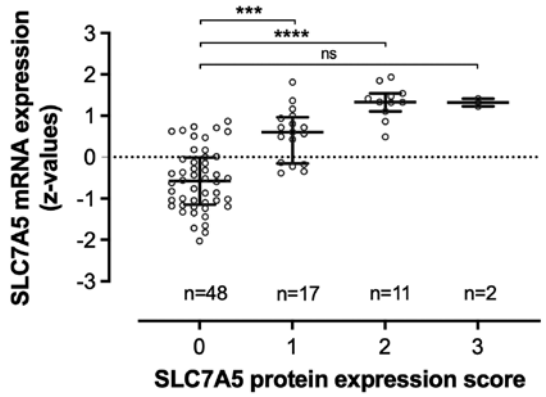

Figure 2. Association between SLC7A5 mRNA expression and SLC7A5 protein expression score in the Örebro cohort patients (n=78). SLC7A5, solute carrier family 7 member 5. ***p≤0.001, ****p≤0.0001 and ns, non-significant (p>0.05) values.

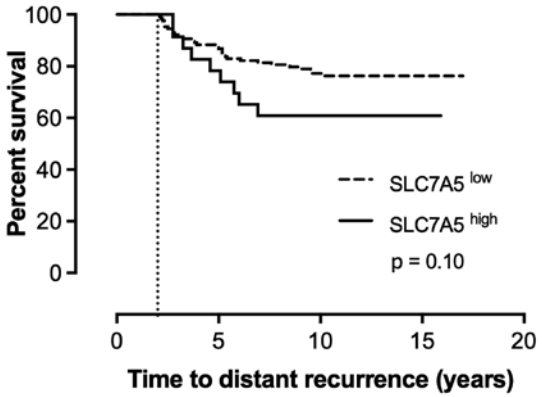

Figure 3. Association between SLC7A5 protein expression and time to distant recurrence. SLC7A5^{low} (n=129; score 0-1) compared with SLC7A5^{high} (n=23; score 2-3). P-value was calculated using the log-rank test. Vertical dotted line symbolizes the 24 months up to when all included patients were recurrence free. SLC7A5, solute carrier family 7 member 5.

in intensity within and between tumors. Representative images of protein staining are provided in Fig. 1, where ~15% of the cases were found to be positive (2+ and 3+) for SLC7A5 protein expression (Table SI).

SLC7A5 mRNA z-values from the microarray analysis (n=80, of which 78 cases were available for IHC staining) were found to be normally distributed (Table SII). The gene expression of SLC7A5 was significantly higher in tumors with high protein expression score (Fig. 2).

SLC7A5 protein expression in association with clinicopathological variables. Associations between SLC7A5 expression and tumor characteristics in ER$^+$ breast cancer are presented in Table SI. The results demonstrated that the positive protein expression of SLC7A5 was significantly associated with histopathological grade in the form of Nottingham Histologic Score (P=0.014).

To further evaluate the prognostic value of tumor SLC7A5 protein expression, survival analysis was performed with regards to distant recurrence. When comparing tumors that were positive and negative for SLC7A5, a trend could be observed in which positive protein expression concurred with lower survival rate and earlier distant recurrence; however, this association was not statistically significant (Fig. 3). The estimated hazard ratio for patients with a SLC7A5- positive tumor was 1.85 (95% CI 0.88-3.90; P=0.11).

SLC7A5 mRNA expression and clinicopathological tumor characteristics. Microarray data with z-value conversion were available from 80 patients in the Örebro cohort. In these patients, increased SLC7A5 mRNA expression was

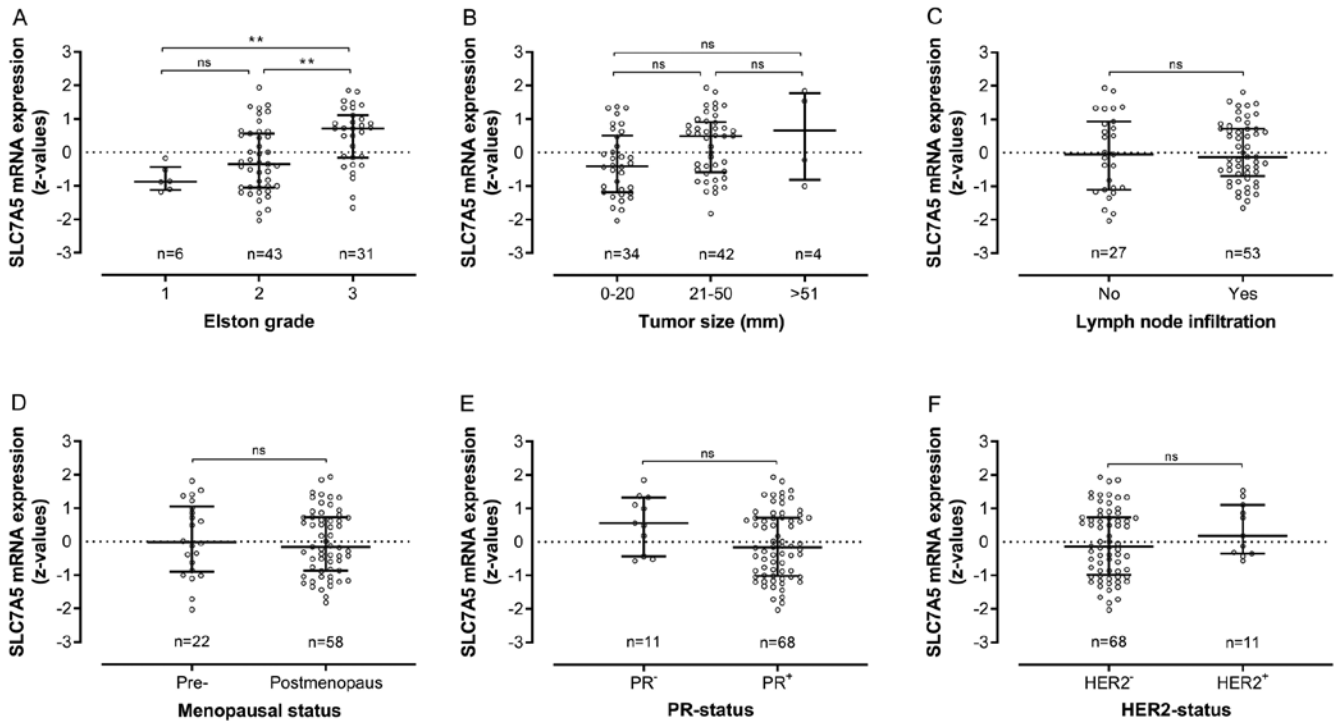

Figure 4. Association between SLC7A5 mRNA expression and clinicopathological variables in estrogen-receptor-positive tumors as assessed via immunohistochemistry (Örebro cohort, n=80). Association between SLC7A5 expression and (A) histopathological grade (Elston grade), (B) tumor size, (C) lymph node infiltration, (D) menopausal status, (E) PR-status and (F) HER2-status. SLC7A5, solute carrier family 7 member 5; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2. $^{**}p \leq 0.01$ and ns, non-significant ($p > 0.05$).

significantly associated with histopathological grade (Fig. 4A), thus adhering with the association between increased expression of SLC7A5 protein and histopathological grade (Table SI). There was no significant association between SLC7A5 mRNA expression and tumor size, lymph node infiltration, menopausal status or PR-status or HER2-status in this cohort (Fig. 4B-F).

For validation of the results obtained from the Örebro cohort, SLC7A5 mRNA expression was also assessed using data on breast cancers categorized as ER$^+$ in the METABRIC dataset (n=1,445). Increased SLC7A5 mRNA expression was similarly demonstrated to be significantly associated with histopathological grade in these tumors (Fig. 5). However, there were also insignificant associations between increased SLC7A5 mRNA expression and lymph node infiltration, as well as progesterone receptor (PR) negativity and HER2-positivity. When investigating differences regarding SLC7A5 mRNA expression in ER$^+$ tumor subtypes, higher z-values could be observed in tumors classified as the more aggressive luminal B subtype compared with luminal A tumors (Fig. 5).

SLC7A5 mRNA expression and breast cancer molecular subtype. By including all primary breast cancer cases in the METABRIC dataset, regardless of ER status (n=1,903), it became possible to further evaluate SLC7A5 expression with regards to clinicopathological factors in the context of molecular breast cancer profiles. The results showed a higher SLC7A5 mRNA expression among tumors with higher histopathological grade. In addition, increased genomic expression of SLC7A5 was more strongly coupled to ER- molecular subtypes, with higher expression of SLC7A5 mRNA z-values in HER2-enriched and triple-negative breast cancer cases compared with tumors classified as ER$^+$ (Fig. 6).

SCL7A5 mRNA expression in correlation with genes related to breast cancer biology, hypoxia and cell metabolism. To also evaluate the biological role of SLC7A5, mRNA expression was assessed in connection with the expression of several other breast cancer related genes and genes related to hypoxia and cell metabolism (Table II). This assessment was performed both on the local Örebro cohort (Fig. S1) and on METABRIC data (Figs. S2-S4). In the Örebro cohort, positive correlations were found between SLC7A5 expression and both MKI67 and HIF1A expression (Table II). However, when testing for correlations between SLC7A5 expression and the other genes aforementioned, no significant correlations could be found.

ER$^+$ cancers in the METABRIC dataset (n=1,445) showed concordant statistically significant correlations between SLC7A5 expression and increased expression of MKI67 and HIF1A (Table II). In this dataset, SLC7A5 was also positively correlated with expression of VEGFA, MYC, ERBB2, and of genes linked to metabolism (LDHA, SLC2A1 and CA9), and negatively correlated with ESR1 expression. When including all tumors in the METABRIC dataset (n=1,903), positive correlations were similarly found between SLC7A5 expression and MKI67, HIF1A, VEGFA, MYC, ERBB2, LDHA, SLC2A1 and CA9 expression. Positive correlations in this dataset were also found between SLC7A5 expression and both MTOR and NDUFA4L2 expression. Negative correlations were demonstrated between SLC7A5 expression and ESR1 and CCND1 expression (Table II). When categorizing the data by ER status, as based on IHC staining, ER$^+$ tumors tended to follow a general pattern of lower relative expression of all assessed genes compared with SLC7A5 mRNA expression (Fig. S4).

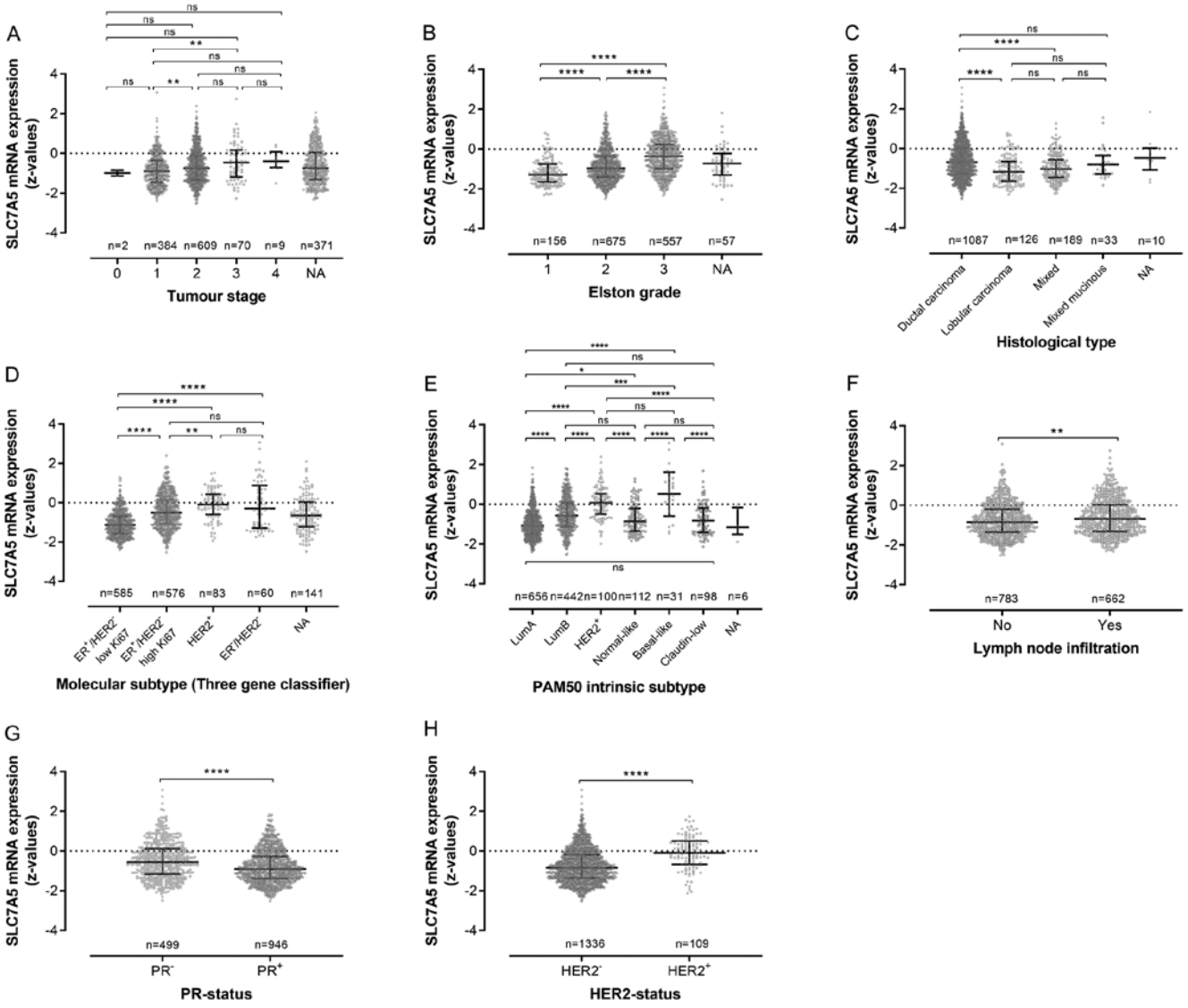

Figure 5. Association between SLC7A5 mRNA expression and clinicopathological variables in estrogen-receptor-positive tumors (METABRIC, n=1,445). Association between SLC7A5 expression and (A) tumor stage, (B) histopathological grade (Elston grade), (C) histological type, (D) molecular subtype classified via three gene expression profiling, (E) genetic subtype based on PAM50 gene expression analysis, (F) lymph node infiltration, (G) PR-status according to genetic analysis, (H) HER2-status according to genetic analysis. SLC7A5, solute carrier family 7 member 5; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2. $^{*}p<0.05$, $^{**}p\leq0.01$, $^{***}p\leq0.001$, and $^{****}p\leq0.0001$ and ns, non-significant ($p>0.05$) values.

Discussion

ER^{+} breast cancers present with varying clinical characteristics and prognosis. Current therapy targets aberrant estrogen-signaling, in most cases with good effect; however, a high number of patients see a progression of the disease, possibly due to endocrine resistance. To overcome therapy resistance, finding novel diagnostic markers and therapeutic targets has become increasingly important (19). The amino acid transporter SLC7A5 (or LAT1) was attributed some pro-oncogenic properties and is overexpressed in various types of cancer, including breast cancer, and may therefore constitute a potential new marker and treatment target.

The results from the present study demonstrated that SLC7A5 protein expression was associated with histopathological grade in ER^{+} breast cancers, which may suggest the role of the amino acid exchanger in breast cancer biology and tumor progression. We were not able to find statistically significant associations between SLC7A5 protein expression and other prognostic markers (e.g. lymph node infiltration or tumor size) or patient outcome (i.e. distant recurrence) in the Örebro cohort. However, Mihály *et al* (20) reported that increased SLC7A5 expression is associated with shorter recurrence free survival in a larger study population of tamoxifen treated patients (n=1,044). Considering the small size of the Örebro cohort and the risk of failing to achieve statistical significance due to inadequate study power, the results from the present study were in concordance with those from El Ansari *et al* (21). El Ansari *et al* (21) also reported that SLC7A5 protein expression is an independent risk factor for worsened patient outcome in highly proliferative ER^{+} tumors and HER2-enriched breast cancer subtypes. SLC7A5 protein expression has been evaluated and reported in different ways, which complicates comparison between studies. In our present cohort, 15% of the tumors were defined as SLC7A5 positive, which was similar to the 17% positivity from the 2,664 breast

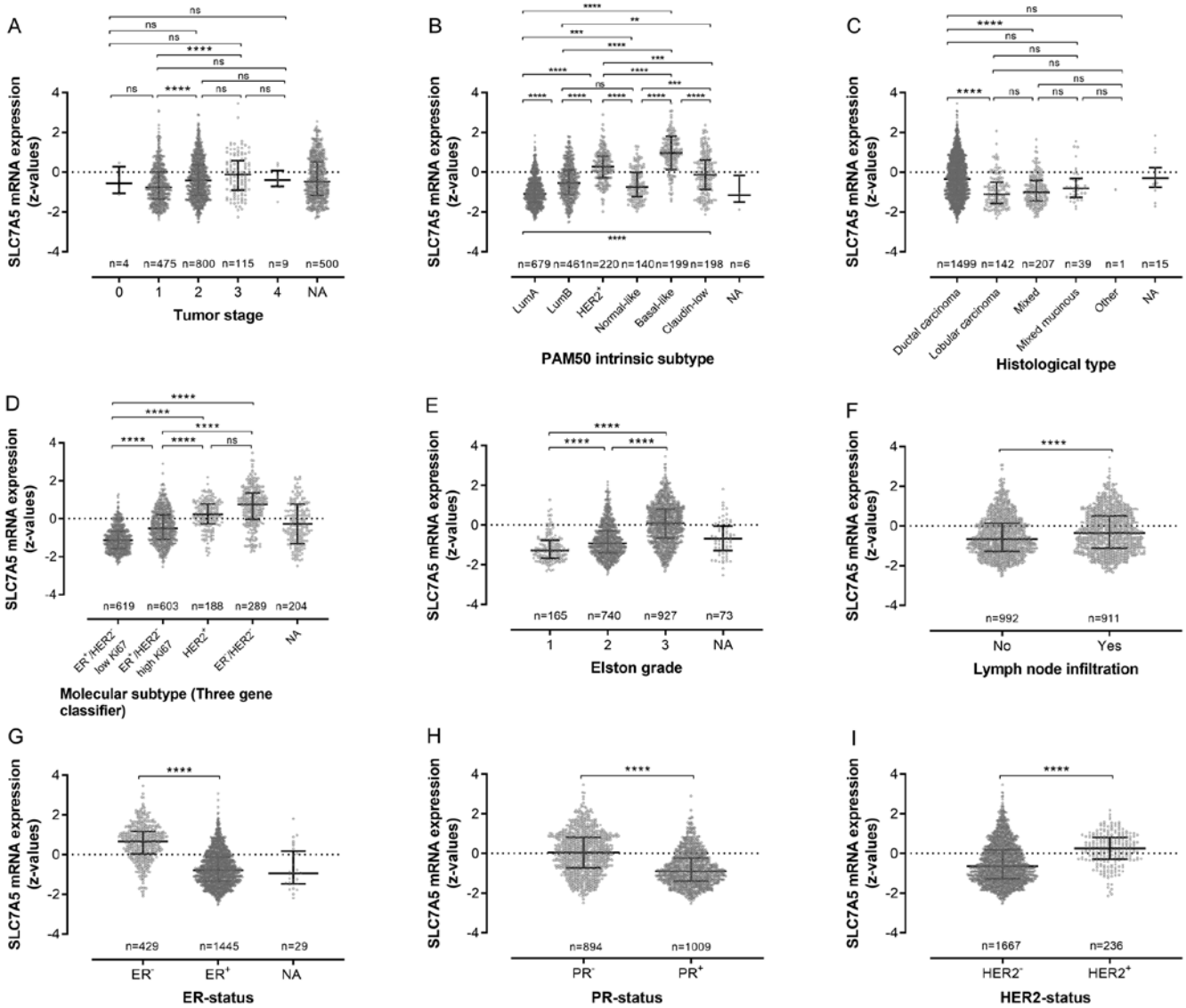

Figure 6. Association between SLC7A5 mRNA expression and clinicopathological variables in all tumors regardless of ER-status (METABRIC, n=1903). Association between SLC7A5 and (A) tumor stage, (B) genetic subtype based on PAM50 gene expression analysis, (C) histological type, (D) molecular subtype classified via three gene expression profiling, (E) histopathological grade (Elston grade), (F) lymph node infiltration, (G) ER-status according to genetic analysis, (H) PR-status according to genetic analysis and (I) HER2-status according to genetic analysis. SLC7A5, solute carrier family 7 member 5; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; ER, estrogen receptor. $^{**}p\leq0.01$, $^{***}p\leq0.001$, $^{****}p\leq0.0001$ and ns, non-significant ($p>0.05$) values.

cancer cases of El Ansari *et al* Bodoor *et al* (22) demonstrated that 84.4 and 64.3% of luminal A (n=32) and luminal B/triple positive (n=42) tumors, respectively, were SLC7A5 positive, and that SLC7A5 expression was overall negatively correlated with HER2 expression. However, this study used a polyclonal antibody, which also stains cytoplasmic structures, as well as a scoring system developed by the authors. El Ansari *et al* used an H-score of >15 as cut-off for positivity, whereas we applied the HER2 evaluation guideline identifying score 2+ and 3+ as SLC7A5 positivity in the present study. The HER2 evaluation guideline was established for pathological assessment of HER2 status and could be adapted for future clinical examination of SLC7A5, which is also a membrane-bound protein.

Comparing SLC7A5 protein expression and mRNA expression in the same patients can be seen as arbitrary. This issue needs to be addressed, as protein expression scoring is based upon tumor cell membrane expression while mRNA expression is based upon analysis of tumor tissue samples, which also contain stromal cells and parts of normal tissue. In the present study, being graded as SLC7A5-negative in protein expression (0 or 1+) meant that <10% of all tumor cells in the specimen were positively stained. Furthermore, there was a variability in SLC7A5 expression, as some specimens with low protein expression still showed substantial mRNA expression levels (Fig. 2). A spatial expression of SLC7A5 could possibly explain this phenomenon and must be taken into consideration.

In the present study, high SLC7A5 protein expression was found to correspond to high mRNA expression, thus providing a rationale for validating our findings in the METABRIC cohort. Furthermore, associations between SLC7A5 gene expression and clinical variables, such as histopathological grade, was connected to poor outcome, which has previously been described in the METABRIC data set (21). In METABRIC

Table II. Association between SLC7A5 mRNA expression and genes related to breast cancer, hypoxia and cell metabolism in the Örebro cohort and the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset.

Gene name	Örebro cohort (n=80)			METABRIC ER+ (n=1,445)			METABRIC (n=1,903)		
	Spearman r	95% CI	P-value	Spearman r	95% CI	P-value	Spearman r	95% CI	P-value
ESR1	-0.023	-0.248 to 0.204	0.8399	-0.136	-0.188 to -0.083	<0.0001	-0.448	-0.484 to -0.410	<0.0001
ERBB2	-0.043	-0.266 to 0.185	0.7071	0.078	0.025 to 0.130	0.0031	0.010	-0.036 to 0.056	0.668
MKI67	0.410	0.202 to 0.582	0.0002	0.348	0.301 to 0.394	<0.0001	0.480	0.444 to 0.515	<0.0001
MYC	0.150	-0.079 to 0.363	0.1851	0.046	-0.008 to 0.098	0.084	0.137	0.091 to 0.182	<0.0001
MTOR	-0.042	-0.266 to 0.185	0.7089	0.013	-0.040 to 0.066	0.6139	0.084	0.038 to 0.129	0.0003
CCND1	-0.117	-0.334 to 0.112	0.3007	0.040	-0.013 to 0.093	0.1297	-0.186	-0.231 to -0.141	<0.0001
HIF1A	0.263	0.039 to 0.462	0.0184	0.097	0.044 to 0.150	0.0002	0.192	0.147 to 0.236	<0.0001
VEGFA	0.062	-0.166 to 0.284	0.585	0.243	0.192 to 0.292	<0.0001	0.355	0.314 to 0.395	<0.0001
EPAS1	0.191	-0.036 to 0.400	0.089	-0.031	-0.084 to 0.022	0.239	-0.039	-0.085 to 0.008	0.0929
LDHA	0.013	-0.214 to 0.238	0.9083	0.066	0.014 to 0.119	0.0118	0.149	0.103 to 0.194	<0.0001
SLC2A1	-0.213	-0.419 to 0.014	0.0579	0.261	0.211 to 0.310	<0.0001	0.381	0.341 to 0.420	<0.0001
CA9	-0.010	-0.235 to 0.217	0.9304	0.136	0.084 to 0.188	<0.0001	0.340	0.298 to 0.380	<0.0001
NDUFA4L2	-0.037	-0.261 to 0.190	0.7416	0.026	-0.028 to 0.079	0.3314	0.121	0.075 to 0.166	<0.0001

SLC7A5, solute carrier family 7 member 5; ER, estrogen receptor; ESR1, estrogen receptor 1; ERBB2, Erb-B2 receptor tyrosine kinase 2; MYC, MYC proto-oncogene, BHLH transcription factor; MTOR, mechanistic target of rapamycin; MKI67, marker of proliferation Ki-67; CCND1, cyclin D1; HIF1A, hypoxia inducible factor 1 subunit alpha; EPAS1, endothelial PAS domain-containing protein 1; VEGFA, vascular endothelial growth factor A; LDHA, lactate dehydrogenase A; SLC2A1, solute carrier family 2, facilitated glucose transporter member 1; CA9, carbonic anhydrase 9; NDUFA4L2, NDUFA4 mitochondrial complex associated like 2.

ER$^+$ breast cancers from the present study, SLC7A5 expression was associated with HER2-positivity and PR negativity. Furthermore, a negative correlation between SLC7A5 expression and ESR1 expression was demonstrated. Among the luminal tumors, it is well known that these observations correspond to a lower likelihood of endocrine treatment response as they influence estrogen signaling. Expression of SLC7A5 seems to vary in luminal cancers and is of great interest in terms of endocrine response (23). Guan *et al* (23) compared differentially expressed genes in tamoxifen-sensitive and tamoxifen-resistant cell lines as well as in clinical breast cancer samples, and developed a two-gene pair signature (TOP2A, SLC7A5; NMU, PDSS1) that could predict tamoxifen therapy outcome. However, in the Örebro cohort, which consisted of tamoxifen-treated patients only, there was no significant difference in distant recurrence between SLC7A5-positive and SLC7A5-negative cases; however, there was a trend for SLC7A5-positive cases to have lower survival rates.

SLC7A5 has previously been featured as one of the five immunohistochemical markers constituting the Mammostrat® risk assessment tool, which is intended to predict the risk of recurrent disease in cases of tamoxifen treated ER$^+$ cancer (24). Using this tool, Bartlett *et al* (24) demonstrated that increased SLC7A5 expression is on its own significantly associated with shorter recurrence-free survival. However, previous studies reported that the analysis of individual expression of SLC7A5 is insufficient to predict the prognosis and response to endocrine therapy in these patients (25-27). These studies suggested that SLC7A5 should be assessed together with SLC3A2, which is a key helper of SLC7A5 membrane transportation, to predict patient outcome in ER$^+$ breast cancer. The role of SLC7A5 as a marker in endocrine disease is therefore disputed, and the present findings further highlight this.

To determine the biological role of SLC7A5, the present study evaluated the correlation between SLC7A5 expression and the expression of genes involved in cell proliferation, metabolism, and hypoxia. The results demonstrated that SLC7A5 gene expression was positively correlated with HIF1A and MKI67 expression in both the Örebro cohort and the METABRIC dataset, indicating the importance of SLC7A5 expression regarding the proliferative capabilities of breast cancer cells. Our findings also suggested a connection between SLC7A5 expression and tissue hypoxia in breast cancer, which has been previously reported in renal cell carcinoma (9). HIF1A overexpression is known to be positively correlated with numerous unfavorable carcinogenic characteristics, such as increased angiogenesis, cell invasion and metastasis (28). Regarding SLC7A5, increased expression together with co-expression of proliferative and hypoxic genes, in primary and metastatic tumor sites could indicate adverse disease (29). SLC7A5 expression has previously been positively associated with expression of hypoxia related genes such as HIF2A in breast cancer, although it was only seen in luminal B tumors (21). The present study provided further insight into the possible connection between amino acid transporters such as SLC7A5 and hypoxia-inducible factors in ER$^+$ disease. In the future, validation of protein expressions in breast cancer will be required, alongside functional *in vitro* investigation. HIF1A signaling has been reported to stimulate the expression of several glycolytic enzymes as well as pH-regulating factors in cancer, including LDHA, SLC2A1 and CA9 (30-32). In the present study, we also observed positive correlation between the expression

of SLC7A5 and LDHA, which encodes the enzyme lactate dehydrogenase, and of SLC2A1, which encodes the glucose transporter GLUT1. Both are crucial to accelerate glycolysis and stimulate the Warburg effect (33-35). SLC7A5 expression was also significantly correlated with CA9 expression, which encodes the enzyme carbonic anhydrase. This enzyme is involved in cellular pH buffering and has been demonstrated to provide a more favorable tumor microenvironment in breast cancer (36,37). These results suggested that SLC7A5 may either be part of or be crucial to the metabolic reprogramming of breast cancer cells. Tumors may upregulate SLC7A5 together with other factors such as LDHA, SLC2A1 and CA9, as a way to counteract the negative effects of oxygen and substrate starvation that arises as the tumor progressively grows.

Regarding the spatial expression of SLC7A5, its expression was demonstrated to differ between and within individual tumors, suggesting that SLC7A5 expression may vary with certain tumor growth conditions. A previous study on basal cell carcinoma reported that SLC7A5 tends to be confined to centrally located tumor cells rather than surrounding cells in the tumor margins (15). In this context, SLC7A5 protein expression patterns and SLC7A5 mRNA association with MKI67, HIF1A, LDHA, SLC2A1 and CA9 could further suggest that SLC7A5 upregulation may be crucial for cell proliferation or survival in certain unfavorable settings, such as for example, in rapidly growing tumor masses where blood flow successively becomes inadequate towards the tumor core or as a response to anti-tumor agents. Sato *et al* (38) reported that SLC7A5 protein expression is higher following chemotherapy, suggesting an enhanced amino acid metabolism when glucose metabolism is impaired from treatment. Saito *et al* (39) also demonstrated that endocrine resistance in breast cancer is associated with adaption to nutrient stress, and that SLC7A5 is an important factor and a potential target to overcome treatment resistance. The presence of SLC7A5 could be a sub-marker for endocrine resistance, suggesting a possible benefit from using SLC7A5 inhibitors such as JPH203, which is currently undergoing a phase II drug trial (UMIN000034080). It is reasonable to think that the expression of amino acid transporters might vary with changing cellular growth conditions. According to our hypothesis, SLC7A5 expression may be induced as a result of unfavorable tumor microenvironment.

In summary, the results from the present study suggested that SLC7A5 expression in ER^+ tumors may be of importance for tumor cell proliferation and survival. However, the biological role as well as the prognostic and predictive values of SLC7A5 expression require further investigation in different subtypes of breast cancer.

Acknowledgements

Not applicable.

Funding

The present study was funded by the Lions Cancer Research Fund for Region Uppsala Örebro (2020), the research committee of Region Örebro County (grant no. OLL-939134) and ALF grants from Region Örebro County (grant no. OLL-555621, OLL-935730).

Availability of data and materials

Data from the Örebro cohort are available from the corresponding author on reasonable request. The Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset is accessible via cBioPortal for Cancer Genomics.

Authors' contributions

RT wrote the original draft and contributed to the methodology and data analysis. AGE and ET contributed to the methodology, data analysis, reviewing and editing of the manuscript, and were responsible for the project supervision and acquisition of funding. All authors confirm the authenticity of all raw data and have read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the regional Ethics Committee of Uppsala, Sweden (approval no. 2011/070) and patients provided written consent for the use of their clinical material.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F: Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 71: 209-249, 2021.
2. Goldhirsch A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thürlimann B, Senn HJ, Albain KS, André F, Bergh J, *et al*; Panel members: Personalizing the treatment of women with early breast cancer: Highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013. Ann Oncol 24: 2206-2223, 2013.
3. Harvey JM, Clark GM, Osborne CK and Allred DC: Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. J Clin Oncol 17: 1474-1481, 1999.
4. Duffy MJ, Harbeck N, Nap M, Molina R, Nicolini A, Senkus E and Cardoso F: Clinical use of biomarkers in breast cancer: Updated guidelines from the European Group on Tumor Markers (EGTM). Eur J Cancer 75: 284-298, 2017.
5. El Sayed R, El Jamal L, El Iskandarani S, Kort J, Abdel Salam M and Assi H: Endocrine and targeted therapy for hormone-receptor-positive, HER2-negative advanced breast cancer: Insights to sequencing treatment and overcoming resistance based on clinical trials. Front Oncol 9: 510, 2019.
6. Häfliger P and Charles RP: The L-type amino acid transporter LAT1 - An emerging target in cancer. Int J Mol Sci 20: 2428, 2019.
7. Scalise M, Galluccio M, Console L, Pochini L and Indiveri C: The human SLC7A5 (LAT1): The intriguing histidine/large neutral amino acid transporter and its relevance to human health. Front Chem 6: 243, 2018.
8. Hanahan D and Weinberg RA: Hallmarks of cancer: The next generation. Cell 144: 646-674, 2011.
9. Elorza A, Soro-Arnáiz I, Meléndez-Rodríguez F, Rodríguez-Vaello V, Marsboom G, de Cárcer G, Acosta-Iborra B, Albacete-Albacete L, Ordóñez A, Serrano-Oviedo L, *et al*: HIF2α acts as an mTORC1 activator through the amino acid carrier SLC7A5. Mol Cell 48: 681-691, 2012.

10. Gatenby RA, Smallbone K, Maini PK, Rose F, Averill J, Nagle RB, Worrall L and Gillies RJ: Cellular adaptations to hypoxia and acidosis during somatic evolution of breast cancer. Br J Cancer 97: 646-653, 2007.
11. Salisbury TB and Arthur S: The regulation and function of the L-type amino acid transporter 1 (LAT1) in cancer. Int J Mol Sci 19: 2373, 2018.
12. Adamaki M: Cancer and the cellular response to hypoxia. Pediatr Ther S1: 002, 2012.
13. Cormerais Y, Giuliano S, LeFloch R, Front B, Durivault J, Tambutté E, Massard PA, de la Ballina LR, Endou H, Wempe MF, *et al*: Genetic disruption of the multifunctional CD98/LAT1 complex demonstrates the key role of essential amino acid transport in the control of mTORC1 and tumor growth. Cancer Res 76: 4481-4492, 2016.
14. Göthlin Eremo A, Lagergren K, Othman L, Montgomery S, Andersson G and Tina E: Evaluation of SPP1/osteopontin expression as predictor of recurrence in tamoxifen treated breast cancer. Sci Rep 10: 1451, 2020.
15. Tina E, Prosén S, Lennholm S, Gasparyan G, Lindberg M and Göthlin Eremo A: Expression profile of the amino acid transporters SLC7A5, SLC7A7, SLC7A8 and the enzyme TDO2 in basal cell carcinoma. Br J Dermatol 180: 130-140, 2019.
16. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, *et al*: The cBio cancer genomics portal: An open platform for exploring multidimensional cancer genomics data. Cancer Discov 2: 401-404, 2012.
17. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, *et al*: Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal 6: pl1-pl1, 2013.
18. Curtis C, Shah SP, Chin S-F, Turashvili G, Rueda OM, Dunning MJ, Speed D, Lynch AG, Samarajiwa S, Yuan Y, *et al*; METABRIC Group: The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature 486: 346-352, 2012.
19. Rondón-Lagos M, Villegas VE, Rangel N, Sánchez MC and Zaphiropoulos PG: Tamoxifen resistance: Emerging molecular targets. Int J Mol Sci 17: 1357, 2016.
20. Mihály Z, Kormos M, Lánczky A, Dank M, Budczies J, Szász MA and Győrffy B: A meta-analysis of gene expression-based biomarkers predicting outcome after tamoxifen treatment in breast cancer. Breast Cancer Res Treat 140: 219-232, 2013.
21. El Ansari R, Craze ML, Miligy I, Diez-Rodriguez M, Nolan CC, Ellis IO, Rakha EA and Green AR: The amino acid transporter SLC7A5 confers a poor prognosis in the highly proliferative breast cancer subtypes and is a key therapeutic target in luminal B tumours. Breast Cancer Res 20: 21, 2018.
22. Bodoor K, Almomani R, Alqudah M, Haddad Y and Samouri W: LAT1 (SLC7A5) overexpression in negative Her2 group of breast cancer: A potential therapy target. Asian Pac J Cancer Prev 21: 1453-1458, 2020.
23. Guan Q, Song X, Zhang Z, Zhang Y, Chen Y and Li J: Identification of tamoxifen-resistant breast cancer cell lines and drug response signature. Front Mol Biosci 7: 564005, 2020.
24. Bartlett JM, Thomas J, Ross DT, Seitz RS, Ring BZ, Beck RA, Pedersen HC, Munro A, Kunkler IH, Campbell FM, *et al*: Mammostrat as a tool to stratify breast cancer patients at risk of recurrence during endocrine therapy. Breast Cancer Res 12: R47, 2010.
25. Yan R, Zhao X, Lei J and Zhou Q: Structure of the human LAT1-4F2hc heteromeric amino acid transporter complex. Nature 568: 127-130, 2019.
26. Alfarsi LH, El-Ansari R, Craze ML, Masisi BK, Mohammed OJ, Ellis IO, Rakha EA and Green AR: Co-expression effect of SLC7A5/SLC3A2 to predict response to endocrine therapy in oestrogen-receptor-positive breast cancer. Int J Mol Sci 21: 1407, 2020.
27. El-Ansari R, Craze ML, Alfarsi L, Soria D, Diez-Rodriguez M, Nolan CC, Ellis IO, Rakha EA and Green AR: The combined expression of solute carriers is associated with a poor prognosis in highly proliferative ER$^+$ breast cancer. Breast Cancer Res Treat 175: 27-38, 2019.
28. Semenza GL: Targeting HIF-1 for cancer therapy. Nat Rev Cancer 3: 721-732, 2003.
29. Kaira K, Oriuchi N, Imai H, Shimizu K, Yanagitani N, Sunaga N, Hisada T, Tanaka S, Ishizuka T, Kanai Y, *et al*: l-type amino acid transporter 1 and CD98 expression in primary and metastatic sites of human neoplasms. Cancer Sci 99: 2380-2386, 2008.
30. Semenza GL: HIF-1: Mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol (1985) 88: 1474-1480, 2000.
31. Rathmell WK and Chen S: VHL inactivation in renal cell carcinoma: Implications for diagnosis, prognosis and treatment. Expert Rev Anticancer Ther 8: 63-73, 2008.
32. Lock FE, McDonald PC, Lou Y, Serrano I, Chafe SC, Ostlund C, Aparicio S, Winum JY, Supuran CT and Dedhar S: Targeting carbonic anhydrase IX depletes breast cancer stem cells within the hypoxic niche. Oncogene 32: 5210-5219, 2013.
33. Valvona CJ, Fillmore HL, Nunn PB and Pilkington GJ: The regulation and function of lactate dehydrogenase A: Therapeutic potential in brain tumor. Brain Pathol 26: 3-17, 2016.
34. Szablewski L: Expression of glucose transporters in cancers. Biochim Biophys Acta 1835: 164-169, 2013.
35. Vander Heiden MG, Cantley LC and Thompson CB: Understanding the Warburg effect: The metabolic requirements of cell proliferation. Science 324: 1029-1033, 2009.
36. Swietach P, Hulikova A, Vaughan-Jones RD and Harris AL: New insights into the physiological role of carbonic anhydrase IX in tumour pH regulation. Oncogene 29: 6509-6521, 2010.
37. Lou Y, McDonald PC, Oloumi A, Chia S, Ostlund C, Ahmadi A, Kyle A, Auf dem Keller U, Leung S, Huntsman D, *et al*: Targeting tumor hypoxia: Suppression of breast tumor growth and metastasis by novel carbonic anhydrase IX inhibitors. Cancer Res 71: 3364-3376, 2011.
38. Sato M, Harada-Shoji N, Toyohara T, Soga T, Itoh M, Miyashita M, Tada H, Amari M, Anzai N, Furumoto S, *et al*: L-type amino acid transporter 1 is associated with chemoresistance in breast cancer via the promotion of amino acid metabolism. Sci Rep 11: 589, 2021.
39. Saito Y, Li L, Coyaud E, Luna A, Sander C, Raught B, Asara JM, Brown M and Muthuswamy SK: LLGL2 rescues nutrient stress by promoting leucine uptake in ER$^+$ breast cancer. Nature 569: 275-279, 2019.