Introduction

Lung cancer is the leading cause of cancer-related deaths in the United States (1). Non–small cell lung cancer (NSCLC) accounts for about 80% of all lung cancers. Although advances have been made in the diagnosis and treatment strategies in the last decade, the prognosis of patients with NSCLC remains poor, with a 5-year overall survival of 15% to 20% (2). A small subset of tumors has been found to be driven by mutated oncogenes for which treatment strategies exist, but still noncurative, therapies are available. Yet, the vast majority of tumors have a complex pathogenesis that is still poorly understood (3). Thus, new molecular diagnostic and therapeutic targets are urgently needed to improve the quality of care and survival of patients with lung cancer.

Although genomic profiling has given important new insights into the mechanisms of carcinogenesis, therapeutic targets and most biomarkers with clinical use are protein products. Advances in proteomic techniques in recent years have allowed for an in-depth analysis of the changes in protein expression and posttranslational modifications associated with lung cancer (4, 5). These studies have yielded large inventories of proteins that can potentially be translated to molecular targets or biomarkers of lung cancer, but few of these candidates have been validated or associated with functional relevance to the disease process.

Given the urgent need for a reliable and noninvasive diagnostic test for lung cancer, we have previously compared shotgun proteomic profiles of fresh-frozen stage I NSCLC to matched control lung specimens and identified several candidates that were significantly overexpressed in NSCLC (6). Solute linked carrier family 1 member A5 (SLC1A5) emerged as a top candidate. SLC1A5 acts as a high-affinity transporter of L-glutamine (Gln) in rapidly growing epithelial and tumor cells in culture (7). Neutral amino acids, including Gln, can be transported by 4 main
families of amino acid transporter systems including sodium-dependent systems A, ASC, N, and sodium-independent system L (8–11). These transporters are classified on the basis of tissue distribution and affinities for different amino acids. System ASC is the most commonly expressed amino acid transporter in human tumor-derived cells, suggesting it might play a major role in cellular transformation and mediation of Gln-dependent growth (10, 12, 13). Because SLC1A5 belongs to the Na\(^+\)-dependent ASC family of amino acid transporters, we examined whether glutamine is transported in lung cancer cells in a Na\(^+\)-dependent manner. In lung cancer, the dependency on Gln for growth, survival, and cell-cycle progression is well documented (14–16). More recently, Gln conversion into the tricarboxylic acid cycle intermediate \(\alpha\)-ketoglutarate through glutaminase was shown to be essential for Kras-induced anchorage-independent growth in A549 cells (17). Other studies showed that reductive carboxylation of glutamine is key for the metabolic reprogramming that enables cancer cells to survive and proliferate under hypoxia in several cancer cellular models, including lung cancer (18–20). Collectively, these studies provide strong evidence for a major role of Gln metabolism in supporting lung cancer cell growth and survival. However, the identity of the transporter(s) that capture Gln in lung cancer cells and how Gln transport is linked to cell growth and survival is still largely unknown. Therefore, the 3 main objectives of this study were first, to evaluate the expression pattern of SLC1A5 protein in different lung cancer pathologic subtypes, second, to assess its relevance to the clinical outcomes in lung cancer, and finally, to investigate the functional contribution of SLC1A5 to glutamine uptake and its role in supporting the cell growth and viability of lung cancer cells.

\section*{Materials and Methods}

\subsection*{Patient selection and tissue microarray}

Tissue microarrays (TMA) of 98 lung cancer tumor tissues were prepared from paraffin-embedded formalin-fixed (FFPE) blocks. The TMA consisted of 46 adenocarcinomas, 37 squamous cell carcinomas, 4 bronchioalveolar carcinomas, 4 large cell carcinomas, 2 NSCLC, 5 carcinoids, and 1 each of adenosquamous, sarcoma, and small cell lung cancer (SCLC). These arrays were constructed according to protocols previously described (21). Archived tissue blocks from consecutive anatomic resections between 1989 to 2002 were retrieved from the files of the Vanderbilt University Medical Center and the Nashville Veterans Administration Medical Center pathology departments (Nashville, TN). Demographic and clinical characteristics of the 98 patients represented in both TMAs are summarized in Table 1, for details in immunohistochemical (IHC) analysis, refer to Supplementary Materials.

\section*{Cell culture}

Human lung cancer cell lines A549 (ADC), H1819 (ADC), HCC15 (SCC), H520 (SCC), and H727 (Carcinoid; American Type Culture Collection) were maintained in RPMI-1640 medium (Gibco by Life Technologies) containing 10\% heat-inactivated FBS (Gibco by Life Technologies), at 37\°C, 100\% humidity, and 5\% CO\(_2\). Cells were passaged every 2 to 3 days to maintain exponential growth.

\subsection*{Glutamine uptake assays}

Measurement of Gln uptake in monolayer cultured NSCLC cells was carried out via the cluster-tray method originally described by Gazzola and colleagues (22). Briefly, cells were plated at 10\(^4\) cells per well in 24-well culture plates (Costar) and allowed to adhere overnight. Before transport

\begin{table}[h]
\centering
\caption{Characteristics of the patients in the TMAs cohort according to the cancer status and SLC1A5 expression}
\begin{tabular}{|l|c|c|c|}
\hline
\textbf{Patients demographics} & \textbf{Total} & \textbf{SLC1A5} & \textbf{SLC1A5} \\
\hline
\textbf{N} & \textbf{98} & \textbf{(-)} & \textbf{(+)} \\
\hline
\textbf{Age, Mean (SD)} & 56.4 ± 11.5 & & \\
\hline
\textbf{Race} & & & \\
\textbf{African American} & 7 (7) & 0 & 7 (100) \\
\textbf{Caucasian} & 91 (93) & 17 (17) & 81 (83) \\
\hline
\textbf{Gender} & & & \\
\textbf{Men} & 56 (57) & 6 (11) & 50 (98) \\
\textbf{Women} & 42 (43) & 13 (31) & 29 (69) \\
\hline
\textbf{Smoking status} & & & \\
\textbf{Ex. never-smoker} & 71 (72) & 13 (18) & 56 (82) \\
\textbf{Current-smoker} & 27 (28) & 3 (11) & 24 (88) \\
\hline
\textbf{Pack-years, mean (SD)} & 55.78 ± 33 & & \\
\hline
\textbf{Histology} & & & \\
\textbf{ADC} & 46 (47) & 12 (26) & 34 (74\%) \\
\textbf{SCC} & 37 (38) & 2 (5) & 35 (95\%) \\
\textbf{Other NSCLC} & 9 (9) & 0 & 9 (100\%) \\
\textbf{Neuroendocrine tumors} & 6 (6) & 3 (50) & 3 (50\%) \\
\hline
\end{tabular}
\end{table}

\textbf{Abbreviations: Neuroendocrine tumors, SCLC + large cell Neuroendocrine + Atypical carcinoid; Other NSCLC, LCC + adenosquamous carcinoma + NSCLC; PKY, pack year (pack per day × number of years smoked).}
assays, the cells were rinsed twice with warm Na\(^+\)-free Krebs-Ringer Phosphate Buffer (cholKRP) in which choline chloride and choline phosphate iso-osmotically replaced the corresponding Na\(^+\) salts, to remove extracellular Na\(^+\) and amino acids. The radiotracer used was [\(3,4\)C\(_2\)Gln-\(^3\)H\(_2\)] glutamine (Amersham) at 500,000 dpm/microl of the specific activity (5 \(\mu\)Ci/mL). For kinetic studies, the amount of unlabeled glutamine in the transport buffer varied from 400 \(\mu\)mol/L to 6.4 mmol/L. Transport values were obtained either in the absence of extracellular Na\(^+\) (diffusion and Na-independent uptake) using cholKRP or in the presence of Na\(^+\) (total uptake), using NaKRP buffer to determine the Na-dependent rates, reported in units of picomol per milligram protein per min. All transport measurements were carried out at 37°C and were terminated after 3 minutes by adding ice-cold PBS saline (PBS) followed by 3 rapid washes with an ice-cold PBS. Intracellular glutamine was extracted with 0.2 mL per well of 0.2% SDS in 0.2 N NaOH; after 1 hour at room temperature, 0.1 mL of the lysate was neutralized with 2N HCl and subjected to liquid scintillation spectrophotometry. The remaining lysate was used for the determination of cellular protein by the Pierce BCA Protein assay. Rates of glutamine transport were calculated from the counts per minute (cpm) per sample, and the specific activity of the uptake mix (in cpm/mmol). These results were then normalized to cellular protein content using Microsoft Excel. For pharmacologic targeting by \(\gamma\)-Glutamyl-p-Nitroanilide (GPNA), Glu uptake assays were conducted in the presence of 0, 100, and 300 \(\mu\)mol/L of GPNA in NaKRP buffer. The transport velocities were calculated from radioactive counts and protein concentration and subsequently expressed as pmol of Gln transported per milligram of protein per minute. Each data point represents the average \pm SEM of at least 3 separate determinations.

Glutamine-dependent proliferation assays

To test the growth dependency on Gln, cells were plated in 12-well tissue culture plates at a density of 2 \(\times\) 10\(^4\) cells per well. The following day, the cells were rinsed once with serum-free Gln-free medium and replaced with RPMI-1640 (Gibco by Life Technologies) with the following variations: supplementation with EGF (25 ng/mL) and 1 \(\times\) growth factors cocktail (Invitrogen) that includes insulin, selenium, and transferein (Sup), Sup + 2 mmol/L Gln (+Gln + Sup), RPMI-1640 + Gln, but no supplements (+Gln – Sup), no Gln but with supplements (−Gln + Sup) or with neither Gln nor supplements (−Gln – Sup). Cultures were left to grow for 3 days with media changes every 48 hours. To test whether SLC1A5 activity mediates the Gln depletion effect, A549 and H520 were cultured in media containing (+Gln + Sup) + 1 mmol/L GPNA, and left to grow for 2 days. To further validate the antigrowth effects of SLC1A5 blockade by GPNA, A549 and H1819 cells were cultured in the optimum growth media (+Gln + Sup) in the presence of increasing doses of GPNA (0, 1, 10, 100, 1,000 \(\mu\)mol/L) for 2 days. Cell growth was monitored by measuring the OD\(_{490}\) by the CellTiter 96-Aqueous colorimetric assay (Promega) at day 0 and after 48 hours of culture. Relative growth rates were expressed as % growth from day 0 and were calculated using the following equation: (Ti-Tz)/(C-Tz) \(\times\) 100, where Ti is the cell number after 48 hours (i = inhibition), Tz is the cell number at time 0 and C is the cell number of the control cells that were cultured in optimum growth conditions (+Gln + Sup; ref. 23).

Transfection of siRNA

A mixture of four 21-nucleotide siRNAs that target human SLC1A5 was synthesized (Thermo Scientific) and was provided as single reagent. The 4 siRNA sequences targeting human SLC1A5 in the siRNA pool were:

1) UGAUAAGUGAAGAGUGA
2) GCAAGGAGGUGGCUCCGAUUC
3) GGUCGACCAUAUCUCUUG
4) GUCUUGGUCCUCUAAGCUAC

A nontargeting control siRNA of scrambled nucleotide sequence used as a negative control for nonspecific binding (siRNACont) was purchased from the same company. All-Stars Hs Cell Death Control siRNA was used as a positive control to verify both the transfection efficiency and as a positive control for cell viability (Qiagen). A549 and H520 cells were transfected using DharmaFECT1-reagent (0.2 \(\mu\)L/well) at 1.0 and 2.5 mmol/L siRNAs for 72 hours before assessing cell growth, viability, and cell-cycle progression as described in Supplementary Materials.

Statistical analysis

The association between SLC1A5 expression and clinical variables was analyzed using the Kruskal–Wallis or Wilcoxon rank-sum tests. Kinetic data was fitted to Michaelis–Menten kinetics with data points equal to mean \pm SEM of n experiments minus nonspecific binding. Statistical analysis for the glutamine uptake kinetics and cell growth assays was conducted with GraphPad Prism (GraphPad Software). Data comparing 2 experimental conditions were statistically analyzed by 2-tailed Student t test and only results with P < 0.05 were considered to be statistically significant. All experimental data are presented as the mean \pm SEM of n independent measurements (as indicated in each figure legends). All treatments within each experiment were conducted in triplicate wells and repeated on 3 independent days. To assess the extent of the dose response treatment of GPNA, ordinary regression analysis was used to evaluate the linear trend between log and log (percent of growth inhibition).

Results

SLC1A5 is overexpressed at the cytoplasmic membrane and is associated with squamous lung cancer histology and male gender

Our shotgun proteomic analysis showed that SLC1A5 protein is overexpressed in tissue extracts of both stage I adenocarcinoma (ADC; \(N = 20\)) and squamous cell carcinoma (SCC; \(N = 20\)) of the lung compared with control lung samples (\(N = 20\)) from fresh-frozen tissue and from...
formalin-fixed paraffin-embedded FFPE tissues ($N = 5$ of each tissue type; Supplementary Fig. S1A and S1B). We confirmed these results in a separate set of tissue lysates collected from 3 normal lung tissues and 3 NSCLC tumors (Supplementary Fig. S1C). To evaluate the pattern of expression for SLC1A5 protein in lung tumors, we conducted immunohistochemical (IHC) analysis using 2 TMAs of archival primary lung cancer tissues collected from patients diagnosed with different histologic subtypes of lung cancer using a validated human anti-SLC1A5 IgG (http://www.proteinatlas.org/). SLC1A5 signals were seen in 35 of 38 (95%) of SCC and of 34 of 46 (74%) ADC subtypes. Other NSCLCs, including large cell carcinoma (LCC), adenosquamous, and NSCLC, not otherwise specified were all positive (9/9; 100%). Neuroendocrine tumors, which included atypical and typical carcinoid and SCLC, were also represented in our TMA but only 50% (3/6) stained positive for SLC1A5 (Table 1). The pattern of SLC1A5 staining in both adenocarcinomas and squamous cell carcinomas was along the cytoplasmic membrane, with less intense cytoplasmic staining. Of the normal cellular components, we found 0 to 1+ cytoplasmic membrane staining of ciliated respiratory epithelial cells, 2+ to 3+ cytoplasmic membrane staining of basal bronchial/bronchiolar cells, 2+ cytoplasmic membrane staining of bronchial submucosal glandular cells, 1+ to 2+ cytoplasmic membrane staining of reactive type 2 pneumocytes (but no staining in type 1 pneumocytes), 2+ cytoplasmic staining of alveolar macrophages, and 1+ to 2+ cytoplasmic and cytoplasmic membrane staining of plasma cells. There was no staining of stromal fibroblasts, smooth muscle, cartilage, endothelial cells, or lymphocytes (Fig. 1A). The occurrence of SLC1A5 expression was significantly higher in SCC than in ADC ($P < 0.001$; Fig. 1B). The most significant correlation between SLC1A5 and clinical
variables was observed with gender, being higher in males than females \( (P < 0.001; \text{Fig. 1C}) \) and with histologic subtype, being higher in SCC than ADC \( (P < 0.001; \text{Fig. 1C and Table 1}) \). No significant correlation was observed with SLC1A5 expression and the overall survival, pack year history of smoking or age.

The immunohistochemistry results from lung cancer cell lines showed that all but one adenocarcinoma cell line (H2009; Supplementary Table S1, not shown in immunohistochemistry) and one SCLC cell line (H345) stained positive for SLC1A5 (Supplementary Table S1 and Supplementary Fig. S2). Similar to lung primary tumors, we found that the subcellular location of SLC1A5 was predominantly membranous (Supplementary Fig. S2A) in all lung cancer cell lines. Western blot analysis using a rabbit polyclonal anti-SLC1A5 antibody \( (24, 25) \) confirmed that the expression of this protein is higher in malignant lung cancer cell lines, A549 (ADC), HCC15 (SCC), H520 (SCC), and H460 (LCC; Supplementary Fig. S2B and S2C). The specificity of this antibody and the confirmation of the SLC1A5 expression in A549 and H1819 cell lines were verified using 3 different antibodies (Supplementary Fig. S3A). One ADC cell line, (H1819), was negative for SLC1A5. H727, a carcinoid cell line, had low levels of the protein. The Western blot results from lung cancer cell lines are consistent with the pattern of expression of SLC1A5 from shotgun proteomics (Supplementary Fig. S1A–S1C) and from immunohistochemistry of tumor TMA (Fig. 1A). These results show strong cell membrane immunostaining for SLC1A5 in most lung cancers, more so in SCC and in men.

**Glutamine uptake in lung cancer cells is mediated in part by SLC1A5**

We tested the \( \text{Na}^+ \) dependency of the cellular uptake of Gln in A549 cells using \(-[\text{G}-^3\text{H}]\) in Krebs–Ringer solution (Fig. 2A and B; ref. 26). Our results depicted in Fig. 2A and Supplementary Table S2 showed that 68% of cellular Gln uptake occurs in a \( \text{Na}^+ \)-dependent manner. To test the contribution of SLC1A5 in Gln uptake by lung cancer cells, we conducted Gln uptake assays in A549 cells in the absence or in the presence of the glutamine analogue, GPNA, a competitive inhibitor of Gln uptake that binds specially to SLC1A5 \( (27, 28) \). Gln uptake kinetics in A549 cells showed a dose-dependent inhibition of Gln uptake of 15% \( (P < 0.05) \) and 32% \( (P < 0.005) \) by incubating at 100 and 300 \( \mu\text{mol/L} \) of GPNA for 3 minutes, respectively (Fig. 2B). No further inhibition of Gln uptake was observed when concentrations of GPNA were increased up to 900 \( \mu\text{mol/L} \) under the same conditions (data not shown). These results suggest that the majority of the cellular Gln uptake in A549 occurs in a \( \text{Na}^+ \)
metabolism-dependent lung cancer cell growth has not been investigated. We therefore cultured 5 lung cancer cell lines that varied in their level of protein expression of SLC1A5 (Fig. 3A and Supplementary Fig. S3), and cultured them in media that varied in Gln and growth factors concentrations for 72 hours as described in the Methods section. Under Gln-deprived condition, cell growth was significantly decreased in cell lines that had high levels of SLC1A5 expression (A549, HCC15, and H520), but no significant effect was observed in cell lines that had low (H727) or undetectable levels of SLC1A5 (H1819; Fig. 3B). Interestingly, H1819 (SLC1A5 null) grew much slower even under optimum growth conditions (+Gln+Sup) compared with A549, H520, and HCC15, all of which overexpress SLC1A5. Pharmacologic treatment of A549 cells (overexpresses SLC1A5) with increasing doses of 6-diazo-4-oxo-L-norleucine (DON), a glutamine antagonist (29) for 4 days resulted in significant growth inhibition in these cells, whereas H1819 (SLC1A5 null) was unaffected (Fig. 3E). These results suggest that SLC1A5 expression regulates at least in part lung cancer cell growth dependency on glutamine. To examine the specific contribution of SLC1A5 in lung cancer cell dependency on glutamine, we treated A549 and H520 cells (overexpress SLC1A5) with 1 mmol/L GPNA for 48 hours. Our results depicted in Fig. 3C and

SLC1A5 expression regulates growth dependency on glutamine in NSCLC cells

The role of SLC1A5 expression in regulating glutamine metabolism-dependent lung cancer cell growth has not been investigated. We therefore cultured 5 lung cancer cell lines that varied in their level of protein expression of SLC1A5 (Fig. 3A and Supplementary Fig. S3), and cultured them in media that varied in Gln and growth factors concentrations for 72 hours as described in the Methods section. Under Gln-deprived condition, cell growth was significantly decreased in cell lines that had high levels of SLC1A5 expression (A549, HCC15, and H520), but no significant effect was observed in cell lines that had low (H727) or undetectable levels of SLC1A5 (H1819; Fig. 3B). Interestingly, H1819 (SLC1A5 null) grew much slower even under optimum growth conditions (+Gln+Sup) compared with A549, H520, and HCC15, all of which overexpress SLC1A5. Pharmacologic treatment of A549 cells (overexpresses SLC1A5) with increasing doses of 6-diazo-4-oxo-L-norleucine (DON), a glutamine antagonist (29) for 4 days resulted in significant growth inhibition in these cells, whereas H1819 (SLC1A5 null) was unaffected (Fig. 3E). These results suggest that SLC1A5 expression regulates at least in part lung cancer cell growth dependency on glutamine. To examine the specific contribution of SLC1A5 in lung cancer cell dependency on glutamine, we treated A549 and H520 cells (overexpress SLC1A5) with 1 mmol/L GPNA for 48 hours. Our results depicted in Fig. 3C and

Figure 3. Glutamine is required for growth of lung cancer cells in vitro. To test whether SLC1A5 expression is correlated with Gln-dependent growth of lung cancer cells in vitro, 5 lung cancer cell lines that vary in their expression level of SLC1A5 protein (A) were grown in culture media that are supplemented with EGF, insulin, selenium and transferein, and 2 mmol/L of Gln (+Gln+Sup), or Gln but no supplements (+Gln−Sup), or no Gln but with supplements (−Gln+Sup) or not supplemented with neither Gln nor supplements. B, the fold change in cell growth was analyzed at day 3 by Cell-Titer 96-Aqueous Colorimetric Assay as a change of optical density (OD) at 490 nm signal from day 0. Sensitivity of growth inhibition to Gln deprivation was significant in cell lines that overexpress SLC1A5, A549, H520, and HCC15, but not in cell lines that have low or undetectable level of the protein H1819 and H727. C, the effect of Gln deprivation on cell viability in SLC1A5-expressing cell lines A549 and H520 were measured by Trypan blue Exclusion Dye method after 48 hours of culturing in media that either supplemented or deprived of Gln. D, the antiggrowth effect of Gln deprivation was measured by calculating the net increase or decrease of cellular growth after 48 hours of culturing under media that was either supplemented or deprived of Gln or fully supplemented media + 1 mmol/L of GPNA using the following equation: (Ti−Tz)/(C−Tz) × 100. Where Ti is the cell number after 48 hours of treatment of growth stimuli or inhibitors (i = inhibition), Tz is the cell number at time zero, and C is the cell number of the control cells that were cultured in optimum growth conditions (+Gln+Sup). E, dose-response decline in cell growth after treatment of A549 and H1819 with increasing concentrations of DON for 48 hours. Statistical significance was assessed by Student t test and was denoted as *: P ≤ 0.05; **: P ≤ 0.005 from 3 independent assays.
D show a significant decrease in cell growth in both cell A549 and H520 cell lines, whereas viability was less affected.

**Blockade of SLC1A5-related Gln uptake in lung cancer cells increases release of intracellular ROS**

Our shotgun proteomic data from fresh-frozen tissue of stage I lung cancer showed an increased expression of both alanyl amino peptidase (ANPEP) and glutathione synthetase (GSS), 2 key enzymes in the glutathione synthesis pathway (data not shown). Because glutamine acts as a precursor in the biosynthesis of glutathione (GSH; ref. 30) and because GSH functions as a scavenger for intracellular excess of reactive oxygen species (ROS; refs. 31, 32), we tested whether inhibiting SLC1A5-mediated Gln uptake in A549 cells would increase the intracellular level of ROS. When treated with increasing doses of GPNA for 24 hours, A549 cells exhibited rising levels of intracellular ROS as measured by the relative fluorescence signal of the oxidized form of H<sub>2</sub>DCFDA (Fig. 2D). To test whether the antiproliferative effect and the increase of intracellular ROS release were mediated by SLC1A5-Gln activity, we measured Gln uptake, cell growth, and intracellular ROS release in A549 cells which express high levels of SLC1A5 compared with H1819 (Supplementary Fig. S2B). The Gln uptake of H1819 was significantly lower (by 40%; P < 0.005) than that of A549 and was comparable with the same uptake level of A549 cells when treated with SLC1A5 inhibitor GPNA at 300 μmol/L (P < 0.05). GPNA did not affect Gln uptake in H1819 compared with A549 cells, which are inhibited by 33% at 300 μmol/L. Pharmacologic blockade of SLC1A5 with increasing doses of GPNA for 48 hours caused a dose-dependent increase in intracellular ROS release in A549 but not in H1819 (Supplementary Fig. S3C). Similarly, a dose-dependent decrease of cell growth was observed in A549 (overexpressing SLC1A5) but not in H1819 (SLC1A5 null) when cells were cultured in media containing increasing concentrations of SLC1A5 inhibitor GPNA (Supplementary Fig. S3D). Collectively, these results suggest that Gln uptake is mediated in part by SLC1A5 transport activity in lung cancer cells and that blockade of this activity decreases cell growth and increases release of intracellular ROS.

**Targeting SLC1A5 causes G<sub>1</sub> arrest by inhibiting mTOR signaling**

To evaluate the effects of glutamine depletion and glutamine-dependent uptake by SLC1A5 on cell-cycle progression, A549 cells were cultured for 48 hours with either supplemented growth media (+Gln+Sup), growth supplement-depleted media (+Gln–Sup), glutamine-depleted medium (–Gln+Sup), media that lacked both glutamine and growth supplements (–Gln–Sup), or in (+Gln+Sup) media + 5 mmol/L GPNA. Glutamine depletion only (–Gln +Sup) resulted in complete inhibition of growth as illustrated by the increased percentage of cells arrested at G<sub>1</sub> phase and decreased percentage of cells at S and G<sub>2</sub>–M phases but cell viability was unaffected (Fig. 4A and B). A similar effect was observed in cells treated with GPNA. To test whether the antiproliferative effect of Gln depletion can be attributed to SLC1A5 activity, we targeted SLC1A5 genetically for downregulation by using a specific siRNA. A significant knockdown of SLC1A5 protein was confirmed by Western blotting after 72 hours incubation with 2 different concentrations of anti-SLC1A5 siRNA (Fig. 5A). Similar to Gln depletion and GPNA treatment, a significant reduction in viability and growth were observed when SLC1A5 was downregulated by siRNA (Fig. 5B and C). The downregulation of SLC1A5 by siRNA also resulted in cell-cycle arrest at G<sub>1</sub>. Although the literature suggests that increased cell surface expression of SLC1A5 can be explained by cancer dependency on glutamine metabolism to support its high...
demand for energy and macromolecular biosynthesis (33–35), more recent studies revealed that SLC1A5 mediation of glutamine transport is independent from glutamine metabolism and necessary for activating critical survival and cellular growth signaling cascades including mTOR, and ERK pathways in some cancer types (24, 28, 36). To test whether Gln uptake into lung cancer cells induces mTOR signaling, we first starved H520 cells of Gln and growth factors for 24 hours. The next day, cells were supplemented with medium with glutamine (+Gln-Sup) or glutamine-free (−Gln-Sup) but with growth factors (EGF and Insulin) or full growth media (Sup) or glutamine-free (SLC1A5-expressing lung cancer cells is unknown. Whether this mechanism is universal in all SLC1A5-expressing lung cancer cells is unknown.

Discussion

We report for the first time the clinical relevance as candidate diagnostic biomarker and the biologic function of SLC1A5 in lung cancer. Our results show that SLC1A5 mediates glutamine transport required for lung cancer cell growth and survival. Specifically, we report that (i) SLC1A5 is overexpressed at the cytoplasmic membrane and is associated with squamous lung cancer histology and male gender, (ii) glutamine uptake in lung cancer cells is mediated in part by SLC1A5, (iii) SLC1A5 expression regulates growth dependency on glutamine in NSCLC cells, (iv) blockade of SLC1A5-related Gln uptake in lung cancer cells increases release of intracellular ROS, and (v) targeting SLC1A5 causes G1 arrest by inhibiting mTOR signaling.

SLC1A5 expression is significantly associated with but not restricted to SCC histology (Fig 1 and Supplementary Fig. S1) and is significantly correlated with higher tumor expression levels in men (Fig. 1B and Table 1). The biologic reasons for this gender correlation are not fully understood. One potential explanation is that sex hormones may regulate SLC1A5 and other amino acids transporters may explain these findings and deserve further investigation. The expression pattern of amino acid transporters in different solid tumors including liver (10, 12), breast (12) and colon cancer (37) is emerging in the literature. In lung cancer, a recent histologic study in a cohort of 160 NSCLCs found that Lat1, a sodium-independent amino acid transporter was expressed in 79.6% (43/54) of nonadenocarcinoma and in 15.1% (16/106) of adenocarcinomas (38). The same study also showed that the expression of Lat1 is significantly correlated with markers of glycolysis, angiogenesis, phosphoinosistide-3 kinase (PI3K)/Akt, EGFR receptor (EGFR), and mTOR pathways. Furthermore, the same group found Lat1 to be correlated with chemoresistance and...
poor prognosis in NSCLC (39). Short of functional data, this report provides preliminary evidence for the potential importance of amino acids transporters in lung cancer progression. Future functional studies should be designed to determine the relative contribution of Lat1 to glutamine uptake in lung cancer cells.

SLC1A5 has known functions in normal and cancer cells: as a retroviral receptor during placental development and cancer–endothelial cell fusion in breast cancer (25, 40), and as a neutral amino acid transporter with high affinity for glutamine (11, 41). Earlier studies in lung cancer models in vitro and in vivo showed that glutamine is essential for both growth and viability of the cells (14–16). Therefore, we focused our efforts on investigating the contribution of SLC1A5 to glutamine transport in lung cancer cell lines. We hypothesized that the enhanced expression of SLC1A5 in lung tumors and cell lines could be an adaptation mechanism that enables lung cancer cells to efficiently capture the overly abundant Gln from the extracellular milieu. Our results indicate that most of the Gln uptake by A549 cells occurs in a Na\(^+\)-dependent fashion and that half of that is mediated by SLC1A5. Pharmacologic and genetic targeting of SLC1A5 significantly attenuates cell growth by forcing the cells to arrest at G\(_1\) phase. Because SLC1A5 can transport aliphatic neutral amino acids including glutamine (10, 41), we anticipate that a part of the growth inhibitory effect of its blockade can be attributed to reducing the uptake of other neutral amino acids. Nonetheless, because SLC1A5 has high affinity to Gln, which is the most abundant amino acid in circulation, and because SLC1A5 contributes to 50% of the Na\(^+\)-dependent Gln transport (Fig. 2 and Supplementary Table S2), SLC1A5 activity is most likely responsible for the phenotypic effects observed. These data also suggest that other Na\(^+\)-dependent transporters such as SN1 (SLC38A3) and/or SN2 (SLC38A5) may contribute to Gln uptake in cancer cells. Future studies are needed to evaluate the relative contribution of other amino acids transporters in lung cancer.

The antigrowth effect of siRNA downregulation of SLC1A5 in lung cancer cells shown in our results is consistent with previous studies that used antisense methods to downregulate SLC1A5 in liver cancer cell lines (24). The direct impact of SLC1A5 downregulation on cell-cycle progression in lung cancer provides the first strong evidence that SLC1A5 is a link between Gln availability and cell division. In addition and consistent with the emerging prosurvival role of \(\gamma\)-glutamine in cancer progression (18, 19), we found that the protein level of GSS, a rate-limiting

Figure 6. SLC1A5-mediated Gln uptake activates mTOR signaling. mTOR, AKT, and ERK signaling activation was evaluated using Western blot analysis of the phosphorylation of p-mTOR signaling in H520 (SCC) and H1819 (ADC) cell lines in response to Gln deprivation or SLC1A5 blockade. Cells were deprived of growth factors and Gln (starved) for 24 hours and were treated with RPMI-1640 media that were supplemented EGF, insulin, selenium and transferein, and 2 mmol/L of Gln (+Gln + Sup), Gln but no supplements (+Gln - Sup), or no Gln but with supplements (-Gln + Supp), or not supplemented with neither Gln not supplements, or the full growth media + 5 mmol/L of GPNA (=Gln + Sup) for 60 minutes. A, Gln deprivation or GPNA treatment has no effect on mTOR or ERK signaling in H1819 cells (SLC1A5 null). B, in H520 (SLC1A5 overexpressed) the activation of mTOR signaling cascade was attenuated as evident by the levels of phosphorylated mTOR and the phosphorylation of its p70S6K and p85S6K p. Gln deprivation or GPNA treatment has no effect in either AKT or ERK signaling in H520 cells.
enzyme which catalyzes the conversion of γ-glutamyl-cysteine to glutathione (42), was higher in A549 than H1181. Interestingly, the expression pattern of GSS mirrors that of SLC1A5 in these 2 cell lines. This is consistent with our shotgun proteomic data that showed GSS to be over-expressed in both SCC and ADC stage I NSCLC tissues compared with control counterpart (data not shown). To test the hypothesis that Gln transported by SLC1A5 contributes to GSH synthesis, we inhibited the transporter activity with GPNA. Blockade of SLC1A5 resulted in an increase of intracellular ROS release in A549 (SLC1A5 positive), but not in H1181 (SLC1A5 negative) with increasing doses of GPNA (Supplementary Fig. S3C). These results suggest that SLC1A5 expression may be one component of a wider metabolic reprogramming scheme that is adopted by lung cancer cells to combat oxidative stress in their microenvironment.

Recent studies revealed a new role of SLC1A5 activity independent from glutamine metabolism and necessary for activating critical survival and cellular growth signaling cascades including mTOR and ERK pathways in some cancer types (24, 28). This role for Gln and its transporter in cancer cells goes beyond traditional metabolic functions of amino acids. Consistent with these reports, our results (Fig. 6B) suggest that glutamine uptake via SLC1A5 can activate mTOR signaling independent from growth factors in H520 SCC cells. Altogether, the results of this study suggest that growth dependency on glutamine is linked to SLC1A5 expression and activity and that inhibition of its Gln uptake activity has cytostatic effects in lung cancer cells in vitro.

In conclusion, our results provide for the first time, a functional link between SLC1A5 activity and the growth dependency on glutamine observed in lung cancer cells. The cytostatic consequences of targeting SLC1A5 activity could be explained in part by the inactivation of mTOR signaling in addition to the depletion of the intracellular glutamine pool necessary for biosynthesis of macromolecules in lung cancer cells. The differential expression of SLC1A5, its cell surface location, and its function as an amino acid transporter make it an attractive target in lung cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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