Histology and Histopathology
From Cell Biology to Tissue Engineering

ORIGINAL ARTICLE   **Open Access**

Upregulation and epigenetic modification of the creatine transporter SLC6A8 in non-small cell lung cancer

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Summary. Introduction. Lung cancer is a major cause of cancer-related death worldwide and effective therapies, besides surgery, are available only for a small proportion of patients. Since cellular respiration is known to be broadly altered in malignant tumors, the cellular processes of respiration can be a potential therapeutic target. One important element of cellular respiration is creatine and its transport by the creatine transporter SLC6A8. Here we describe the expression of SLC6A8 at the RNA and protein level, epigenetic modifications as well as survival analysis in NSCLC tissues and matched controls.

Materials and Methods. We analyzed epigenetic modifications of the *SLC68A* gene in 32 patients, of which 18 were additionally analyzed by transcriptome analysis. The expression of SLC6A8 at the protein level was assessed by immunohistochemistry using an independent cohort and correlated with clinico-pathological data including survival. Kaplan-Meier analysis was performed to analyze the possible effects of the transcriptional levels of SLC6A8 in another separate cohort (n=1925).

Results. *SLC6A8* loci are epigenetically modified in NSCLC compared with tumor-free controls. SLC6A8 is upregulated in NSCLC at the RNA and protein level. High mRNA expression of *SLC6A8* was associated with an overall poor prognosis in lung adenocarcinoma patients and displayed the strongest adverse prognostic effect in male smokers with adenocarcinomas. Results of transcriptome analysis were partially confirmed at the protein level.

Conclusions. Our results suggest an important role of creatine and its transport via SLC6A8 in NSCLC.

Key words: SLC6A8, Creatine Transporter, Cell Respiration, Non-small cell lung cancer (NSCLC), Transcriptome, Methylome, Immunohistochemistry, Survival Analysis

Introduction

Lung cancer is the leading cause of cancer-associated death worldwide (Torre et al., 2016) and is often diagnosed in the metastasized state due to rapid tumor growth and unspecific early symptoms. Non-small cell lung carcinoma (NSCLC) is the most frequent type (85% of patients) with an increasing incidence of the sub-entity adenocarcinoma (adNSCLC), which accounts for approximately 40% of lung cancers, whereas

squamous cell carcinoma (sqNSCLC) accounts for roughly 25%. Even though targeted therapies and immune checkpoint inhibition have substantially improved the therapeutic modalities in NSCLC in recent years, they can only be applied to a limited proportion of patients (Morgensztern and Herbst, 2016; Hendriks et al., 2018). Hence, there still is a great need for further insights into complementary targets.

One of these potential new targets could be cellular respiration as it is broadly altered in malignant tumors compared with tumor-free tissues. We have previously shown that the expression of TKTL1, an enzyme involved in the anaerobic degradation of glucose by transketolases, is enhanced in a large proportion of NSCLC (Schultz et al., 2008). A further important element of cellular respiration is creatine. Physiological-ly, non-phosphorylated creatine is produced inside the liver and kidneys. Upon its distribution to target cells, creatine kinases catalyze the ATP-dependent phosphorylation of creatine. Phosphorylated creatine is then absorbed into the target cell via the creatine transporter SLC6A8, where phosphate is used to regenerate energy by ATP (Loo et al., 2015).

Creatine metabolism has long been the focus of research into autism spectrum disorders and intellectual disability, as the so-called X-linked creatine transporter deficiency is a clinical syndrome that is characterized by developmental delay, impaired intelligence, autistic behavior, and seizures (Cameron et al., 2017). However, so far, little research has been conducted in the context of malignancies.

As carcinomas frequently display largely altered metabolic pathways regarding cellular respiration, which might for example be influenced by dietary habits, we analyzed the *SLC6A8* gene in human NSCLC and the corresponding tumor-free lung tissues from the same donors. Here we describe the expression of SLC6A8 at the RNA and protein level, as well as epigenetic modifications in the gene body and the potential prognostic value of SLC6A8 during risk stratification.

Materials and methods

Tissues for methylome and transcriptome analysis

We collected tissue samples from 32 patients undergoing curative surgical removal of lung cancer, of which 14 had adNSCLC and 18 sqNSCLC. The samples were analyzed together with their matched tumor-free lung tissue. The protocol was approved by the local ethics council at the University of Luebeck (AZ-12-220). The tumor samples were micro-dissected to enrich the tumor content for methylome and transcriptome analysis.

Methylome analysis of the SLC6A8 gene in NSCLC and tumor-free lung tissues

Epigenetic modifications of the *SCL68A* gene were analyzed in 32 patients (19 males, 13 females) by Infinium HumanMethylation450k BeadChips (Illumina Inc., San Diego, CA, USA) as previously described (Marwitz et al., 2014). The analyzed primary NSCLC collective comprised 14 adNSCLC (5 males, 9 females) as well as 18 sqNSCLC (14 males, 4 females). The mean age of the patients at surgery was 65.6 years, 16 were smokers, 14 were ex-smokers, one patient was a never-smoker, and smoking status was unknown in one case. Tumor stages were divided according to the 8th Edition of the Union Internationale Contre le Cancer (UICC) staging system as follows: T1 (n=4), T2 (n=18), T3 (n=7), T4 (n=3).

Hierarchical clustering of methylation levels of *SLC6A8* CpG loci obtained by GenomeStudio software was visualized using the ClustVis web tool (Metsalu and Vilo, 2015), where the heatmap is plotted using the pheatmap R package (version 0.7.7).

Transcriptome analysis of SLC6A8 in NSCLC and tumor-free lung tissue

Of the 32 aforementioned patients, 18 (10 adNSCLC, 8 sqNSCLC) were additionally analyzed by transcriptome analysis as described elsewhere (Marwitz et al., 2017). This cohort consisted of 9 female and 9 male patients. The mean age at diagnosis was 66.06 years, 10 were smokers, 7 were ex-smokers, and one patient was a never-smoker. Tumor stages were divided according to the 8th Edition of the UICC staging system as follows: T1 (n=1), T2 (n=9), T3 (n=6), T4 (n=2).

The relative *SLC6A8* gene expression values at the RNA level were quantile-normalized and extracted from the GEO-dataset GSE74706, then analyzed with GraphPad Prism v.8.

SLC6A8 protein expression assessed by Immunohisto-chemistry

By Immunohistochemistry (IHC), we analyzed tissues from an independent cohort of 165 adNSCLC and 126 sqNSCLC samples from a total of 291 patients. The histological diagnosis including grading was based on the 2015 World Health Organization Classification of Lung Tumors; for determination of the tumor state, the 8th Edition of the UICC staging system was used. Tumor samples were evaluated by two independent pathologists (CK and SP). For IHC, tissue microarrays (TMA) were constructed from Formalin-fixed, paraffin-embedded (FFPE) tissue blocks from tumors and corresponding normal lung tissue. We had clinicopathological data for 242 of these patients, which are summarized in Table 1.

Biomaterials were acquired with the approval of the respective local ethics committees (University of Bonn (No. 036/08) and Cologne (AZ 13-091)). Written informed consent was obtained from all patients.

For TMA construction, each sample was represented in triplicates of 0.6 mm diameter cores. A tumor sample was incorporated into further analyses if at least two

cores were evaluable. IHC staining of SLC6A8 was performed manually. In brief, slides were incubated at room temperature for 1 hour with the primary antibody anti-SLC6A8 (clone 62196, Abcam, Cambridge, USA) at a 1:100 dilution. SLC6A8 staining was considered positive if it was cytoplasmatic. Nuclear or pure membranous staining was not observed. The protein expression of SLC6A8 was assessed by estimation of staining intensity (SI) and estimation of the percentage of positive tumor cells from all tumor cells (Tumor proportion score, TPS). SI was indicated as strongly positive (SI 3), moderately positive (SI 2), weakly positive (SI 1), and negative (SI 0). TPS was scored as high (≥50% positive tumor cells), low (<1-50% positive tumor cells), or absent expression (0% positive tumor cells). Values of SI and TPS for all examined cores of a patient sample were recorded as their mean value. For the definitive scoring, the SI of the majority of tumor cells (cut-off ≥50%) was leading.

IHC staining of Ki-67 (clone 30-9, Ventana Tucson, AZ, USA; RTU) was performed with the Ventana Discovery (Ventana Medical System) automated staining system. The Ki-67 index was estimated per core and the mean was calculated from all cores per case.

For mitotic count, mitoses were counted in all cores per case using H&E-stained slides, and the mean value per core was assessed.

Analysis of survival rates

We performed Kaplan-Meier analyses in a separate collective of patients (n=1925) from data generated using Affymetrix chips and retrieved from kmplot.com. The KM-plotter is an online service capable of assessing the impact of a large number of genes on survival in different cancer types (Győrffy et al., 2013). Sources of this data collection include GEO, EGA, and TCGA. For survival analysis of the IHC cohort, Kaplan-Meier analysis and log-rank tests were performed.

Statistical Analysis

For methylome and transcriptome analysis, GraphPad Prism 8 (GraphPad Software, Boston,) was used. The results are displayed as mean ±95% confidence interval. The relative gene expression values at the RNA level were quantile-normalized and analyzed using an unpaired t-test and the multiple comparison correction of Benjamini, Krieger, and Yekutieli (Q=5%). As part of the Kaplan-Meier analyses, we determined hazard ratios and performed log-rank tests.

For IHC analysis, SPSS (version 26.0, SPSS Inc., Chicago IL) was used. Pearson's chi-squared test and one-way ANOVA were used to correlate the SLC6A8 protein expression score with clinicopathological characteristics. Only two-tailed tests were applied and *P*-values <0.05 were considered statistically significant.

Results

We analyzed epigenetic modifications of the *SLC68A* gene in 32 patients, of which 18 were additionally analyzed by transcriptome analysis. The expression of SLC6A8 at the protein level, on the other hand, was assessed in an independent cohort of 291 patients and correlated with clinicopathological data, including survival.

The creatine transporter SLC6A8 is epigenetically modified in NSCLC

The collective methylome analysis comprised 32 NSCLC samples, 14 being adNSCLC (5 males, 9 females) and 18 sqNSCLC (14 males, 4 females). Concerning the methylation of *SLC6A8* CpG loci, we noticed a clear difference between the NSCLC subtypes and tumor-free lung tissue (Fig. 1). Especially in sqNSCLC, we observed a remarkable hypomethylation

Table 1. Patient baseline characteristics of the independent IHC cohort.

Patients	male	165 (68.2%)
	female	77 (31.8%)
Tumor type*	adNSCLC	165
	sqNSCLC	126
Age at first diagnosis	mean	65 years
Survival time	mean	21 months
Survival status	alive	178
	deceased	62
	unknown	51
UICC**	Ia	29 (12%)
	Ib	67 (27.7%)
	IIa	14 (5.8%)
	IIb	49 (20.2%)
	IIIa	63 (26.0%)
	IIIb	8 (3.3%)
	IV	12 (5%)
Smoking status	current	133 (55.2%)
	former	95 (39.4%)
	never	13 (5.4%)
	unknown	1 (0.4%)

*: According to the 2015 World Health Organization Classification of Lung Tumors; **: According to the 8th Edition of the UICC staging system.

Table 2. SLC6A8 protein expression in the NSCLC cohort.

	Overall		adNSCLC		sqNSCLC	
	n	%	n	%	n	%
High	51	17.5	50	30.3	1	0.8
Moderate	116	39.9	69	41.8	47	37.3
Weak	95	32.6	46	27.9	49	38.8
Negative	29	10.0	0	0.0	29	23.1
Total	291		165		126	

in 13 of the 19 CpG loci, whereas the remaining 6 CpG loci showed a slight increase in methylation levels. Also, when comparing adNSCLC with tumor-free lung tissue, we saw a distinct but slightly less pronounced difference in methylation. Additionally, we examined the effects of sex on methylation, observing sex-related differences in DNA methylation between normal male and female samples at the CpG loci cg00140085 (males: mean: 0.048, SD: 0.014; females: mean: 0.423, SD: 0.025) and cg14349378 (males: mean: 0.091, SD: 0.030; females: mean: 0.378, SD 0.040) on chromosome X, probably because of dose compensation. This sex-specific DNA methylation pattern was impaired in some tumor samples. Nevertheless, sex-related differences were found to be independent of the entity of each sample.

The transcription of the creatine transporter SLC6A8 is upregulated in NSCLC

The described epigenetic changes in *SLC6A8* CpG loci in NSCLC suggested corresponding changes at the transcriptional level. Therefore, we additionally analyzed micro-dissected tumor and lung tissue samples obtained from patients (overall n=18) deriving from the methylome cohort. The collective of tumor samples comprised 10 adNSCLC as well as 8 sqNSCLC. Relative quantile-normalized gene expression values for *SLC6A8* were obtained from the GEO-dataset GSE74706 and analyzed with GraphPad Prism v.8 (Fig. 2). We performed unpaired t-tests with the multiple comparison correction of Benjamini, Krieger, and Yekutieli. The statistical analysis revealed a significant increase in *SLC6A8* expression at the RNA level in adNSCLC compared with tumor-free lung tissue (*P*<0.001). Differences in expression were even more pronounced in sqNSCLC compared with tumor-free lung tissue (*P*<0.0001). Overall, we saw a distinct increase in *SLC6A8* expression at the RNA level in NSCLC compared with tumor-free lung tissue, which hints at an increased capability of tumor cells to import creatine for ATP regeneration and hence, available energy for malignant processes.

SLC6A8 protein expression assessed by IHC in TMAs and its correlation with clinicopathological characteristics

SLC6A8 protein expression was assessed in another, independent cohort of patients. The clinicopathological data of these patients are summarized in Table 1. Via IHC, increased expression of SLC6A8 at the protein level could be confirmed. Overall, 17.5% of tumor samples showed high expression (51 out of 291), 39.9% moderate expression (116 out of 291), 32.6% weak expression (95 out of 291), and 10% (29 out of 291) showed no protein expression of SLC6A8. Comparing the two NSCLC subtypes, we found different expression patterns, with a higher expression in adNSCLC than in sqNSCLC. In brief, in the sqNSCLC cohort, 23.1% were negative (29 out of 126), 38.8% were weakly positive (49 out of 126), 37.3% were moderately positive (47 out of 126) and 0.8% were highly positive (1 out of 126). In contrast, in the adNSCLC cohort, none of the tumor samples were negative (0 out of 165), 27.9% were weakly positive (46 out of 165), 41.8% were moderately positive (69 out of 165) and 30.3% were highly positive (50 out of 165) (Table 2).

The distribution of low (negative and weak) and high (high and moderate) expression patterns was highly statistically significant between the subtypes (*P*<0.0001; two-tailed Chi-square test without Yates correction). Pneumocytes of corresponding normal lung tissues displayed no significant expression of SLC6A8. Furthermore, tumor-associated lymphocytes and macrophages mostly showed SLC6A8 expression. Figure 3 provides examples of different SLC6A8 expression profiles assessed by IHC.

We further assessed if the protein expression of SLC6A8 correlates with clinicopathological data. We
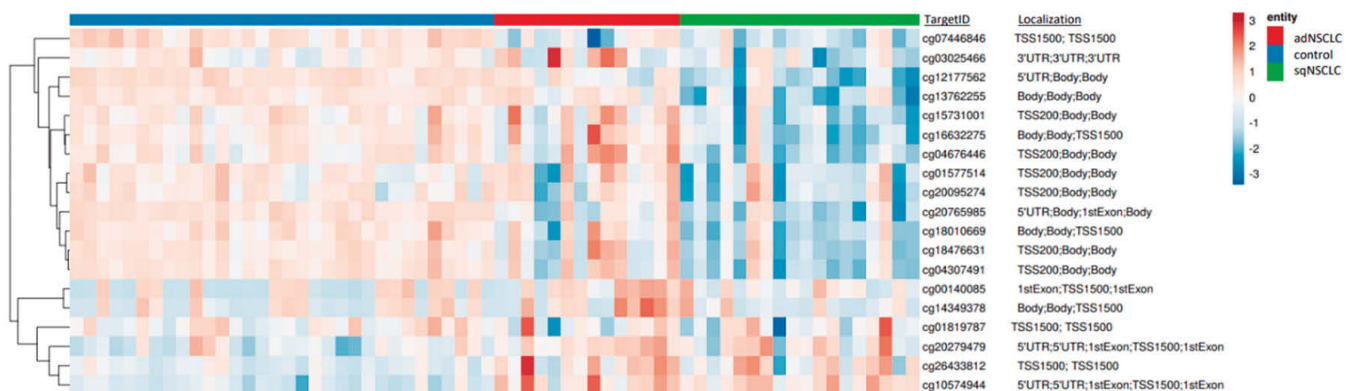
Fig. 1. Hierarchical clustering of the methylation levels of *SLC6A8* CpG loci in human Methylation 450k BeadChip (second upper line: green, tumor-free lung tissue; red, adNSCLC; blue, sqNSCLC; first upper line: pink, female; turquoise, male; heatmap: red, high; blue, low DNA methylation values; mean DNA methylation = 0). The target IDs of the CpG loci and their localization (chr.X, hg19) are named in the right-hand row.

found no significant correlation of SLC6A8 expression with age, UICC status, grading, and smoking status. We found a significant correlation concerning the Ki-67 index, being lower in NSCLC with high SLC6A8 expression (P=0.001), whereas we found no correlation between SLC6A8 expression and mitotic count (P=0.428). Another significant result was observed concerning sex, meaning that SLC6A8 was more expressed in female NSCLC patients (P<0.001).

Since SLC6A8 is involved in cell respiration, we analyzed if SLC6A8 protein expression is associated with tumor growth patterns. The growth pattern of sqNSCLC is solid throughout; however, the growth pattern of adNSCLC differs and is reflected in the grading (lepidic=G1, acinar and papillary=G2, solid and micropapillary=G3). Only one case was graded as G1 and was therefore excluded. Comparing G2 and G3 adNSCLC as regards SLC6A8 protein expression, we found that moderately differentiated adNSCLC showed a proportionally higher protein expression of SLC6A8 (n=60 high vs. n=15 low) than poorly differentiated adNSCLC (n=59 high vs. n=30 low) without being statistically significant (P=0.050006; two-tailed Chi-square test).

Survival analysis: Association of SLC6A8 with survival of NSCLC patients

We next analyzed whether SLC6A8 protein expression is associated with overall survival of the independent IHC cohort (Table 1). Samples were
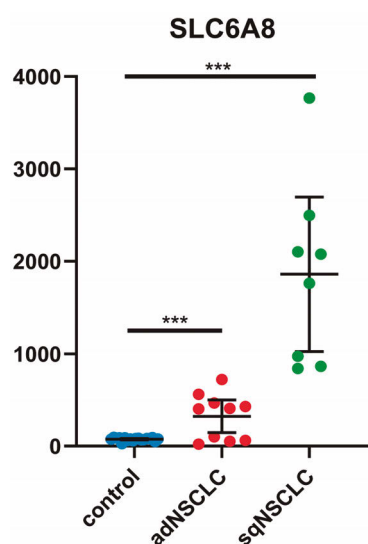
Fig. 2. Relative gene expression level of *SLC6A8* as quantile-normalized expression values of tumor-free lung tissues and matched tumors depicting the mean value with 95% confidence interval with error bars (blue: controls, red: adenocarcinomas, green: squamous cell carcinomas). Unpaired t-tests and multiple comparison correction of Benjamini, Krieger, and Yekutieli (Q=5%) were used with p≤0.001 (***) regarded significant.

dichotomized into low and high expressing groups, defining negative and weak SLC6A8 expression as low, and high and moderate SLC6A8 expression as high. Here, we found a trend toward favorable overall survival for patients with high SLC6A8 expressing NSCLC than for patients with low SLC6A8 expressing NSCLC (log-rank test P=0.28, data not shown). When survival data were analyzed separately for the two tumor entities, no significant differences were found (P=0.84 for sqNSCLC and 0.221 for adNSCLC).

We further performed survival analyses on publicly available mRNA data from another independent cohort of 1925 patients retrieved from kmplot.com (Fig. 4) to analyze the possible effects of SLC6A8 transcriptional levels on survival.

Here, high levels of SLC6A8 transcription were generally associated with an unfavorable prognosis in NSCLC patients (Fig. 4A) at a hazard ratio of 1.58 (95% CI = 1.39-1.79). Regarding the main NSCLC subtypes, we found that this prognostic effect was stronger in adNSCLC (Fig. 4B) and not detectable when solely considering sqNSCLC (Fig. 4C). In stage 1 NSCLC patients, the separation was more distinct compared with all NSCLC patients (Fig. 4D). The same holds for stage 1 adNSCLC (Fig. 4E) but not for sqNSCLC (Fig. 4F).

Since *SLC6A8* is located on chromosome X, we analyzed the effect of sex (female Fig. 4G and male Fig. 4H). No significant differences were observed when comparing overall NSCLC tissues. We then analyzed the effects of smoking habits on *SLC6A8* transcription and survival. Smokers with sqNSCLC of all stages presented similar outcomes as stage 1 sqNSCLC (Fig. 4I). Smoking adNSCLC patients of all stages were better separated than adNSCLC patients overall and similar to adNSCLC stage 1 (Fig. 4J).

We stratified these smoking adNSCLC patients by sex. Interestingly, female adNSCLC smokers showed a weaker correlation (Fig. 4K) than male adNSCLC smokers (Fig. 4L). In male smoking patients with adenocarcinomas, the overall strongest adverse prognostic effect was observed with a hazard ratio of 3.49 (95% CI=1.81-6.74).

Discussion

Over the past decade, the discovery of various gene alterations and rearrangements as well as immune checkpoints has led to the development of a multitude of novel prognostic factors and therapies in NSCLC, however, the different entities of NSCLC show a heterogeneous response to these therapeutic options (Osmani et al., 2018). The development of therapeutic regimens might also require an additional in-depth look at tumor metabolism. In particular, cell respiration and the generation of intracellular energy via creatine transport could offer new insights and serve as a supplementary prognostic factor, or maybe even as an additional therapeutic option. Nevertheless, research in this area is still in its infancy and many questions remain
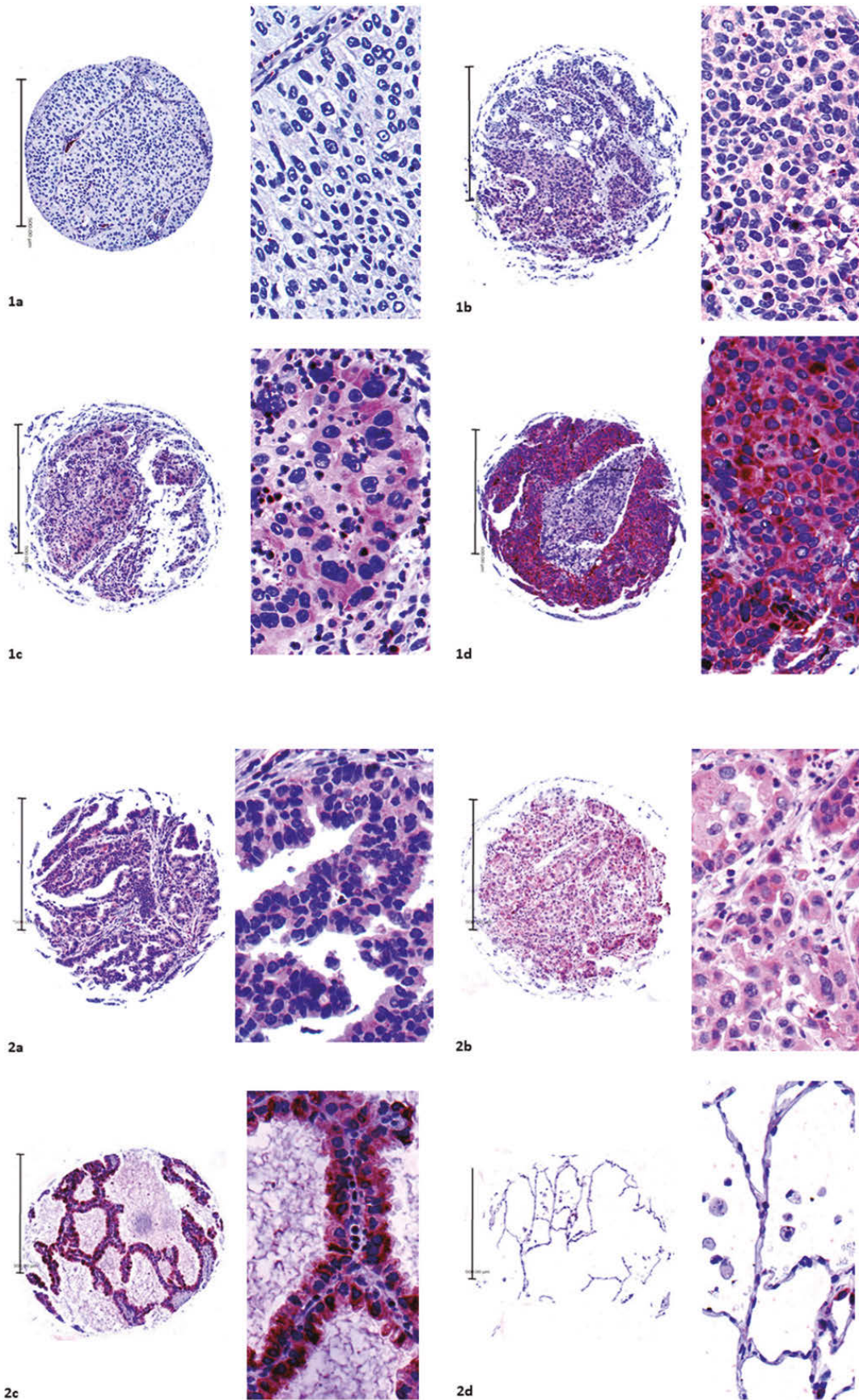
Fig. 3. Exemplary images of SLC6A8 expression patterns in NSCLC. Top: sqNSCLC with absent **(1a)**, weak **(1b)**, moderate **(1c)**, and strong **(1d)** expression. Bottom: adNSCLC with weak **(2a)**, moderate **(2b)**, and strong **(2c)** expression. SLC6A8 expression of lymphocytes is additionally depicted in 1c. Bottom right **(2d)**: lung tissue with mostly negative pneumocytes (×100 and 600, respectively. Scale bars: 500 μm).
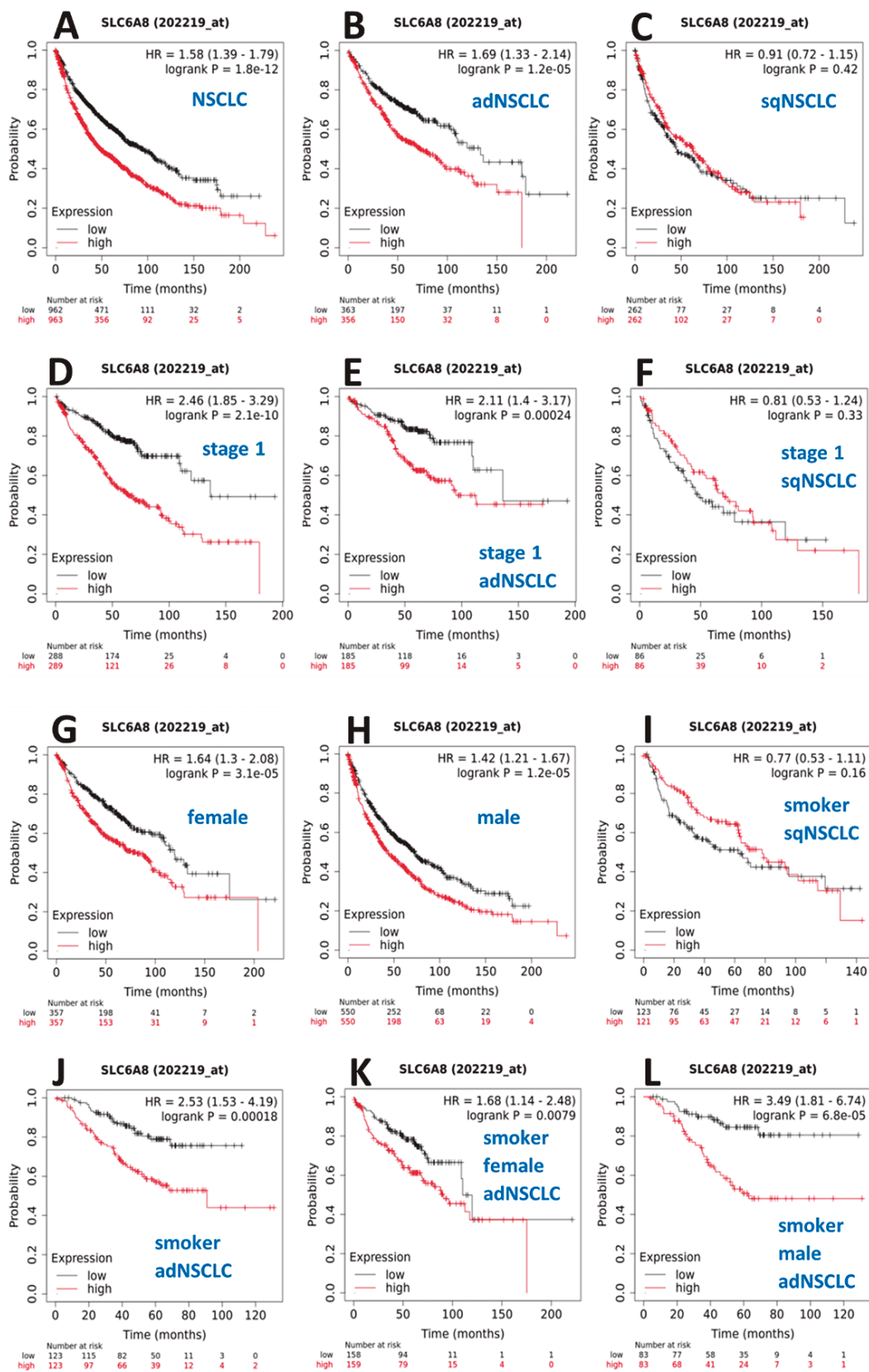
Fig. 4. Kaplan-Meier survival analysis using mRNA data retrieved from kmplot.com (n=1925), generated using Affymetrix chips (black, low expression; red, high expression). The results are stratified by NSCLC subtype, tumor stage, smoking habits, and sex. Statistical evaluation is depicted as a hazard ratio (HR) with 95% confidence intervals and *P*-value of the log-rank test (log-rank P).

unclear.

To the best of our knowledge, this is one of the first studies comprehensively describing the role of the creatine transporter SLC6A8 in NSCLC and our results indicate that high SLC6A8 expression is associated with adverse clinicopathological features. We show that SLC6A8 is upregulated in NSCLC tissues compared with tumor-free lung tissues at the RNA and protein level. We also noticed a clear difference in the epigenetic modification of *SLC6A8* CpG loci in NSCLC compared with tumor-free lung tissues. The sex-dependent differences in methylation of two of the CpG loci were seen in the context of X-inactivation for dose compensation in the female sex (Briggs and Pera, 2014). In additional survival analyses, high levels of SLC6A8 transcription were found to be associated with an overall unfavorable prognosis in NSCLC patients. This is in line with the results of Feng et al. (2021) and Fan et al. (2022). This effect was much more pronounced in adNSCLC than in sqNSCLC. Further detailed analysis revealed the strongest adverse prognostic effect in smoking male adenocarcinoma patients.

Interestingly, not all RNA data can be directly transferred to the protein level. We observed an overall significantly higher expression of SLC6A8 in NSCLC than in tumor-free lung tissue, however, results at the RNA level differed in part from the results of IHC staining. At the RNA level, we noticed a remarkably stronger expression of *SLC6A8* in sqNSCLC than in adNSCLC, whereas the ratio was reversed regarding the protein level assessed via IHC. Here we stated a significantly stronger expression of SLC6A8 in adNSCLC than in sqNSCLC. These differences could be explained primarily by the posttranscriptional changes and regulation of SLC6A8 expression. One example of this effect is the percentage of non-coding RNA, which is also known to be an important regulator in oncogenesis (Anastasiadou et al., 2018). Furthermore, there are a variety of kinases known to regulate SLC6A8. For example, Janus-activated kinase-2 (JAK2) and -3 (JAK3) are known to downregulate the activity of SLC6A8 (Shojaiefard et al., 2012; Fezai et al., 2015), whereas other kinases, such as the transmembrane Klotho protein, serum, and glucocorticoid inducible kinases 1 (SGK1) and 3 (SGK3), as well as protein kinase mTOR, enhance the activity of SLC6A8 (Shojaiefard et al., 2005, 2006; Almilaji et al., 2014).

Furthermore, we have not identified a significant correlation with other meaningful clinicopathological data. We found a significant correlation concerning the Ki-67 index with a significantly lower Ki-67 index in NSCLC with high SLC6A8 expression (P=0.001). However, since we did not find a significant correlation between SLC6A8 expression and mitotic count (P=0.428), this might be a phenomenon without causal significance.

In contrast to transcriptomic data, we found no significant correlation between protein expression of SLC6A8 and overall survival (P=0.28).

Feng et al. (2021) and Fan et al. (2022) could also confirm the protein expression of SLC6A8 in NSCLC tissue via IHC and found high levels of SLC6A8 transcription to be associated with an overall unfavorable prognosis in NSCLC patients. However, their cohorts were smaller (n=30 and n=36/51), respectively. Feng et al. have not divided their cohort into adNSCLC and sqNSCLC, and Fan et al. only investigated adenocarcinomas.

The presented differences in SLC6A8 expression between the two NSCLC sub-entities may be due to known differences in cell metabolism and proliferation rate. The metabolite creatine is known to be increased in NSCLC in general but more markedly so in sqNSCLC, whereas the activity of creatine kinases has been previously described to be lower in both sqNSCLC and adNSCLC compared with tumor-free lung tissue (Rocha et al., 2015). Besides, adNSCLC are known to primarily rely on aerobic glycolysis, whereas sqNSCLC predominantly rely on anaerobic glycolysis (Meijer et al., 2012). Therefore, sqNSCLC seem to be particularly dependent on phosphocreatine as a source for generating ATP. In this context, one could assume that SLC6A8 expression is associated with tumor growth, especially in adenocarcinomas, which show different growth patterns (lepidic, acinar, papillary, solid, micropapillary) and, with that, different relationships with their environment. However, for adNSCLC, we found no significant association between SLC6A8 expression and grading (G2 vs. G3), the latter reflecting the growth pattern.

Even though little is known about the role of creatine in malignant diseases, there is some work on the role of the SLC6A8 transporter in immune cells and, thus, in the tumor microenvironment. Macrophages are known to have high intracellular amounts of creatine with concentrations comparable to tissues such as muscle or brain. They obtain creatine primarily through SLC6A8-mediated active uptake and, interestingly, creatine inside the macrophage has a potent inhibitory effect on Interferon γ (Ji et al., 2019). A modification in creatine metabolism, therefore, also means a change in the Interferon γ signaling cascade within the tumor microenvironment. Furthermore, creatine uptake by SLC6A8 has been reported to be highly important for the antitumor immunity of CD8+ cytotoxic T cells (Di Biase et al., 2019). In the current study, protein expression of SLC6A8 in lymphocytes and macrophages could also be observed via IHC. Fan et al. suggest a close relationship between SLC6A8 and the tumor immune microenvironment in lung adenocarcinomas (Briggs and Pera, 2014). They found high SLC6A8 expression to be associated with less immune cell infiltration based on TCGA data using the ESTIMATE algorithm. Malignant cells often develop special mechanisms to use cellular metabolites to maintain rapid proliferation; one example of this circumstance is the increase in creatine metabolism (Kazak and Cohen, 2020). In the current study, the enhanced SLC6A8

expression we have demonstrated indicates an increased creatine uptake of the tumor cells. Such metabolic changes offer opportunities for new targeted therapeutic approaches.

Our study has several limitations. First, our methylome/transcriptome and protein data do not derive from the same cohort, which is why they are not readily transferable to each other. Second, the cohort used for methylome and transcriptome data was not as large and as well characterized in terms of clinicopathological data, especially survival data, as the IHC cohort. Therefore, we needed to resort to publicly available mRNA data deriving from another independent cohort from kmplot.com to analyze the possible effects of SLC6A8 transcriptional levels on survival. Despite the aforementioned limitations, based on our results, one could suggest that creatine and its transport via SLC6A8 might present an important target in tumor metabolism. Nevertheless, there is still a substantial need for additional research, especially concerning the complex interaction of creatine metabolism between tumor cells and the tumor microenvironment.

Conclusions

Lung cancer represents the leading cause of cancer death worldwide and even though targeted therapies have improved the therapeutic modalities for NSCLC during the last decade, they can only be applied to a small proportion of patients. There is still a great need for further insights into complementary targets. As carcinomas frequently display largely altered physiology regarding cellular respiration, we examined the creatine transporter SLC6A8 in human NSCLC and the corresponding tumor-free lung tissues. We demonstrated that SLC6A8 is epigenetically modified in NSCLC, which results in an upregulation of transcription compared with tumor-free lung tissue. Upregulation of SLC6A8 at the protein level could further be confirmed via IHC in an independent cohort, indicating a biological activity of SLC6A8. Regarding survival, we described a correlation between high levels of SLC6A8 transcription with an overall poor prognosis in NSCLC patients. Further detailed analysis revealed a pronounced adverse prognostic effect in adNSCLC and the strongest adverse prognostic effect in smoking male adenocarcinoma patients.

Our results suggest a substantial role of creatine and its transport via SLC6A8 in NSCLC. However, further validation and research are required, especially regarding the complex interplay of creatine metabolism between tumor cells and the tumor microenvironment.

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