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Original Article

High expression of SLC6A10P contributes to poor prognosis in lung adenocarcinoma

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Abstract: Purpose: To investigate the expression profile and prognostic value of SLC6A10P in patients with non-small cell lung cancer (NSCLC). Patients and methods: TCGA datasets were used to investigate the differential expression of SLC6A10P in NSCLC, lung adenocarcinoma (LUAD), and lung squamous cell carcinoma (LUSC). Expression of SLC6A10P was measured by in situ hybridization in tissue microarrays containing 136 NSCLC (51 LUAD and 85 LUSC) patients. The prognostic value of SLC6A10P was then evaluated. Results: SLC6A10P was highly expressed in tumor tissues compared with normal lung tissues. High SLC6A10P expression was associated with lymph node metastasis (NSCLC, P = 0.0054; LUAD, P = 0.0149), more advanced tumor stage (NSCLC, P = 0.0126; LUAD, P = 0.0416) and poor overall survival (NSCLC, P = 0.0248; LUAD, P = 0.0316) in NSCLC and LUAD. Multivariate analysis revealed that SLC6A10P was an independent prognostic factor in LUAD patients (P = 0.017). SLC6A10P showed no association with clinicopathological parameters and no prognostic value in LUSC. Conclusion: SLC6A10P is highly expressed in tumor tissues and its high expression predictspoor survival in patients with LUAD. SLC6A10P might serve as a novel therapeutic target and prognostic biomarker in LUAD patients in the future.

Keywords: SLC6A10P, lung adenocarcinoma, pseudogene, prognosis, bioinformatics

Introduction

Over the past few decades, the incidence and mortality of lung cancer has dramatically increased in China [1]. Currently, lung cancer is one of the most common malignancies and the leading cause of cancer-related deaths in China [2]. Non-small cell lung cancer (NSCLC), which contains two main histological types: lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC), are the main histological types of lung cancer. Despite the development of diagnosis and treatment methods, the 5-year overall survival rate of lung cancer has not been significantly improved. Therefore, further investigation of the molecular mechanisms involved in carcinogenesis and progression of NSCLC is urgently needed.

In recent years, open data resources have been widely used for screening and identifying cancer-related genes [3]. By analyzing the TCGA database and carefully reviewing the literature, we found a novel lung cancerrelated candidate gene, SLC6A10P (Solute carrier family 6 member 10, pseudogene).

The SLC6A10P gene mapped to chromosome 16p11.2 [4, 5]. SLC6A10P belongs to the solute carrier family 6 (SLC6) family, which contains nineteen functional members and four pseudogenes [6]. Aberrant expression of SLC6A10P has been observed in some malignancies [7]. However, to the best of our knowledge, the role of SLC6A10P in lung cancer has not been reported. In the present study, we aimed to investigate the gene's expression profile and prognostic value in NSCLC.

Materials and methods

Bioinformatics

Three datasets named *TCGA_LUNG_exp_HiSeqV2-2015-02-24*, *TCGA_LUAD_exp_HiSeqV2-2015-02-24* and *TCGA_LUSC_exp_HiSeq-*

Table 1. Clinical and histologic features of the 140 patients

Patient demographics	Number of of patients	Percentage
Age (years)		
≤60	67	49.3%
>60	69	50.7%
Sex		
Male	108	79.4%
Female	28	20.6%
Pathological type		
LUAD	51	37.5%
LUSC	85	62.5%
Differentiation		
Poorly	55	40.4%
Moderate or well	81	59.6%
Tumor size		
T1	15	11.0%
T2-3	121	89.0%
Lymph node status		
N0	79	58.1%
N1-2	57	41.9%
Tumor stage		
I	64	47.1%
II-III	72	52.9%

LUAD: lung adenocarcinoma; LUSC: lung squamous cell carcinoma.

V2-2015-02-24 were downloaded at the website of the UCSC Cancer Browser (https://genome-cancer.ucsc.edu/). These datasets contained 94 paired NSCLC tissue samples, 46 paired LUAD tissue samples and 48 paired LUSC tissue samples, respectively. Files named *"genomic Matrix"* in these datasets were used to obtain the mRNA expression levels of SLC6A10P (using Editplus® software). Expression Atlas (http://www.ebi.ac.uk/gxa/home/) was used to determine the expression profile of SLC6A10P in human tissues or cell lines.

Ethics, consent and permissions

This study was reviewed and approved by the Research Ethics Committee of Zhongshan Hospital, Fudan University. Written informed consent was obtained from all of the patients. All specimens were handled and made anonymous based onethical and legal standards.

Patients and construction of tissue microarray (TMA)

A total of 140 tissue samples were obtained from patients with NSCLC who were operated on at the Thoracic Surgical Division of Zhongshan Hospital, Fudan University in the year 2005. A total of 10 normal lung tissues were obtained from patients, whose illness was diagnosed as benign lung tumor, undergoing operation at the Thoracic Surgical Division of Zhongshan Hospital, Fudan University in the year 2005. All patients were staged according to the international staging system and were graded according to the recent 2004 World Health Organization criteria. In the present study, no patients received adjuvant or neoadjuvant chemotherapy or radiation treatment prior to surgery, and no other malignancy within 5 years prior to diagnosis was present. As previously described [8], tissue samples from 140 primary NSCLC cases and 10 normal lung tissues were arranged in rows and columns to construct a TMA.

In situ hybridization (ISH) and evaluation

Detection of SLC6A10P in FFPE TMA samples was performed using a SLC6A10P ISH Kit (Boster, Pleasanton, CA, USA). Antisense SLC6A10P probes were as follows: 5'-CCCAGCACACTTGGCTCTCTAGGTAGGTCCTACTATTACT-3', 5'-TCCATGGTGATCGTTTTCTACTGCAACACCTACTACATCA-3' and 5'-TCTGCTGGACCTGGTCTGGCCATCTGTTACCTGCCTATCT-3'. ISH was performed, essentially, as described by Nuovo et al. [9].

Briefly, 6 μm sections were deparaffinized, rehydrated and submitted to protease digestion followed by postfixation for 10 minutes with 1% paraformaldehyde (PFA). Then, hybridization was done at 37°C overnight with a mixture containing target probes to SLC6A10P. Posthybridization wash was done at 37°C in SCC, followed by incubation with blocking buffer at 37°C for 30 minutes. The blue color was developed by incubating the slides with DAB at 37°C.

SLC6A10P expression levels were determined as follows: The TMA was scanned with Aperio CS2 scanner (Leica Biosystems, San Diego, CA, USA), and representative images for each sample were captured under ×200 magnification.

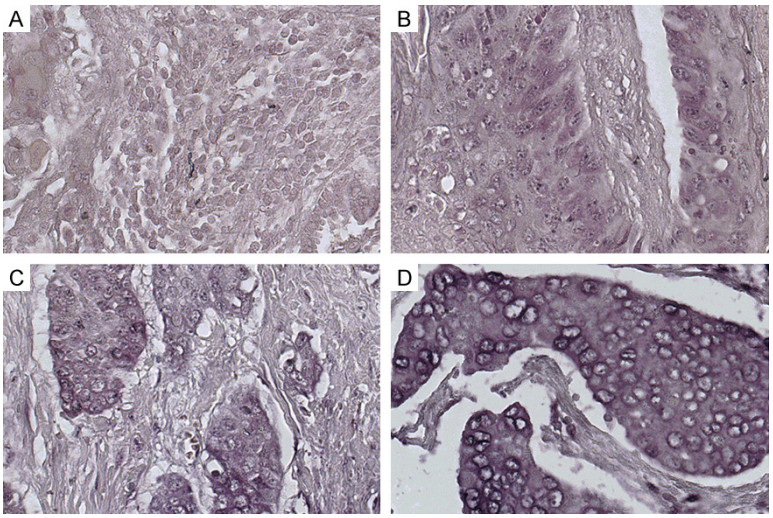

Figure 1. *In situ* hybridization analysis of 140 lung cancer patients. A. Negative SLC6A10P staining in normal lung tissues. B-D. Positive SLC6A10P staining in NSCLC tumor tissues, and the cytoplasmic expression varied.

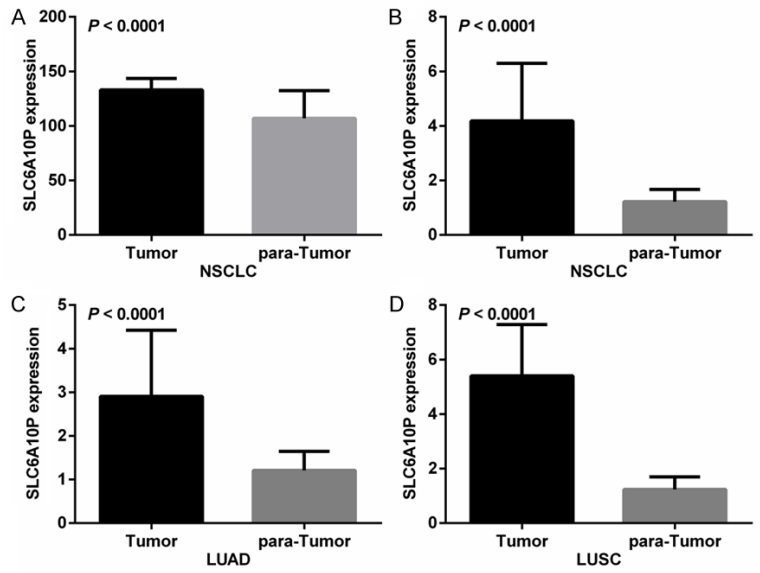

Figure 2. SLC6A10P is more highly-expressed in tumor tissues than in normal lung tissues. (A) The expression level of SLC6A10P in NSCLC tissues is significantly higher than normal lung tissues ($P < 0.0001$). TCGA datasets further verified that SLC6A10P is an average 3.95-fold high-expressed in NSCLC tissue samples ($P < 0.0001$) (B), 2.80-fold higher-expressed in LUAD tissue samples ($P < 0.0001$) (C) and 5.05-fold higher-expressed in LUSC tissue samples ($P < 0.0001$) (D).

Then, by using Image-Pro Plus v6.0 software (Media Cybernetics, Bethesda, MD, USA), integrated optical density (IOD) of all the positive staining of SLC6A10P in each photograph was measured, and its ratio to total area of each photograph was calculated as SLC6A10P density.

Statistical analysis

Data were presented as mean ± S.D. Overall survival (OS) times were defined as the period from primary surgery until the death of the patient or the latest followup. To calculate the optimum cutoff point for OS, the X-tile statistical package (Version 3.6.1, Yale University School of Medicine, USA) was used. Student's t-test, χ^2 test, Kaplan-Meier survival analysis,and Cox regression analysis were performed using SPSS 23.0 software (IBM Corp, Armonk, NY, USA). The graphs were made by GraphPad Prism 6.02 software (GraphPad Prism Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

The 136 patients' characteristics

The 140 NSCLC cases contained 51 LUAD (adeno squamous cell carcinoma was also included), 85 LUSC (squamous adenocarcinoma was also included), 1 sarcomatoid carcinoma and 3 neuroendocrine carcinoma. We aimed to investigate the expression and prognostic value of SLC6A10P in LUAD and LUSC, therefore, only 136 NSCLC (51 LUAD and 85 LUSC) cases were included in this study. **Table 1** presents an overview of the clinicopathological parameters of the 136 patients. The mean age of the patients was 60 years, ranging from 26 to 79 years. Most NSCLC cases were in males and more than half of the tumors were LUSC. Clinical followups were recorded until July 2013. The median followup time was 42.5 months (range, 3~101 months). By the end of the followup, 89 patients had died.

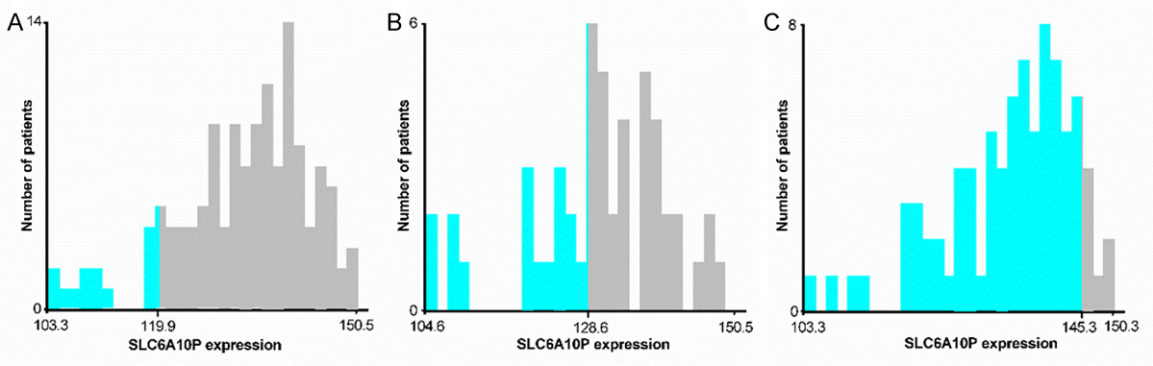

Figure 3. The optimum cutoff points are calculated by the X-tile statistical package. A. The optimal cutoff point of SLC6A10P for NSCLC is 119.90. B. The optimal cutoff point of SLC6A10P for LUAD is 128.60. C. The optimal cutoff point of SLC6A10P for LUSC is 145.30.

Table 2. Correlation between SLC6A10P expression and clinicopathological variables in NSCLC patients

Characteristics	Low-expression	High-expression	*P* value
Age (years)			0.9536
≤60	7	60	
>60	7	62	
Sex			0.4354
Male	10	98	
Female	4	24	
Pathological type			0.3077
LUAD	7	78	
LUSC	7	44	
Differentiation			0.1788
Poorly	8	47	
Moderate or well	6	75	
Tumor size			0.1897
T1	3	12	
T2-3	11	110	
Lymph node status			0.0054*
N0	13	66	
N1-2	1	56	
Tumor stage			0.0126*
I	11	53	
II-III	3	69	

LUAD: lung adenocarcinoma; LUSC: lung squamous cell carcinoma; *Significant correlation.

The expression profile of SLC6A10P

Using the method previously described, we measured the *in situ* SLC6A10P mRNA in NSCLC patients. SLC6A10P was expressed in the cytoplasm of most tumor cells and to a lesser extent expressed in the cytoplasm of normal lung tissue cells. (**Figure 1**). The cytoplasmic expression varied among tumors. The expression levels of SLC6A10P were measured as previously described.

Compared with the 10 normal lung tissues, SLC6A10P is significantly higher expressed in the 136 NSCLC tissues ($P < 0.0001$) (**Figure 2A**). TCGA datasets were used to further verify this conclusion. By analyzing the TCGA datasets, we found that SLC6A10P is an average 3.95-fold higher expressed in NSCLC tissue samples ($P < 0.0001$) (**Figure 2B**). Further analysis shows that SLC6A10P is an average 2.80-fold higher expressed in LUAD tissue samples ($P < 0.0001$) (**Figure 2C**) and 5.05-fold higher expressed in LUSC tissue samples ($P < 0.0001$) (**Figure 2D**).

SLC6A10P confers poor prognosis in NSCLC

The X-tile statistical package was used to calculate the optimum cutoff point. For all NSCLC patients, expression levels of more than 119.90 are regarded as high expression and less than or equal to 119.90 are regarded as low expression of SLC6A10P (**Figure 3A**).

We then investigated the association between SLC6A10P expression and clinicopathological variables. In NSCLC, SLC6A10P high expression was significantly correlated with lymph node metastasis ($P = 0.0054$) and more advanced tumor stage ($P = 0.0126$) (**Table 2**).

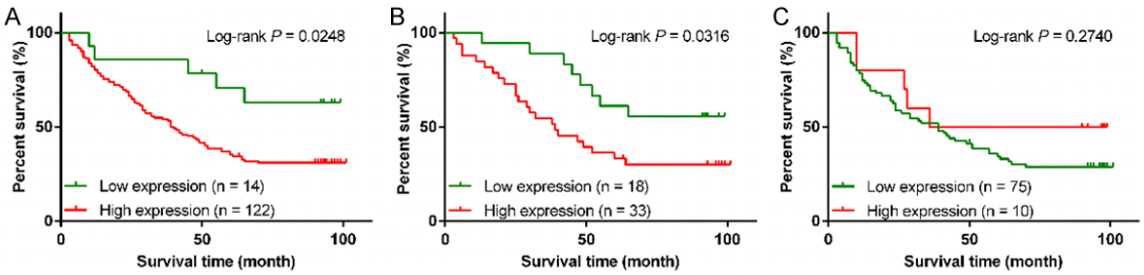

Figure 4. Kaplan-Meier survival curves according to SLC6A10P expression in NSCLC patients. Univariate survival analysis indicates that high SLC6A10P is associated with poorer prognosis in NSCLC (*P* = 0.0248) (A), as well as in LUAD patients (*P* = 0.0316) (B). (C) There wasno association between SLC6A10P and overall survival in patients with LUSC (*P* = 0.2740).

Table 3. Correlation between SLC6A10P expression and clinicopathological variables in LUAD patients

Characteristics	Low-expression	High-expression	*P* value
Age (years)			0.7262
≤60	10	20	
>60	8	13	
Sex			0.2025
Male	15	22	
Female	3	11	
Differentiation			0.3693
Poorly	10	14	
Moderate or well	8	19	
Tumor size			0.5267
T1	4	5	
T2-3	14	28	
Lymph node status			0.0149*
N0	15	16	
N1-2	3	17	
Tumor stage			0.0416*
I	13	14	
II-III	5	19	

*Significant correlation.

However, SLC6A10P expression showed no correlation with age (*P* = 0.9536), sex (*P* = 0.4354), pathological type (*P* = 0.3077), tumor differentiation (*P* = 0.1788) or tumor size (*P* = 0.1897) in NSCLC (**Table 2**). Further, the Kaplan-Meier survival curves were plotted to evaluate the prognostic value of SLC6A10P. The survival analysis reveals that high expressed SLC6A10P is significantly correlated with worse OS in NSCLC patients (*P* = 0.0248) (**Figure 4A**).

Further study shows that SLC6A10P confers poor prognosis in LUAD

We further explored the correlation between SLC6A10P expression and clinicopathological variables in LUAD and LUSC. For LUAD and LUSC patients, we used the grouping method described previously. The cutoff value for LUAD was 128.60, and for LUSC it was 145.30. **Table 3** shows that similar with NSCLC, in LUAD, high SLC6A10P expression is concluded to be significantly correlated with lymph node metastasis (*P* = 0.0149) and more advanced tumor stage (*P* = 0.0416), but not correlated with any other clinicopathological parameters. The survival analysis reveals that high expressed SLC6A10P is significantly correlated with worse OS in LUAD patients (*P* = 0.0316) (**Figure 4B**). As shown in **Table 4**, in LUSC, no significant correlations are observed between SLC6A10P and any of the clinicopathological parameters evaluated in this study. Equally, SLC6A10P expression is found to have no correlation to OS in LUSC patients (*P* = 0.2740) (**Figure 4C**). Univariate and multivariate analysis further revealed that high expression of SLC6A10P is an independent prognostic factor for worse OS in LUAD (*P* = 0.017) (**Table 5**).

Discussion

NSCLC is a highly malignant and aggressive tumor type and has shown a poor 5-year overall survival rate [2]. Therefore, it was urgent that we investigate the potential therapeutic targets of NSCLC. SLC6A10P (alias *CT2*) is located on 16p11.2 and isa pseudogene of the human creatine transporter gene, SLC6A8 (solute carrier family 6 member 8, alias *CT1*) [4, 5, 10]. Historically, pseudogenes have been believed

Table 4. Correlation between SLC6A10P expression and clinicopathological variables in LUSC patients

Characteristics	Low-expression	High-expression	*P* value
Age (years)			0.6604
≤60	32	5	
>60	43	5	
Sex			0.7487
Male	63	8	
Female	12	2	
Differentiation			0.3440
Poorly	26	5	
Moderate or well	49	5	
Tumor size			0.6991
T1	5	1	
T2-3	70	9	
Lymph node status			0.2634
N0	44	4	
N1-2	31	6	
Tumor stage			0.1101
I	35	2	
II-III	40	8	

to represent nonfunctional genomic fossils, however, emerging evidence has suggested that many of them may be biologically active [11]. To the best of our knowledge, the role of SLC6A10P in lung cancer is still unclear. In our present study, we mainly investigated the expression profile and prognostic value of SLC6A10P in patients with NSCLC.

It was reported that SLC6A10P is detected in testis [4], brain [12] and ovaries [7]. By browsing the site (Expression Atlas, http://www.ebi.ac.uk/gxa/home/), we found that SLC6A10P is detected in many human tissues (heart, lung, prostate, etc.) and differential expressed in some cell lines (MCF-10A, etc.) (http://www.ebi.ac.uk/gxa/genes/ENSG00000214617?bs). In our present study, three TCGA datasets were downloaded and used to access the expression profile of SLC6A10P. Then, by analyzing the TCGA datasets, we found that SLC6A10P is significantly higher expressed in tumor tissues when compared with normal lung tissues.

Based on the differential expression, we believe that there are functional roles associated with SLC6A10P. This hypothesis has been supported by some studies. A study done by Bayou et al. showed that SLC6A10P may be involved in the autistic phenotype in patients with autism [12]. Ndika et al. has provided evidence that SLC6A10P has promoter activity and the promoter activity seems to be stronger than the parent gene, SLC6A8 [13]. A recent study showed that SLC6A10P was lower expressed in ovarian cancer and the low expression was associated with longer TTR (time-to recurrence) [7]. In our present study, the expression levels of SLC6A10P in 140 NSCLC patients were detected using an ISH Kit and measured using computer software. In patients with NSCLC, high SLC6A10P expression is found significantly correlated with lymph node metastasis and more advanced tumor stage, and the high expression results in worse OS. To deeply explore the prognostic value of SLC6A10P, we further investigated the role of SLC6A10P in patients with LUAD or LUSC, respectively. High SLC6A10P expression correlates with lymph node metastasis and more advanced tumor stage, leading to worse OS and is an independent prognostic factor for worse OS in LUAD patients, while SLC6A10P shows no correlation with any clinicopathological parameters and no prognostic value in LUSC. On the whole, SLC6A10P may be a useful marker for poor prognosis and a potential therapeutic target in NSCLC, especially in LUAD patients. However, it still takes time to explore the exact biological mechanisms of SLC6A10P in NSCLC.

In conclusion, our study indicates that SLC6A10P is higher expressed in tumor tissues and its high expression predicts a poor survival in patients with LUAD. In the future, SLC6A10P may become a novel therapeutic target and prognostic biomarker in LUAD patients but that awaits further investigation.

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Table 5. Cox regression analysis of overall survival in LUAD patients

Characteristics	Univariate analysis			Multivariate analysis		
	P value	HR	95% CI	*P* value	HR	95% CI
Age (years) (≤60 vs >60)	0.245	1.521	0.750-3.087	0.051	2.211	0.998-4.901
Gender (Male vs Female)	0.382	0.687	0.296-1.594	0.078	0.439	0.176-1.095
Differentiation (Moderate or well vs Poorly)	0.746	0.889	0.438-1.806	0.896	1.050	0.505-2.185
Tumor size (T2-3 vs T1)	0.689	1.216	0.467-3.171	0.995	0.997	0.361-2.750
Lymph node status (positive vs negative)	0.125	1.745	0.857-3.553	0.977	1.023	0.215-4.860
Tumor stage (II-III vs I)	0.260	1.500	0.741-3.039	0.791	1.229	0.268-5.632
SLC6A10P expression (high vs low)	0.038*	2.359	1.051-5.295	0.017*	2.964	1.217-7.218

HR: hazard ratio; CI: confidence interval; *Significant correlation.

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Disclosure of conflict of interest

None.

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