Int J Clin Exp Pathol 2018;11(8):3835-3845
www.ijcep.com /ISSN:1936-2625/IJCEP0080116

Original Article

The association of UNC5A expression with the clinicopathologic features and prognosis of radiotherapy in patients with non-small-cell lung cancer

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Received May 22, 2018; Accepted June 29, 2018; Epub August 1, 2018; Published August 15, 2018

Abstract: UNC5A is widely known as a neuronal axonal guide factor and was found to have a low expression in a variety of tumors. In our study, we investigated the expression of UNC5A in non-small cell lung cancer (NSCLC) and analyzed its association with the clinical features and prognosis of NSCLC radiotheray patients. Methods: We collected 169 NSCLC patients' clinical and pathological data for the study. Immunohistochemical staining was evaluated to analyze the expression of UNC5A in NSCLC tissues. The expressions of UNC5A in normal and NSCLC tissues were analyzed using the Oncomine database. We investigated the overall prognostic value of UNC5A in NSCLC patients through the Kaplan-Meier plotter database. Results: The low expression rate of UNC5A was 55.0% (93/169) in NSCLC by immunohistochemical analysis. The overall survival (OS) of NSCLC radiotheray patients with a low expression of UNC5A was shorter than that in patients with a high expression (P = 0.000). The expression of UNC5A was strongly and significantly associated with the TNM stage (P = 0.013) but not associcated with other clinicopathologic features. The results of COX regression showed that the expression of UNC5A, general condition and TNM stage were independent prognositic factors of NSCLC patients. ROC analysis showed a high area under the curve for UNC5A expression in NSCLC (AUC = 0.746). At a cut-off level of > 1027, the UNC5A expression, general condition and TNM stages, could be used for the prognosis of NSCLC with high sensitivity and specificity. The Oncomine database showed that UNC5A expression was found to significantly decline in NSCLC tissues compared to normal tissues (P = 0.029). We used the Kaplan-Meier plotter database to analyze NSCLC patients with a high expression of UNC5A and found they had better OS than patients with a low expression (P = 0.0492). Conclusion: The expression of UNC5A may be a potential prognostic biomarker of NSCLC.

Keywords: UNC5A (UNC5H1), non-small cell lung cancer, predictor

Introduction

Lung cancer is the most common human cancer in the world. It is one of the top three most common cancers in both men and women, according to a 2016 survey. The highest mortality rates for cancer in males were from lung, prostate and colorectal cancer. For cancer in females, the highest mortality rates were from lung, breast and colorectal cancer. Of these four cancers, over 1/4 (27%) of the resulting deaths were caused by lung cancer [1]. According to histopathology, lung cancer can be divided into non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC), which accounts for about 87% and 13% of all lung cancers, respectively. Squamous cell carcinoma (SCC), adenocarcinoma (Ade), and large cell carcinoma are the main types of NSCLC [2]. Although advances in clinical examination, drug therapy, surgery, radiotherapy, and comprehensive treatment have improved survival rates [3, 4], the prognosis is still poor, and average 5-year over-all survival rates range between 5.5 and 15.7% in NSCLC patients [5].

Uncoordinated-5-homolog (UNC5H), known as an axon-guidance molecule, contains four members, UNC5H1 (UNC5A), UNC5H2 (UNC5B), UNC5H3 (UNC5C), and UNC5H4 (UNC5D). UNC5H plays a critical role in the development and differentiation of nerve cells [6, 7]. UNC5H1 (UNC5A) acts as a netrin-1 receptor in nerve cells and plays an important role in axonal guidance. It also regulates apoptosis in non-neuronal cells. As a novel downstream target of

Table 1. Demographic and clinicopathological parameters of enrolled patients

Clinicopathological parameters	169 patients (%)
Age (median, rage)	61.0 (28-85)
< 61	76 (45)
≥ 61	93 (55)
Sex	
Male	132 (78.1)
Female	37 (21.9)
Smoking	
Yes	132 (78.1)
No	37 (21.9)
TNM stage	
IIA	8 (4.7)
IIB	32 (18.9)
IIIA	59 (34.9)
IIIB	70 (41.4)
Histological Type	
Squamous cell carcinoma	105 (62.1)
Adenocarcinoma	56 (33.1)
Other types of carcinoma	8 (4.7)
KPS (median, rage)	90 (70-90)
< 90	30 (17.8)
≥ 90	139 (82.2)
Induction chemotherapy	
Yes	104 (61.5)
No	65 (38.5)
Sequential chemotherapy	
Yes	119 (70.4)
No	50 (29.6)
Radiotherapy	
3D-CRT	43 (25.4)
IMRT	117 (69.2)
SBRT	9 (5.3)
Dose of radiotherapy (median, rage)	60 (40-66)
< 60 Gy	33 (19.5)
≥ 60 Gy	139 (80.5)

p53, UNC5A plays a significant role in p53-dependent apoptosis in non-neuronal cells and tumor cells [8]. The expression of UNC5A is usuallylow in multiple non-nervous system cancers, such as colorectal, gastric, breast, ovary, uterus, and kidney [9, 10]. In colorectal and rectal cancer, the down-regulation is partially associated with loss of heterozygosity [11]. In bladder cancer, UNC5A as a tumor-suppressor gene participates in DNA damage-repair [9]. UNC5A is associated with tumorigenesis and progression in a variety of tumors.

The purpose of our study was to find out whether UNC5A expression was related to the malignancy of NSCLC. At the same time, we sought to learn whether the immunohistochemical staining of UNC5A can predict the prognosis of NSCLC radiotherapy patients.

Materials and methods

Patient selection

In our study, 169 patients were enrolled. All the patients were diagnosed as having NSCLC by pathologists in China Medical University First Affiliated Hospital from 2010 to 2014. The study population included 132 (78.1%) male and 37 (21.9%) female patients, with a median age of 61 (range, 28-85 years). The histology types included 56 adenocarcinoma, 105 squamous cell carcinoma, and 8 other types of carcinoma. According to the Seventh edition of the Tumor-Node-Metastasis (TNM) system of cancer staging adopted by the World Health Organization (UICC 2009), there were 8 patients in stage A, 32 in stage B, 59 in stage A and 70 in stage B. We used the Karnofsky performance status (KPS) to evaluate the general conditions. Clinicopathological data are listed in **Table 1**. The tissue samples were obtained by bronchoscopy or CT-guided transthoracic and the embedded in paraffin. Subsequently, all patients underwent induction chemotherapy + radiotherapy + sequential chemotherapy or concurrent chemoradiotherapy + sequential chemotherapy or simple radiotherapy. No patients received surgical treatment. All the patients participated in outpatient follow-up at the end of their treatment.

Radiotherapy

All 169 patients received radiotherapy between 2010 and 2014 at China Medical University First Affiliated Hospital Radiotherapy Department. All treated patients underwent a planning CT and 6-MV x-ray was used. We applied the Pinnacle 3D treatment planning system. The planning target volume (PTV) required covering the macroscopic disease with an edge of 0.5-1 cm for the primary. The prescribed dose was the mean dose in the PTV and its minimum dose of 90% was related to the isocenter. The conventional radiotherapy model is once per day and 5 days a week. Three-dimensional conformal radiotherapy (3D-CRT) was used in 43 patients, with 2.0 Gy daily fractions with a con-

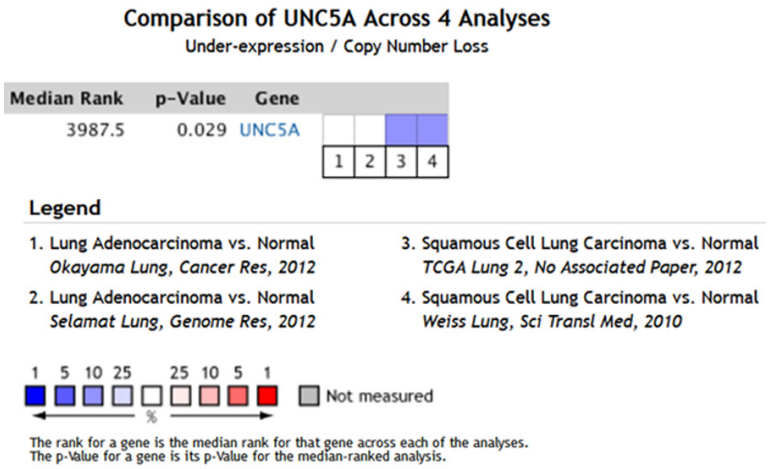

Figure 1. The difference of UNC5A expression between tumor tissues and normal tissues in NSCLC from the publicly available Oncomine database (P = 0.029).

ventional treatment model, 117 patients were treated with intensity modulated radiation therapy (IMRT) with a 1.8-2.2 Gy daily fraction with a conventional treatment model, and 9 patients adopted stereotactic body radiation therapy (SBRT) with a 6-10 Gy daily fraction once per day for 3 days a week. The applied radiation dose was 40-66 Gy. The median radiotherapy dose was 60 Gy. The median treatment time was 42 days, ranging between 18 (treatment was continuous) and 47 days.

Chemotherapy

The patients were treated in chemoradiotherapy with a platinum-based regimen. The induction and sequential chemotherapy regimen included docetaxel plus cisplatin or vinorelbine plus cisplatin, or etoposide plus cisplatin intravenously. Some patients with adenocarcinoma were given pemetrexed induction or sequential chemotherapy.

Immunohistochemistry (IHC)

Paraffin embedded tissues were cut into 4 μm cross sections and mounted on Superfrost Plus slides. The sections were dewaxed in a xylene solution, rehydrated through a graded series of ethanol solutions, then washed with phosphate buffer solution (PBS). High temperature and high pressure induced epitope retrieval was performed using the laboratory pressure cooker at 105°C for 3 min in a citrate buffer (PH 6.0). After retrieval, the tissue sections were allowed to cool in the buffer and then were rinsed with PBS. The sections were immersed in 3% hydrogen peroxide at room temperature for 10 minutes to block endogenous peroxidase activity and then rinsed with PBS. Subsequently, the slides were incubated with a rabbit anti-UNC5A antibody (100 μg/ml, 1:50 dilution, cut. no. 22068-1-AP Proteintech Group USA) at 4°C overnight. Then we rinsed the slides with PBS three times and incubated them in a biotin-labeled goat anti-rabbit secondary antibody solution for 15 min at room temperature. The slides were incubated with the streptavidin-horseradish peroxidase conjugate complex for 10 min at room temperature then rinsed the slides three times with PBS. All slides were stained with the DAB chromogen system (ZSGB Beijing China). Finally, the sections were counterstained with hematoxylin, rehydrated through a graded series of ethanol solutions and mounted. Immunohistochemical staining was evaluated in the cytoplasm and cytomembrane of the tumor cells.

The stained slides were scanned using a VENTANA Image Viewer. The expression of UNC5A was determined by the double-blind method. The stains were scored by two experienced pathologists. Each section was selected with five representative high vision. By multiplying the intensity fraction and the percentage area, the expression of UNC5A was obtained [1]. The staining intensity scores were divided into four groups: no staining marked 0; weak marked 1; moderate marked 2, and strong marked 3. For the percentage of positively stained cells, we divided them into groups labeled from 0 to 4 (< 5% labeled 0; 5%-25% labeled 1; 25%-50% labeled 2; 51%-75% labeled 3; 75% labeled 4). After making the calculations, we separated the specimens according to expression into four groups (negative, weak, moderate, and strong), 0 and 1 were negative, 2-4 were weak, 5-8 were moderate and 9-12 were strong. Moderate and strong were evaluated as positive results.

Database analysis

Oncomine (www.oncomine.org) is a cancer microarray database and data mining platform

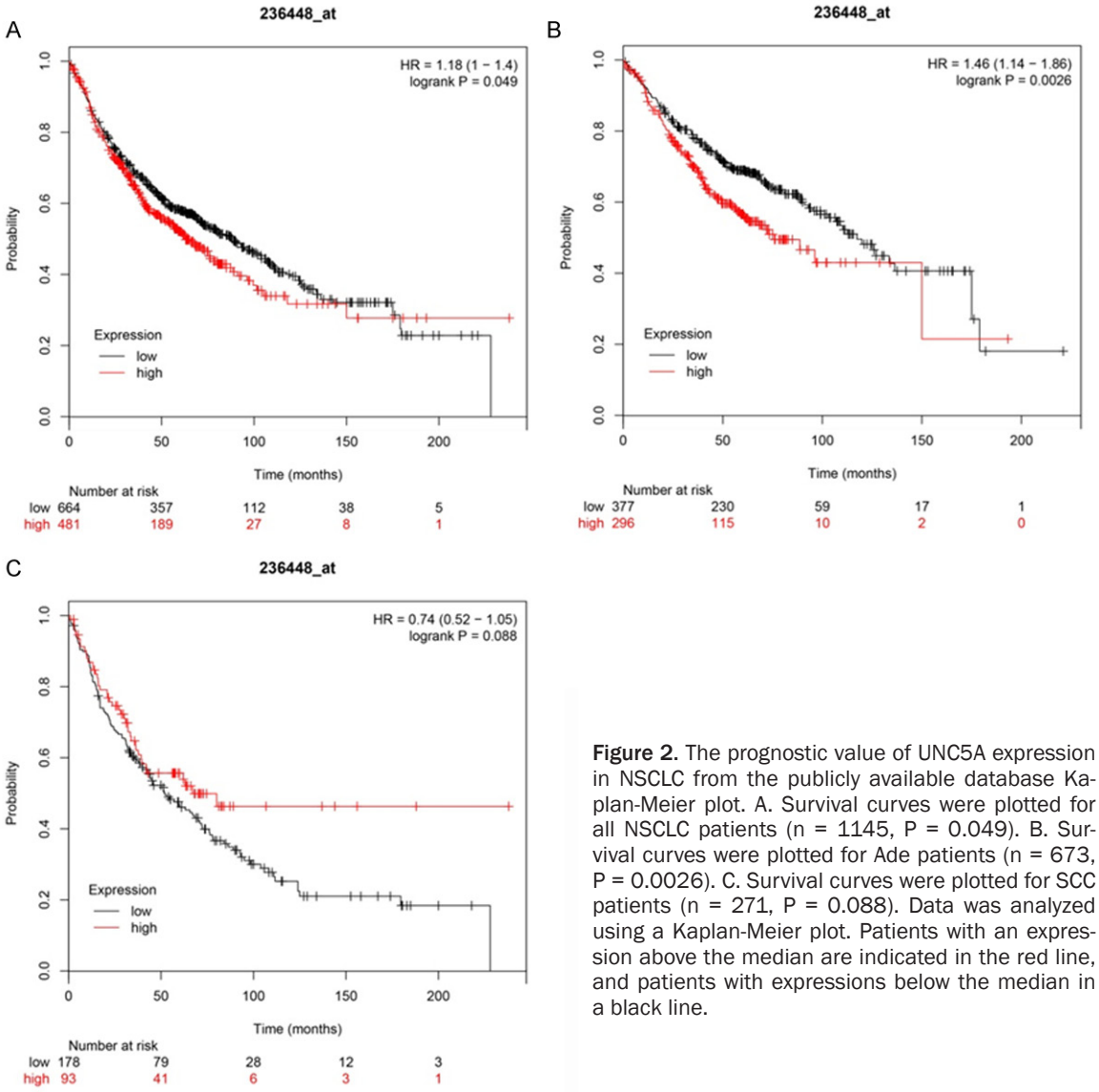

Figure 2. The prognostic value of UNC5A expression in NSCLC from the publicly available database Kaplan-Meier plot. A. Survival curves were plotted for all NSCLC patients (n = 1145, P = 0.049). B. Survival curves were plotted for Ade patients (n = 673, P = 0.0026). C. Survival curves were plotted for SCC patients (n = 271, P = 0.088). Data was analyzed using a Kaplan-Meier plot. Patients with an expression above the median are indicated in the red line, and patients with expressions below the median in a black line.

based on the web. It is based on the whole genome found in the expression of the new contacts and includes most of the major types of cancer of the transcriptome data compared with their corresponding normal tissue [12, 13]. We analyzed UNC5A gene expression level using Oncomine. We compared the gene expression level of cancer vs. normal patient datasets. In this research, we selected all fold change, *P*-value = 0.05, and the top 10% gene rank as threshold.

A Kaplan-Meier plot (www.kmplot.com) was used to evaluate the prognostic significance of UNC5A mRNA expression in NSCLC, which is an online database, including gene expression data and clinical data. In this database, researchers can get lung cancer [14], ovarian cancer [15], gastric cancer [16], and breast cancer [17] data. We used a Kaplan-Meier survival plot to analyze the OS of NSCLC patients. The database automatically divided UNC5A mRNA expression into two groups: high expression and low expression. The survival curve, log-rank *P*-value and hazard ratio (HR) with 95% confidence intervals were calculated and displayed in the results by the computer.

Statistical analysis

Data were analyzed with a computer using Statistical Package for the Social Sciences (SPSS Version 19.0) statistical software. The survival data analysis was performed using Kaplan-Meier and log-rank tests. When two groups were compared, the χ^2 test was used. Univariate and multivariate Cox analyses were used to examine the independent influence of

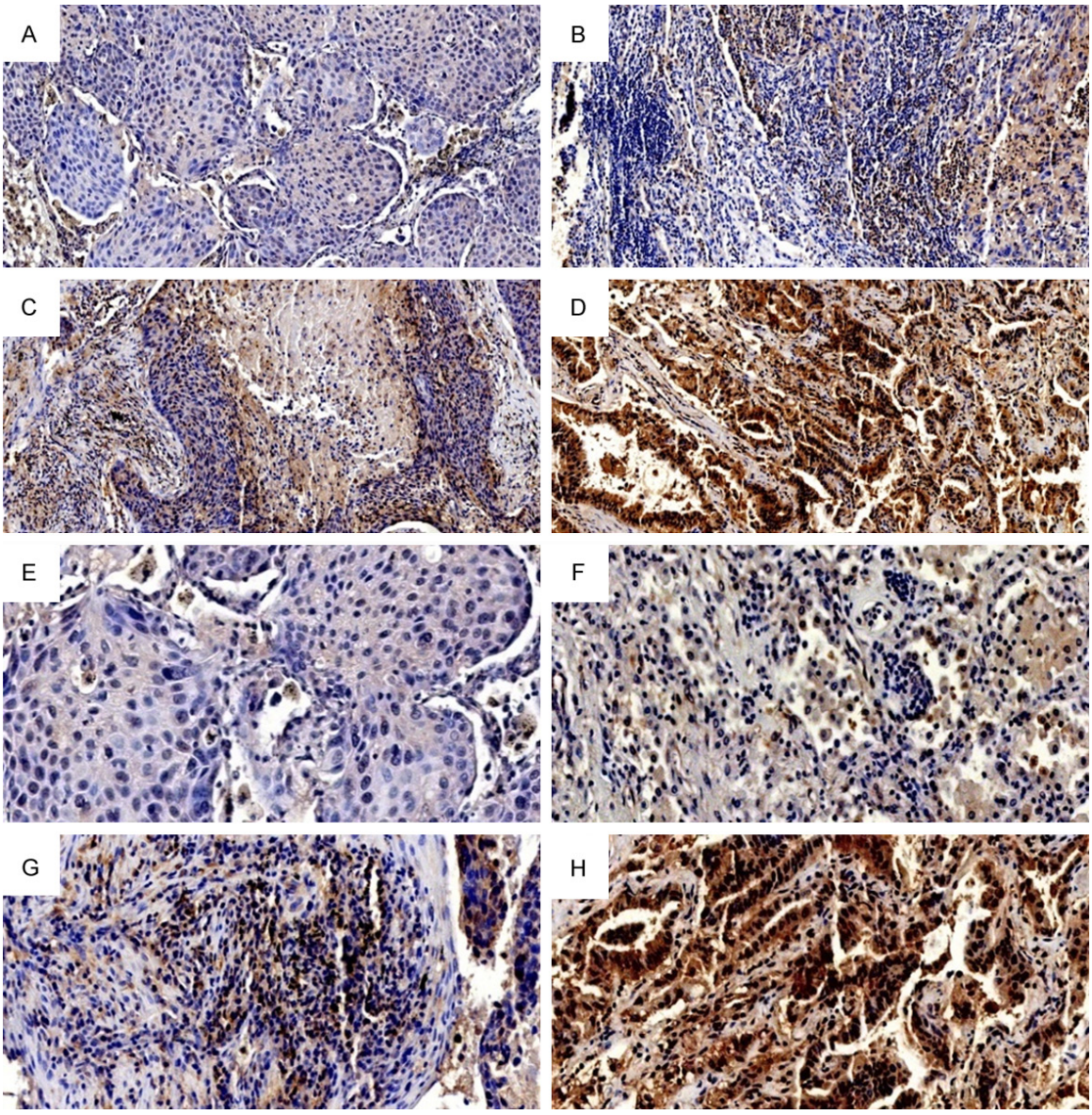

Figure 3. An immunohistochemical detection of UNC5A expression in NSCLC tissues. (A) NSCLC tissues UNC5A staining was negative, (B) was weakly positive, (C) was moderately positive, and (D) was strongly positive (A-D Magnification × 100). (E) was negative, (F) was weakly positive, (G) was moderately positive, and (H) was strongly positive (E-H Magnification × 200).

potential prognostic factors on OS. Receiver operating characteristic (ROC) analysis was performed to compare the sensitivity and specificity of the parameters to enable a prediction of OS. $P < 0.05$ was considered statistically significant.

Results

Database analysis reveals UNC5A has a low expression in NSCLC

UNC5A levels in NSCLC were investigated using the Oncomine database (www.oncomine.org). The expression of the UNC5A gene was summarized in 2190 samples of 4 databases. The results showed that the expression of UNC5A decreased significantly in NSCLC tissues compared with normal tissues ($P = 0.029$) using the Weiss [18], TCGA, Okayama [19] and Selamat [15] databases (**Figure 1**).

Database analysis reveals the correlation between UNC5A expression and prognosis in NSCLC

We then examined the prognostic value of UNC5A mRNA expression in a Kaplan-Meier

Table 2. Relationship Between the UNC5A Expression and Clinicopathologic Features in NSCLS

	Negative (n = 38)	Weak (n = 55)	Moderate (n = 26)	Strong (n = 50)	χ^2	*p* value
SEX						
Male	28 (77.7%)	42 (76.4%)	21 (80.8%)	41 (82.0%)	1.083	0.781
Female	10 (26.3%)	13 (23.6%)	5 (19.2%)	9 (18.0%)		
Age						
< 61	19 (50.0%)	31 (56.4%)	8 (30.8%)	18 (36.0%)	7.018	0.071
≥ 61	19 (50.0%)	24 (43.6%)	18 (69.2%)	32 (64.0%)		
Histological Type						
SCC	19 (50.0%)	31 (56.4%)	19 (73.1%)	36 (72.0%)	10.742	0.097
Ade	16 (42.1%)	19 (34.5%)	7 (26.9%)	14 (28.0%)		
Other	3 (7.9%)	5 (9.1%)	0	0		
TNM stage						
IIA	0	2(3.6)	1 (3.8%)	5 (10.0%)	20.950	0.013
IIB	2 (5.3%)	9 (16.4%)	5 (19.2%)	16 (32.0%)		
IIIA	14 (36.8%)	19 (34.5%)	8 (30.8%)	18 (36.0%)		
IIIB	22 (57.9%)	25 (45.5%)	12 (46.2%)	11 (22.0%)		
Smoke						
Yes	31 (81.6%)	41 (74.5%)	20 (76.9%)	40 (80.0%)	0.802	0.849
No	7 (18.4%)	14 (25.5%)	6 (23.1%)	10 (20.0%)		
KPS						
≥ 90	11 (28.9%)	6 (10.9%)	4 (15.4%)	10 (20.0%)	5.126	0.163
< 90	27 (71.1%)	49 (89.1%)	22 (84.6%)	40 (80.0%)		

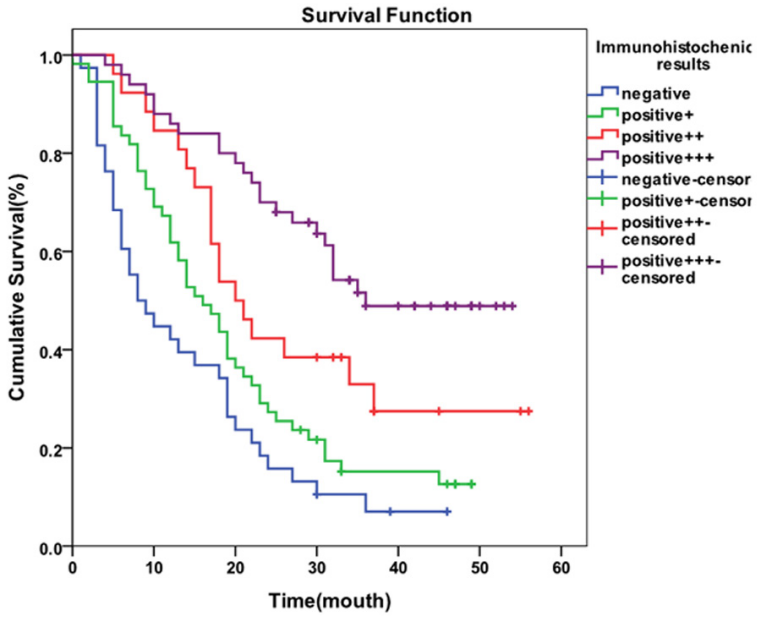

Figure 4. Kaplan-Meier analysis for correlation bewteen OS and UNC5A expression in NSCLC patients (P = 0.000).

plot (www.kmplot.com. The desired Affymetrix ID was valid: 236448_at (UNC5A). Survival curves were plotted for total NSCLC patients (**Figure 2A**), Ade patients (**Figure 2B**), and SCC patients (**Figure 2C**). In all the NSCLC patients, a high expression of UNC5A mRNA was found to be associated with better OS (HR 1.18 [1-1.4], P = 0.0492) (High expression = 481, low = 664). The same result was found in the Ade patients (HR 1.46 [1.14-1.86], P= 0.0026) (high = 377 low = 296). But in SCC, the same results were not obtained (HR 0.74 [0.52-1.05], P = 0.088) (high = 178 low = 93).

UNC5A expression in NSCLC by HIC

In this study, we evaluated the correlation between the expression of UNC5A and clinicopathologic features. In the 169 cases, the immunohistochemical analysis for UNC5A showed there were 38 negative for staining, 55 were weak, 26 moderate, and 50 had strong staining. In the 169 cases, the negative or weak were 93

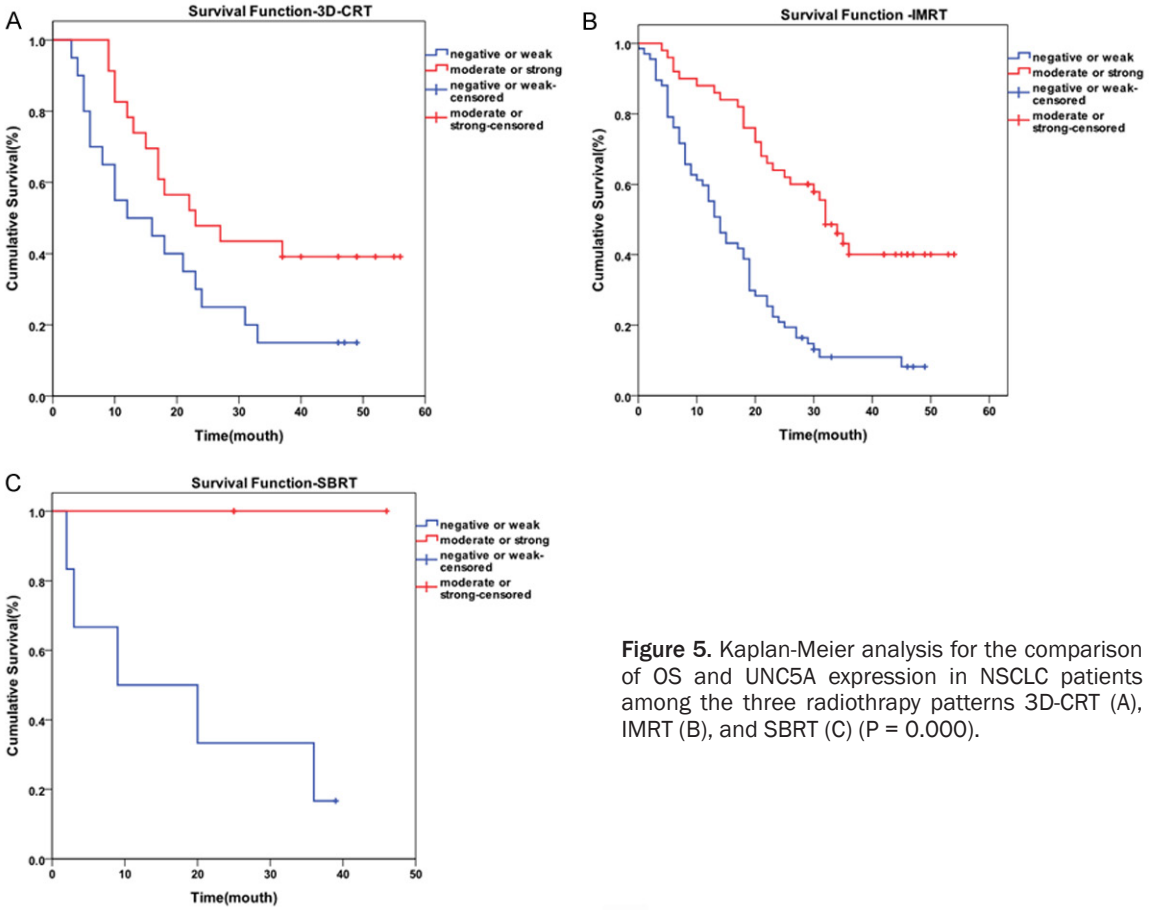

Figure 5. Kaplan-Meier analysis for the comparison of OS and UNC5A expression in NSCLC patients among the three radiothrapy patterns 3D-CRT (A), IMRT (B), and SBRT (C) (P = 0.000).

(55.0%), and the moderate or strong were 76 (45.0%), and the majority were observed in the cytoplasms and cytomembranes as brown granules (**Figure 3**). There is a significant correlation between UNC5A expression and TNM pathology stages (P = 0.013). But there is no statistical relationship was found among the different NSCLC histology sub-types in UNC5A expression. However, no statistical correlations were found between UNC5A expression and the remaining clinicopathologic features, such as age, gender, smoking, or KPS scores. The immunohistochemical findings of UNC5A expression in NSCLC are summarized in **Table 2**.

Low UNC5A expression predicts poor survival in NSCLC radiotherapy patients

At the end of the follow-up period, all 169 patients had died 124 patients, and the overall survival time (OS) was 20.00 months (95% CI = 17.34-22.65), and the 1-year survival rate was 68.0%, the 2-year productivity was 39.6%, the 3-year survival rate was 27.8%, and the 5-year survival rate was 26.6%. The median survival time of the UNC5A expression negative group was 10.9 mouths, the weak group was 17.4 mouths, the moderate group was 21.8 mouths, and the strong group 42.3 mouths. A Kaplan-Meier survival analysis was used to analyze the association between UNC5A expression and the OS of 169 NSCLC patients (**Figure 4**). Patients with a higher UNC5A expression were likely to have longer survival time. There was a significant statistical correlation between UNC5A expression and survival time (P = 0.000). We divided the expression of UNC5A into two groups of strength and weakness. According to the stratified analysis of radiotherapy, the expression of UNC5A in different radiotherapy patterns was closely related to the patient's OS (P = 0.000) (**Figure 5**). The results of COX univariate and multivariate analysis showed that TNM stage, general condition, and UNC5A expression were significant predictors for OS. (**Table 3**).

In order to develop a more sensitive predictive tool, we constructed a radiotherapy pr-ognostic scoring model combining three independent pr-

Table 3. The univariate and multivariate analysis for the clinicopathologic features and survival time

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
SEX	0.953 (0.628-1.446)	0.821		
Age	1.028 (0.720-1.468)	0.877		
Histological Type	1.762 (0.779-1.447)	0.705		
TNM stage	1.771 (1.412-2.223)	0.000	1.537 (1.195-1.976)	0.001
Smoke	1.031 (0.679-1.564)	0.887		
KPS	0.557 (0.373-0.891)	0.013	0.611 (0.382-0.976)	0.039
Radiotherapy dose	0.926 (0.588-1.456)	0.738		
Radiotherapy methods	0.992 (0.710-1.387)	0.965		
Induction chemotherapy	1.110 (0.772-1.597)	0.572		
Sequential chemotherapy	1.016 (0.692-1.494)	0.934		
UNC5A expression	0.605 (0.514-0.714)	0.000	0.631 (0.524-0.759)	0.000

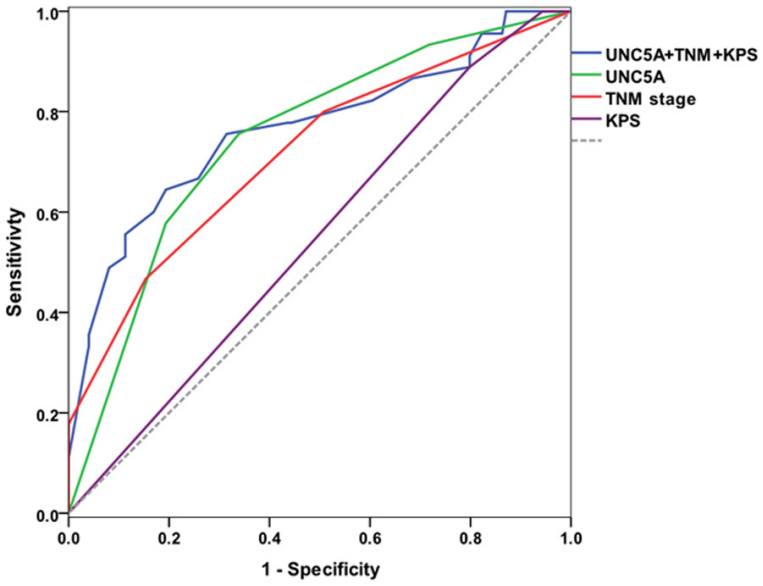

Figure 6. ROC analyses show the highest area under the curve among the combined fators (UNC5A expression, KPS and TNM stage) (AUC = 0.766, P = 0.000), UNC5A expression (AUC = 0.746, P = 0.000), TNM stage (AUC = 0.717, P = 0.000), and KPS (AUC = 0.548, P = 0.337). At a cut-off level of > 1027, the combination factor produced the 64.4% sensitivity and the 80.6% specificity for detecting the prognosis of NSCLC patients.

ognostic factors which were calculated by COX multivariate analysis, UNC5A expression (IHC), KPS, and TNM stage. We compared the above three influencing factors alliance with single factor by ROC analysis. In our study, the ROC curve shows the highest area under the curve for the combination score. A combination of three factors (P = 0.000, AUC [95% CI] = 0.766 [0.676 to 0.855]) showed a better prognostic value than did UNC5A expression (P = 0.000, AUC [95% CI] = 0.746 [0.662 to 0.829]) alone, TNM stage (P = 0.000, AUC [95% CI] = 0.717 [0.625 to 808]) or KPS (P = 0.337, AUC [95% CI] = 0.548 [0.454 to 0.643]). At a cut-off level of > 1027, the combination factor produced the 64.4% sensitivity and 80.6% specificity for detecting the prognosis of NSCLC patients (**Figure 6**).

Discussion

Lung cancer is the most common malignancy in the world [20]. It is the number one cause of cancer deaths worldwide [21]. Among the various types of lung cancer, NSCLC is still one of the most frequency diagnosed and the most fatal human malignancies, with a poor prognosis. Although combined treatment based on surgery, radiotherapy and chemotherapy has been widely used, the prognosis of NSCLC is still poor [22]. With the development of genomics, proteomics, and metabolomics in recent years, a number of new molecular markers of NSCLC have been found.

The UNC5A that belongs to the family of the netrin-1 receptors (UNC5H) is an important neuronal axonal guide factor, but in recent years it has been given more attention in the development of tumors. Netrin-1 concentrations are elevated in advanced NSCLC, and

Mahmut et al. found that the expression of netrin-1 was associated with the prognosis of NSCLC [23]. Thiebault et al. reported that in most non-nervous system tumors, the expression of UNC5A was significantly down-regulated, showing obvious heterozygous deletions (LOH) in colorectal tumors [11]. UNC5A mediated apoptosis that requires an interaction with the MAGE protein NRAGE [24], and dramatically activates caspase-3. Caspase-3 is widely believed to be the most important shear enzyme in cell apoptosis, indicating that UNC5A may be involved in the apoptosis of tumor cells. Previous studies have shown that UNC5A and UNC5B were both transcriptional targets of p53 and played a role in p53-dependent apoptosis [8, 25, 26]. Barnault et all showed that the depletion of UNC5A combined with the hepatic unfolded protein response was associated with the development and prognosis of hepatocellular carcinoma [27]. However, most studies looked at the tumors' mechanistic investigations, yet few studies focused on the relationship between the expression of UNC5A and tumor prognosis. The biomarkers for the prognosis of radiotherapy patients are even fewer.

The purpose of this study was to investigate whether UNC5A could be used as a biomarker for the clinicopathologic prognosis of NSCLC at the protein levels. In this reserch, we found that the expression rate of UNC5A was 55.0% in negative or weak and 45.0% in moderate or strong. This finding was consistent with known effects of UNC5A in other cancers and also consistent with the results of the known database ONCOMINE. UNC5A presented a low expression in NSCLC, and the status of UNC5A expression correlated with the pathology stage. It was found that patients with a high UNC5A expression had a poor tumor stage. But the expression was not associated with other clinicopathological features. To investigate the potential prognostic value of UNC5A expression and prognosis, we analyzed the relationship between expression levels and OS in NSCLC patients. In this study, patients with low UNC5A expression had significantly poorer OS than with those with a high level (P = 0.000). Because of the small number of patients with SBRT, the expression of UNC5A was divided into just two groups: high and low. In different radiotherapy methods, the expression of UNC-5A also showed a close correlation with OS, that is, the patients with low UNC5A expression had poor OS. Furthermore, a multivariate Cox analysis proved that the UNC5A expression and the well-known prognostic factors, tumor staging and general condition were independent prognostic factors for NSCLC patients. Since all of our patients received radiotherapy, and most of the patients received chemotherapy at different periods, the factors such as radiotherapy and chemotherapy did not become independent prognostic factors. In the Kaplan-Meier plotter database, although UNC5A expression in squamous carcinoma cases with overall survival in patients had no statistical significance (P = 0.088), we analysised that mainly due to squamous carcinoma had relatively fewer cases than adenocarcinoma (271 vs. 673), but in general the non-small cell lung cancer and adenocarcinoma, we obtained the same as the experiment results. ROC analysis showed UNC5A expression, general condition and TNM stages were all obtained the curve area greater than 0.5 (the area of UNC5A = 0.746, TNM stage = 0.717, general condition = 0.548). The combined factors of the above three factors obtained the higher area under the curve than the three independent elements (the area of combined factors = 0.766). At a cut-off level of > 1027, the combined factor could be used for the prgnosis of NSCLC with high sensitivity and specificity. Therefore, the expression level of intratumoral UNC5A combined with the existing TNM stage system can improve the predictive value of NSCLC patients' OS. In conclusion, these results suggest that a low expression of UNC5A might indicate poor prognosis in NSCLC patients and the UNC5A expression combined with the existing TNM stage system can improve the predictive value of NSCLC patients' OS.

This study was limited by its small sample size, and the TNM stageing was limited to II, III stage. We didn't carry out qRT-PCR tests for RNA expression studies. Therefore, the profound molecular roles of UNC5A in NSCLC still need to be explored in the context of the correlation.

Disclosure of conflict of interest

None.

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