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ORIGINAL ARTICLE – THORACIC ONCOLOGY

High Expression of CHRNA1 is Associated with Reduced Survival in Early Stage Lung Adenocarcinoma after Complete Resection

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ABSTRACT

Background. Non-small cell lung cancer (NSCLC) is the leading cause of cancer deaths around the world, and a high recurrence rate after complete resection is an important issue reducing the cure rate and survival of patients with early stage NSCLC. Several pathologic biomarkers are associated with recurrence in early stage lung cancer after complete resection.

Methods. We evaluated the expression and prognostic value of the α1 subunit of the nicotinic acetylcholine receptor (CHRNA1) as well as other pathologic features of tumor tissues resected from patients with stage I adenocarcinoma of the lung.

Results. A high ratio (173/185) of CHRNA1 expression (93.5 %) was found in stage I lung adenocarcinoma. In the multivariate survival analysis, tumor necrosis, angiolymphatic invasion, perineural invasion, and CHRNA1 expression were independent poor prognostic factors for both recurrence-free and overall survival (OS). Patients expressing CHRNA1 had worse median recurrence-free survival (60.6 vs. 77.9 months, $P = 0.03$) and OS (65.1 vs. 77.9 months, $P = 0.04$) compared with CHRNA1-negative patients.

Conclusions. CHRNA1 expression could be directly tested from the tumor after complete resection. In early stage NSCLC, it could be a useful prognostic factor for recurrence and survival.

Lung cancer is the leading cause of cancer death around the world, and adenocarcinoma is the most common subtype of non-small cell lung cancer (NSCLC).[1] Complete resection of the tumor is currently the standard treatment for the possibility of a cure in early stage (stage 1) NSCLC. However, the high recurrence rate of about 30 % at 1 year after complete resection decreased 5-year overall survival (OS) to about 40–60 %.[2–4] The reasons for the high recurrence rate in early stage NSCLC remain controversial but could be attributed to its invasive histological features or a specific gene mutation.[5,6]

The epidemiology of NSCLC can be attributed to smoking tobacco in many patients. In addition to smoking, the other causes include radon, air pollution, secondhand smoke, cooking oil fumes, and unknown reasons.[7,8] Evidence demonstrates that 20 or more carcinogens in smoke are proven to induce NSCLC.[9] The best known is nicotine, which plays a confirmed role in carcinogenesis, in addition to smoking behavior, while expression of certain subtypes nicotinic acetylcholine receptors (nAchRs) have questionable roles in carcinogenesis.[9–11] Recently, one study suggested that 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) rather than nicotine played the most important role in promoting migration and invasion of lung

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cancer cells via nAchRs.[12] On the other hand, acetylcholine also acts as an autocrine or paracrine factor in lung cancer to induce nAchR expression.[11,13]

Among the nAchRs, the neuronal heteropentamers containing three α subunits (from α2 to α6) and two β subunits (from β2 to β4) were investigated in three whole genome studies for their variant gene clusters on chromosome 15q25 and risk in lung cancer.[14–16] Alpha 1 subunit of nAchRs, which is also called CHRNA1, belongs to muscle type nAchR different from other neuronal alpha subunits nAchRs. CHRNA1 is detected its expression in NSCLC, and nicotine could activate its downstream MAPK and AKT signaling pathways to induce drug resistance.[17] There is still no large study evaluating the expression of CHRNA1 in lung cancer patients, and most other studies investigated other subtypes of nAchRs, not CHRNA1.[10,15,17] We hypothesized that there is relationship between smoking, CHRNA1 expression, and prognosis in NSCLC. Therefore, we sought to confirm the expression of CHRNA1 in NSCLC patients and further to identify its prognostic value, especially relating to recurrence and survival after complete resection in patients with early stage NSCLC.

MATERIALS AND METHODS

Study Population

In this retrospective study of medical records from 1995 to 2007, we identified 214 patients with histologically documented stage I lung adenocarcinoma who underwent complete resection of their tumors at Taipei Veterans General Hospital, Taiwan. Disease follow-up was evaluated every 3 months with computed tomography or magnetic resonance imaging. Among the 214 patients, 185 had paraffin-embedded tumor samples available for study and were thus enrolled onto the study for CHRNA1 expression and survival evaluation. This study was approved by the institutional review board of Taipei Veterans General Hospital.

Immunohistochemical Study

Tumor specimens were stained using an immunohistochemical (IHC) method to detect CHRNA1 expression and downstream phosphorylated (p-ERK) as well as phosphorylated AKT (p-AKT). The samples were fixed in acetone, air dried, and subsequently bathed in Tris-buffered saline (pH 7.6). Briefly, 6-μm-thick sections of tumor tissue were cut from the frozen specimens for IHC analysis. Each tissue microarray was placed in a 65 °C oven for 15 min. Sections were then deparaffinized in xylene twice for 5 min, then rehydrated through an ethanol gradient to water (100 %, 95 %, 70 % ethanol, water). Endogenous peroxidase activity was blocked by covering the tissue with blocking solution for 30 min. Heat-induced epitope retrieval was performed in antigen retrieval solution at 100 °C for 10 min by microwave. For CHRNA1 IHC staining, a polyclonal anti-CHRNA1 antibody (Sigma-Aldrich) was used at a dilution of 1:10,000 and incubated at 4 °C overnight. p-ERK and p-AKT were stained by polyclonal anti-p-ERK1/2(T202/Y204) and anti-p-AKT1(S473) (Cell Signaling Technology) at a dilution of 1:200 and 1:25, respectively. Immunodetection was performed with a horseradish peroxidase–DAB detection kit (Vector Laboratories). For interpretation of the CHRNA1, p-ERK, and p-AKT IHC results, the intensity of immunoreactivity was graded according to the following scale: 0 (negative), 1 (weakly positive), 2 (moderately positive), and 3 (strongly positive). The percentage score was semiquantitatively assessed by the percentage of positive-stained cells: 0 (0 %), 1 ($\leq$10 %), 2 (11–50 %), and 3 (51–100 %). The IHC score of each specimen was represented by the value of the intensity score multiplied by the percentage score, which ranged from 0 to 9, according to the method described in a previous report.[18] The results were classified into two groups according to the intensity and extent of staining: in the low CHRNA1 group, no staining (IHC score = 0) or weak staining intensity (IHC score < 3) was present; the positive staining group was defined as IHC scores of 3 or more. The same classification was used for p-ERK and p-AKT expression. All of the IHC staining results were reviewed and scored independently by the same pathologist.

Statistical Analysis

The Chi square test was used to characterize differences in demographic data and comorbidities between the study and comparison cohorts. The Kaplan–Meier method was used to estimate survival, and the log-rank test was used to determine the differences between survival curves. OS was defined as time elapsed between diagnosis and date of death. Recurrence-free survival (RFS) was defined as the time between diagnosis and date of recurrence or death. Cox proportional hazard models were used to compare the hazard ratio for mortality between the cancer patients and comparison cohorts after stratification by age, gender, and hospitalization. We adjusted comorbidities in the multivariate analysis. In all analyses, $P < 0.05$ was considered statistically significant. All statistical analyses were performed by SPSS statistical software, version 19.0 for Windows (SPSS, Chicago, IL, USA).

RESULTS

Patient Characteristics

As shown in Table 1, a total of 173 patients had CHRNA1 IHC scores of $\geq$3 (93.5 %) and 12 patients had

TABLE 1 Basic characteristics of stage I lung adenocarcinoma patients with/without CHRNA1 expression

Characteristic	CHRNA1 ≥ 3	CHRNA1 < 3	*P*
Sex			0.209
Male	114 (65.9 %)	6 (50.0 %)	
Female	59 (34.1 %)	6 (50.0 %)	
Age			0.371
>60 years	128 (74.6 %)	10 (83.3 %)	
≤60 years	45 (25.4 %)	2 (16.7 %)	
Histologic differentiation			0.114
Well	13 (7.5 %)	3 (25.0 %)	
Moderate	106 (61.3 %)	6 (50.0 %)	
Poor	54 (31.2 %)	3 (25.0 %)	
Tumor necrosis			0.226
Yes	74 (42.8 %)	7 (58.3 %)	
No	99 (57.2 %)	5 (41.7 %)	
Angiolymphatic invasion			0.602
Yes	57 (32.9 %)	4 (33.3 %)	
No	116 (67.1 %)	8 (66.7 %)	
Perineural invasion			0.579
Yes	8 (4.6 %)	0 (0.0 %)	
No	165 (95.4 %)	12 (100.0 %)	
Pleural invasion			0.082
Yes	101 (58.4 %)	4 (33.3 %)	
No	72 (41.6 %)	8 (66.7 %)	
Smoking			0.564
Yes	78 (47.9 %)	5 (45.5 %)	
No	95 (52.1 %)	7 (54.5 %)	
Recurrence			0.020
Yes	54 (31.2 %)	0 (0.0 %)	
No	119 (68.8 %)	12 (100.0 %)	

CHRNA1 nicotinic acetylcholine receptor alpha 1

scores of <3 (6.5 %). The median follow-up time was 65.3 months (range 0.2–126 months). The basic characteristics between the two groups of patients were similar. Both groups contained elderly patients aged 60 years or more. Most patients (169 of 185; 90.8 %) had moderate histologic differentiation, and about half of the patients had no history of smoking tobacco. In the group with CHRNA1 IHC scores of <3, no patient had recurrence, which was significantly different compared to the group with CHRNA1 IHC scores of ≥3 (0 vs. 31.2 %, $P = 0.02$).

CHRNA1 Expression in Tumor Tissues

CHRNA1 expression in patients with stage I lung adenocarcinoma was demonstrated in tumor samples by immunohistochemistry, resulting in a cytoplasmic staining pattern (Fig. 1). The CHRNA1 intensity was scored as no staining (intensity score = 0, Fig. 1a), weak staining (intensity score = 1, Fig. 1b), moderate staining (intensity score = 2, Fig. 1c), and strong staining (intensity score = 3, Fig. 1d).

CHRNA1 Expression is Associated with RFS

We performed univariate and multivariate survival analysis for the prognostic factors of RFS. Age > 60 years, tumor necrosis, angiolymphatic invasion, perineural invasion, and CHRNA1 expression were significant prognostic factors of RFS (Table 2). After multivariate Cox regression for the significant prognostic factors identified in the univariate analyses, tumor necrosis, angiolymphatic invasion, perineural invasion, and CHRNA1 expression independently predicted RFS.

CHRNA1 Expression is Associated with OS

For OS, univariate analysis revealed that tumor necrosis, angiolymphatic invasion, perineural invasion, and CHRNA1 expression significantly predicted OS. After multivariate Cox regression analysis, all of these factors independently predicted OS (Table 2).

Increased Expression of CHRNA1 is Associated with Reduced Survival

The Kaplan–Meier survival curves revealed that CHRNA1-positive patients had significantly worse RFS (median 60.6 months, range 0.2–126.0 months) compared with that of the CHRNA1-negative patients (median 77.9 months, range 18.1–125.2 months) ($P = 0.03$, Fig. 2a). The CHRNA1-positive patients also exhibited significantly worse OS (median 65.1 months, range 0.2–126.0 months) compared with that of the CHRNA1-negative patients (median 77.9 months, range 18.1–125.2 months) ($P = 0.04$, Fig. 2b). The expression of p-ERK and p-AKT demonstrated no significant difference in survival, although both demonstrated a trend for poor prognosis (Supplementary material Figs. S1, S2, S3 and S4). We also analyzed the correlation between CHRNA1 and downstream phosphorylated signaling expression and found that the CHRNA1-negative patients also had lower p-ERK and p-AKT expression, although without significance (Supplementary material Fig. S5).

Smoking Did Not Influence Survival in Patients with CHRNA1 Expression

Because nicotine and its associated metabolites activate downstream carcinogenic pathways in lung cancer via nAchRs, we further analyzed the relationship between CHRNA1 expression and smoking. As shown in Table 1,

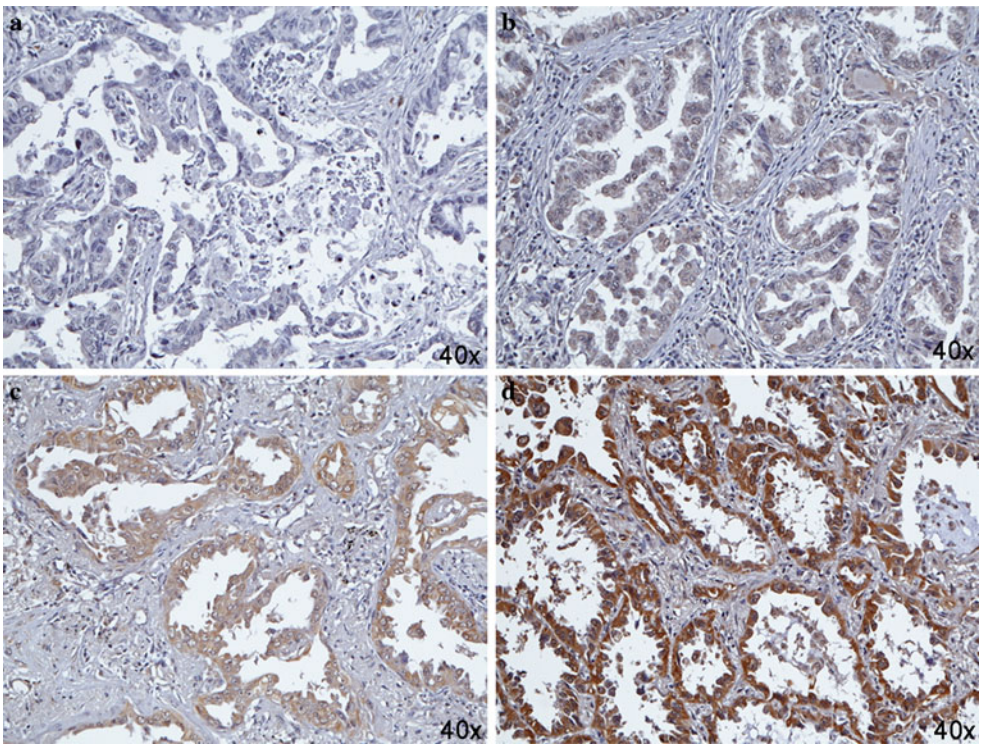

FIG. 1 Immunohistochemical staining for CHRNA1 in stage I adenocarcinoma. **a** No staining (intensity score = 0). **b** Weak staining (intensity score = 1). **c** Moderate staining (intensity score = 2). **d** Strong staining (intensity score = 3)

there was no difference in smoking between the CHRNA1 positive and negative groups. The RFS and OS survival also demonstrated no significant differences between smoking and nonsmoking patients with CHRNA1 expression (Fig. 3).

DISCUSSION

We confirmed the high expression ratio of CHRNA1 and its prognostic significance for RFS and OS in stage I lung adenocarcinoma patients. The roles of nAchRs in cancers have been popular topics of study in recent years. In addition to the previous genome-wide studies focusing on nicotine exposure, nAchRs genetic variants, and lung cancer risk, recent studies also focused on the autocrine and paracrine effects of acetylcholine for the nAchRs expressed in lung cancer cells.[10,11,14,19] The α1 subunit of nAchRs, also known as CHRNA1, has been little studied compared to other subunits of nAchRs in lung cancer. To our knowledge, this study is the first large study of stage I lung cancer patients for whom CHRNA1 and its prognostic value were evaluated during long-term follow-up after complete resection of early stage NSCLC.

The mechanisms for early metastasis of lung cancer cells are still unclear. Early metastasis could be explained by the highly invasive histologic features of NSCLC. In this study, we also identified other well-known invasive pathologic features, such as significant prognostic factors including tumor necrosis, angiolymphatic invasion, and perineural invasion. These factors are associated with the possible mechanisms of cancer invasiveness or metastasis. For example, tumor necrosis is an adverse factor for survival and recurrence in stage I NSCLC, which could be due to hypoxia induced epithelial mesenchymal transformation of lung cancer cells.[20] Angiolymphatic invasion is also identified as a poor prognostic factor for stage I NSCLC, which could be related to tumor angiogenesis and vascular endothelial growth factor expression.[21,22] Perineural invasion independently predicted poor prognosis in early stage NSCLC, comparable to the tumor node metastasis classification system.[23,24] The mechanisms of perineural invasion and their association with metastasis are still unclear. In the current study, we unexpectedly found that no patient had perineural invasion in the CHRNA1-negative group, although this result lacked significance, possibly as a result of the small number of patients (Table 1). We believe the relationship between the expression of neurotransmitter receptors and perineural invasion warrants further study.

Because activation of downstream ERK1/2 and AKT of CHRNA1 has been reported in NSCLC in vitro, we also analyzed the expression levels of phosphorylated ERK and AKT between CHRNA1-positive and -negative patients and determined their associations with prognosis.[17] The

TABLE 2 Univariate and multivariate Cox regression in prognostic factors for recurrence-free survival and overall survival

Characteristic	Univariate analysis		Multivariate analysis	
	HR (95 % CI)	*P*	HR (95 % CI)	*P*
Recurrence-free survival				
Male	1.13 (0.74–1.74)	0.572		
Age > 60 years	1.76 (1.04–2.99)	0.034	1.63 (0.96–2.77)	0.071
Smoking	1.22 (0.80–1.85)	0.365		
Well-differentiated tumor	1.28 (0.90–1.81)	0.170		
Tumor necrosis	1.72 (1.15–2.59)	0.009	1.67 (1.10–2.53)	0.015
Angiolymphatic invasion	1.90 (1.25–2.88)	0.003	1.65 (1.08–2.51)	0.021
Pleural invasion	0.90 (0.60–1.36)	0.624		
Perineural invasion	2.66 (1.28–5.51)	0.009	2.13 (1.02–4.45)	0.045
CHRNA1 expression	4.16 (1.02–16.89)	0.046	4.82 (1.18–19.67)	0.029
p-ERK expression	1.74 (0.93–3.28)	0.085		
p-AKT expression	1.19 (0.75–1.88)	0.466		
Overall survival				
Male	1.12 (0.73–1.74)	0.600		
Age > 60 years	1.65 (0.98–2.81)	0.062		
Smoking	1.22 (0.79–1.87)	0.371		
Well-differentiated tumor	1.28 (0.90–1.84)	0.174		
Tumor necrosis	1.81 (1.19–2.74)	0.005	1.75 (1.15–2.67)	0.010
Angiolymphatic invasion	2.01 (1.32–3.07)	0.001	1.77 (1.15–2.71)	0.010
Pleural invasion	0.95 (0.62–1.43)	0.788		
Perineural invasion	2.80 (1.35–5.81)	0.006	2.21 (1.06–4.64)	0.035
CHRNA1 expression	3.91 (0.96–15.89)	0.057	4.39 (1.07–17.94)	0.040
p-ERK expression	1.48 (0.77–2.86)	0.245		
p-AKT expression	1.16 (0.72–1.86)	0.542		

HR hazard ratio, *CHRNA1* nicotinic acetylcholine receptor alpha 1

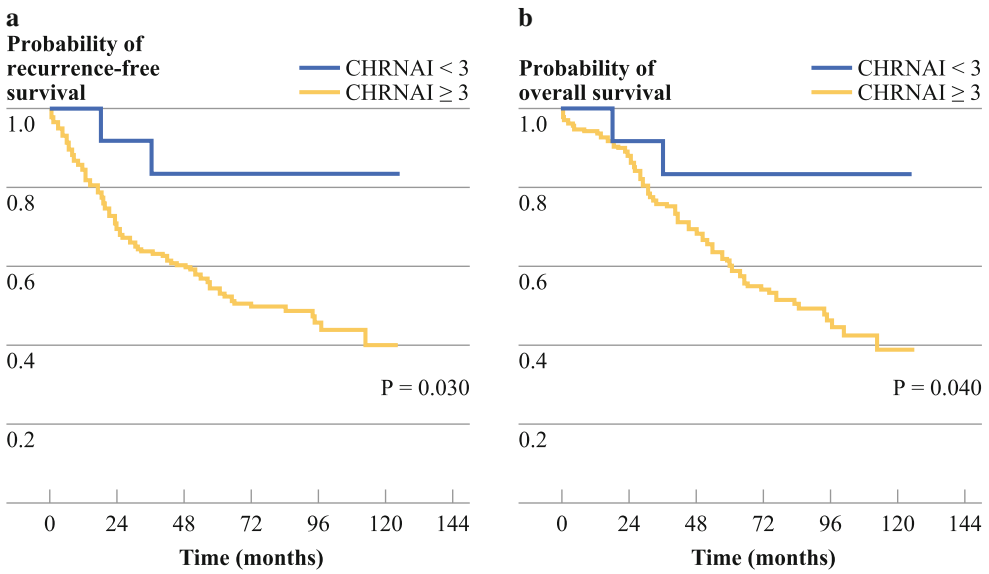

FIG. 2 Kaplan–Meier survival curves for stage I lung adenocarcinoma patients with and without CHRNA1 expression. **a** RFS for patients with CHRNA1 < 3 (median 77.9 months, range 18.1–125.2 months) versus CHRNA1 ≥ 3 (median 60.6 months, range 0.2–126.0 months), $P = 0.03$. **b** OS for patients with CHRNA1 < 3 (median 77.9 months, range 18.1–125.2 months) versus CHRNA1 ≥ 3 (median 65.1 months, range 0.2–126.0 months), $P = 0.04$

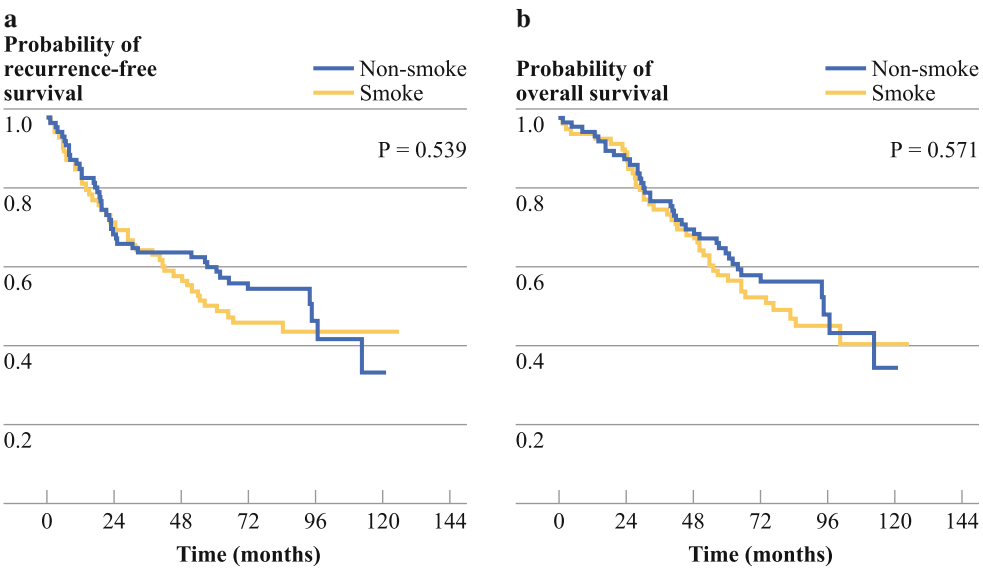

FIG. 3 Kaplan–Meier survival curves for CHRNA1 expressed patients with and without smoking. **a** RFS for patients with smoking (median 56.8 months, range 0.2–125.9 months) versus nonsmoking (median 62.6 months, range 0.3–121.4 months), $P = 0.539$; **b** OS for patients with smoking (median 65.3 months, range 0.2–125.9 months) versus nonsmoking (median 65.1 months, range 0.27–121.43 months), $P = 0.571$

results revealed trends for high p-ERK and p-AKT expression in CHRNA1-positive patients as well as for poor prognosis, but without significance. Such inconsistency of expression for CHRNA1 and phosphorylated downstream signaling could be due to the degradation of phosphorylated proteins in the surgically resected tissues.[25,26] However, activation of ERK or AKT signaling from other upstream receptors, such as EGFR in NSCLC, could also have accounted for the differences in prognosis between CHRNA1 and ERK/AKT activation.[27]

In both the CHRNA1-positive and -negative groups, about half of the study patients were nonsmokers. Why nonsmokers had CHRNA1 expression, despite their lack of nicotine stimulation, is still unknown. We also unexpectedly found that CHRNA1 expression predicted RFS and OS without influence by smoking. The possible explanations for this result include secondhand smoke, air pollution, and cooking oil fumes, which could also stimulate nAchRs for cancer metastasis; other factors such as acetylcholine autocrine–positive feedback to induce or activate nAchR expression and associated carcinogenetic signaling pathways, also warrant further study.[28]

The limitation of this study is that it is retrospective, with its inevitable selection bias. The small number of non-CHRNA1 patients also raised question about its representative role. Therefore, further large prospective studies are required to confirm our study results.

In conclusion, CHRNA1 expression in early stage NSCLC appears to be a useful prognostic factor for recurrence and survival. Recently many reports have focused on nAchR inhibitors as target agents for cancer therapy.[29–31] We provide evidence that CHRNA1, a subunit of nAchRs expressed in lung cancer patients, should be the target of cancer therapeutic development for improving the cure rate or for preventing recurrence and prolonging patient survival.

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DISCLOSURE The authors declare no conflict of interest.

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