p53+/-Rb1-ASCL1^{low} Phenotype Predicts Better Prognosis After Surgical Resection in Large Cell Neuroendocrine Carcinoma: a Molecular Characteristic and Clinical-pathological Investigation of Lung Neuroendocrine Tumors

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Research

Keywords: lung neuroendocrine tumors, large cell neuroendocrine carcinoma, molecular phenotype, lymph node metastasis, prognosis

Posted Date: December 4th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-120207/v1

Abstract

Background: Based on morphology, necrosis degree, and proliferation status, lung neuroendocrine tumors (NETs) are commonly divided into four subtypes: typical carcinoid (TC), atypical carcinoid (AC), large cell neuroendocrine carcinoma (LCNEC), and small cell lung carcinoma (SCLC). However, cases difficult to classify still exist in diagnosis.

Methods: We immunohistochemically investigated the molecular phenotypes in lung NETs and assessed their prognostic value.

Results: After morphological re-analysis of 179 NETs, 19 cases were classified as undefined, which included 3 cases showing carcinoid morphology with high proliferation (Ki67>20% or mitotic count>10/2mm^2), and 16 cases showing intermediate differentiated morphology with Ki67 among 20% to 60%. Furthermore, molecular phenotypes were determined by expression of p53, Rb1, ASCL1, STK11, and gene mutation status of KRAS. Interestingly, p53^{wt}Rb1$^+$ distinguished an unique subcategory from undefined lung NET cases which differ from SCLC or LCNEC in prognosis, indicating WD-NET G3 existed in lung NETs. Additionally, in LCNEC, high ASCL1 expression was only relevant to lymph node metastasis rather than overall survival. However, in p53$^{+/-}$Rb1$^-$ LCNEC phenotype, low ASCL1 predicted better outcomes, along with less risk of lymph node metastasis.

Conclusion: This study provided evidence for existence of WD-NET G3 in NETs and revealed promising prognosis of p53$^{+/-}$Rb1$^-$ ASCL1^{low} subcategory in LCNEC, which could be beneficial to the evaluation of patient status in future clinic practice.

Background

Lung neuroendocrine tumors are considered to originate from the Kulchitsky cells in the bronchial mucosa. In 2015, the World Health Organization (WHO) classified lung neuroendocrine tumors into typical carcinoid (TC), atypical carcinoid (AC), small cell lung cancer (SCLC) and large cell neuroendocrine carcinoma (LCNEC) [1]. TC, AC, SCLC and LCNEC can be mostly distinguished by tumor morphology, necrosis degree and mitotic image. Meanwhile Ki67 is usually used as an auxiliary classification index [2]. In 2018, the International Agency for Research on Cancer (IARC) and WHO proposed a general classification framework for neuroendocrine tumors [3], which jointly defined neuroendocrine tumors in all organs as neuroendocrine neoplasms. In this framwork, lung well-differentiated neuroendocrine tumor (WD-NET) is divided into G1 and G2, in which G1 equals typical carcinoid and G2 equals atypical carcinoid. LCNEC and SCLC are classified into poorly-differentiated neuroendocrine cancer G3 (PD-NEC G3). WD-NET and PD-NEC have significantly different clinical characteristics. Generally, WD-NET patients were younger with an average age about 50 years and high 5-year survival rate (TC: 90%−95%; AC: 60% −70%) [4]. The pathogenesis is not closely related to smoking. The patients are usually not sensitive to radiotherapy or chemotherapy (especially platinum), so surgery was preferred. On the other hand, the average age of PD-NEC patients is about 70 years old. PD-NEC is closely related to smoking. Most PD-

NEC is sensitive to chemotherapy initially, but the development of acquired chemoresistance is ubiquitous, and the 5-year survival rate is only about 10% [5].

In addition to clinic characteristics, WD-NET and PD-NEC are completely different genetically. According to the whole genome sequencing, carcinoid only has a very low mutation load, and about 11%–13% WD-NET are characterized by MEN1 gene mutation, which is not found in PD-NEC [6]. PD-NEC has high mutation load. In SCLC, the TP53 and RB1 mutation are central events in tumor pathogenesis, with a rate of 90–100% and 92%, respectively [7,8]. According to the different expression of ASCL1, SCLC can be divided into three different molecular subtypes [9,10]: the subgroup with high expression of ASCL1 is called classical subtype, which is the most common; the subgroup with low expression of ASCL1 and high expression of NeuroD1 is called variant subtype; the third is the subtype with negative expression of both genes.

For LCNEC, Rekhtman N et.al. [11] and George J et.al. [12,13] considered LCNEC to be heterogeneous, and could be divided into SCLC-Like subtype, NSCLC-Like subtype and unusual Carcinoid-Like subtype. In SCLC-Like subtype, both TP53 and RB1 mutations exist. In NSCLC-Like subtype, STK11 / KRAS / KEAP1 mutations are common. And Carcinoid-Like subtype has MEN1 mutation with low total mutation load. George J et.al. [13] further classified LCNEC into ASCL1low and ASCL1high subtypes. The ASCL1 high NSCLC-Like cases is called LCNEC-Type I while The SCLC-Like $ASCL1^{low}$ group is called LCNEC-Type II. Interestingly, they also noticed individual cases diagnosed as LCNEC had carcinoid-like MEN1 mutations, presented carcinoid-like morphology, but with high KI67 LI (> 20%). However, the prognosis of these cases was not studied in their study. This kind of carcinoid tumor with high proliferative activity has also been reported in previous studies, especially in pancreas NENs, namely WD-NET G3. Pancreas WD-NET G3 is different from PD-NEC, the WD-NET G3 patients were not sensitive to chemotherapy such as platinum drugs but had better overall outcomes similar to WD-NET G1/G2.

These studies above suggest that further molecular typing of NENs not only result in accurate diagnosis, but also contribute to evaluation of prognosis and therapy selection. In this study, 179 cases of lung neuroendocrine tumors in our hospital from February 2012 to December 2018 were collected. The protein expression of TP53, Rb1, ASCL1, STK11 and the mutation status of KRAS genes were evaluated in TC, AC, SCLC, LCNEC and unclassified cases. Based on these markers, we further explored whether WD-NET G3 exists in lung NENs and evaluated the prognosis and clinicopathological characteristics of different lung neuroendocrine tumors after classification.

Materials And Methods

Patient cohort

217 cases of pulmonary neuroendocrine tumorfrom February 2012 to December 2018, were collected and sorted retrospectively. According to the following criteria, 179 cases were included in the study.

Case rejection criteria: When patient has both puncture and operation samples, the puncture samples are removed; The patient has only puncture specimen with less tissue, it is not suitable to be sectioned and stained again; The patient was pathologically diagnosed as mixed neuroendocrine tumor of the lung.

Immunohistochemistry and KRAS mutation test

In this study, the staining was conducted on tissue array. 4 um Sections were de-paraffinized and hydrated, and the endogenous peroxidase was blocked. Antigen retrieval was performed using the Dako Target Retrieval Solution, High PH (Dako Ominis, Agilent Technologies, Santa Clara, CA, USA), in a PTLink set at 98ºC for 25 min. Then slides were incubated with primary antibody for 60 min at room temperature. Immunostaining was achieved by an enzyme-conjugated polymer complex (Dako K8002, Agilent Technologies, Santa Clara, CA, USA) adapted for an autostainer (Dako Autostainer Link 48, Agilent Technologies). The primary antibodies used as follows: anti-Rb1 (Santa Cruz, sc–102, 1:250, USA), anti-P53 (Abcam, ab32389, 1:200, USA), anti-ASCL1 (Abcam, ab74065, 1:250, USA), anti-STK11 (Abcam, ab15095, 1:500, USA).

Interpretation standard of the markers above:

Under normal conditions, the half-life of p53 protein is very short and it is degraded quickly. The normal peripheral lung tissue was used as a wild-type control group, and immunohistochemical examination showed that the nuclei were scattered and brownish yellow. After mutation, the protein accumulated rapidly and stably located in the nucleus. Immunohistochemistry showed that more than 60% of tumor cells had uniform brownish yellow staining. In addition, TP53 gene can also produce nonsense mutation, and the expression of p53 protein in tumor cells is completely absent compared with the positive control in interstitial tissue. 60%–100% of the tumor cells were positive or negative (indicating mutation), the rest were wild type.

Rb1 encoded protein was located in the nucleus. In the normal cells, the nucleus is brownish yellow. After the gene mutation, the fibrovascular tissue was used as the positive control, and the expression was negative. Therefore, the expression of nuclear positive staining protein is normal, and the absence of nuclear staining suggests that Rb1 gene mutation.

STK11 gene encodes proteins that function in the cytoplasm. In adenocarcinoma, about 33% of STK11 can mutate, resulting in the loss of STK11 protein expression. Therefore, the lung adenocarcinoma was taken as the negative control, the tumor stroma as the positive control. The positive expression of STK11 protein indicated that STK11 was not mutated, and the deletion of STK11 protein indicated that STK11 gene mutation might exist.

As a nuclear transcription factor, ASCL1 protein is expressed in the nucleus, and the staining intensity of the nucleus is evaluated.

Immunohistochemical reactivity was scored by three pathologists (Zitian Huo, Yaqi Duan, and Guoping Wang) independently as follows: multi-plication of the intensity of immunostaining (1, weak;2, moderate;

3, strong) and the percentage of positivetumour cells, which resulted in a score of 0–300. Ascore < 10 was considered as 0 (cut-off level < 10), ascore of 10–40 was considered as 1+,41–140 as 2 + and 141–300 as 3+.

KRAS gene detection kit (Amoydx company, China) was used as manufacture's instruction.

Counting of mitosis in 2mm2 field of vision(count/HPF)

The number of mitotic images in the corresponding field of vision was calculated by using Nikon pathological microscope with the field number of eyepiece of 2. (actual field of view diameter = number of field of view / magnification of objective lens)

Follow-up

We obtained the survival data by consulting the original medical record, telephone and follow-up. Overall survival (OS) is defined as: from the day of disease diagnosis to the date of death or the last follow-up, calculated in months, the end date of follow-up is February 26, 2019, a total of 119 cases obtained OS information.

Bioinformatical validation

Integrate George Julies DNA/RNA sequencing data set and clinical prognosis data set,we totally obtained 57 case with Complete data information, including 14 cases of sclc-like type. Patients were divided into ASCL1-high/low group according to the mean fpkm.The survival analysis found that the different expression levels of ASCL1 cannot provide a good guide to the prognosis of patients in ovrall LCNEC levels. However, in sclc-like LCNEC, since there are only 14 patients with survival data, it can still be found that patients with high ASCL1 expression seem to have more Poor prognosis. Based on the statistics of our larger sample size, we can significantly find that patients with high ASCL1 expression in sclc-like LCNEC are more likely to have tumor metastasis and a worse prognosis.

Statistical analysis

The software graphpad prism 7.0 was used for statistical analysis. The overall survival (OS) was calculated by the Kaplan-Meier method and compared by the log-rank test. Two groups were compared using Student's t-test, and multiple groups were compared using one-way analysis of variance (ANOVA). Data were presented as the mean ± SEM. $P < 0.05$ was considered statistically significant.

Results

Re-diagnosis and classification of lung neuroendocrine tumors

Of all 179 cases (Table 1), 29 cases met the criteria of carcinoid diagnosis (typical carcinoid: typical morphological features + mitotic image count < 2 / 2mm2 + Ki67 ≤ 5%; atypical carcinoid: typical morphological features + mitotic image count < 10 / 2mm2 + Ki67 < 20%), and were classified as WD-NET

(Fig. 1A&B). Besides, 43 had typical LCNEC morphology (Fig. 1C), with mitotic image count > 10 / 2mm2 and Ki67 ≥ 60%. 88 cases had classic small cell carcinoma morphology (Fig. 1D), with Ki67 ≥ 50%.

Table 1
Morphological classification of lung neuroendocrine tumors

Morphology	Ki 67 LI	Mitotic image	Cases	Classification
Definite TC	< 5%	< 2/2mm^2	15	TC
Definite AC	< 20%	< 10/2mm^2	14	AC
Definite SCLC	≥ 50%	> 10/2 mm^2	88	SCLC
Definite LCNEC	≥ 60%	> 10/2 mm^2	43	LCNEC
Carcinoid-Like	30%	> 10/2 mm^2	2	undefined
Carcinoid-Like	25%	< 10/2mm^2	1	
Between WD and PD	≥ 20%	> 10/2 mm^2	16	

Note: typical carcinoid (TC), atypical carcinoid (AC), small cell lung cancer (SCLC) , large cell neuroendocrine carcinoma (LCNEC), poorly-differentiated (PD), well-differentiated (WD).

The remaining 19 cases were undefined-lung NETs. Of them, 3 cases had typical carcinoid morphology (Fig. 2A): the tumors were arranged in organ like or palisade like arrangement; most of the cells were uniform in morphology; the cytoplasm was medium and light stained; the cell atypia and nucleocytoplasmic ratio were slightly higher than AC; However, the tumor showed high proliferation activity with mitotic image > 10 / 2mm2, or Ki67 ≥ 20% (Fig. 2C&D). In the other 16 cases, the morphology was between PD and WD (Fig. 2D&E): the tumor showed irregular infiltrative growth mode, intratumoral fibrosis, more tumor necrosis than AC, but the cell nucle-ocytoplasmic ratio was not high, the cell atypia was lower than LCNEC, with trabecula, organ like structure and thin-walled vascular growth mode. Of them, the mitotic image was higher than 10/2mm2 while Ki67 LI was between 20%–60% (Fig. 2F).

Expression of p53、Rb1、STK11、ASCL1 in lung neuroendocrine tumors

Representative IHC staining was shown in Fig. 3A-D. The protein expression of p53 and Rb1 was significantly different in WD-NET (TC / AC) and PD-NEC (LCNEC / SCLC) (Table 2). In 29 cases of WD-NET, there was no absence of Rb1 protein, only one case of AC had the absence of tumor p53 expression. In 43 cases of LCNEC, the abnormal expression or absence rates of p53 and Rb1 were 91% (39 / 43) and 77% (33 / 43), respectively. In 67% (29 / 43) of LCNEC, both abnormal expression of p53 and Rb1 loss happened. In 88 cases of SCLC, the abnormal ratio of p53 and RB1 protein were 93% (82 / 88) and 92% (81 / 88) respectively. In 85% (75 / 88) cases of SCLC, both p53 and Rb1 were abnormal (Fig. 4A).

Table 2
Expression of biomarkers in lung NENs

Biomaker	WD-NET		PD-NEC		P value (WD vs PD)
	TC	AC	LCNEC	SCLC	
p53 $^{+/-}$	0%(0/15)	7%(1/14)	91%(39/43)	93%(82/88)	< 0.0001*
Rb1^{-}	0%(0/15)	0%(0/14)	77%(33/43)	92%(81/88)	< 0.0001*
Stk11^{-}	26%(4/15)	14%(2/14)	9%(4/43)	0%(0/88)	0.027
Ascl1^{high}#	27%(4/15)	35%(5/14)	53%(23/43)	76%(67/88)	0.003*

The expression rate and intensity of ASCL1 in PD-NEC was significantly higher than WD-NET. In WD-NET, 69% (20 / 29) ASCL1 protein was stained as 0–1 +, 21% (6 / 29) as 2 +, and only 10% (3 / 29) as 3+. On the other hand, in LCNEC, 47% (20 / 43) was 0–1 +, 12% (5 / 43) was 2 +, 42% (18 / 43) was 3 +. In SCLC, 24% (21 / 88) is 0–1 +, 13% (12 / 88) is 2 +, 63% (55 / 88) is 3 + (Fig. 4B).

The absence of STK11 expression is more common in WD-NET (6 / 29, 21%) rather than LCNEC (4 / 43, 9%). No absence of STK11 expression was found in SCLC (Fig. 4C).

Molecular phenotype of LCNEC and undefined cases in lung neuroendocrine tumors

According to the expression of p53, Rb1, STK11, ASCL1 and the detection of KRAS mutation, the typical LCNEC and the undefined cases were re-classified (Table 3). In 43 typical LCNEC cases, 29 patients had abnormal expression of both p53 and Rb1, with no STK11 deletion or KRAS mutation. According to Rekhtman N's previous study, they were defined as SCLC-Like LCNEC. Besides, the expression of RB1 was absent in 4 cases. Among them, 1 case showed low expression of ASCL 1, with no deletion of STK11 but rather mutation of exon 2 of KRAS gene c.34G > T (p.G12C). Thus this case was defined as NSCLC-Like type. In the other 3 p53wtRb1-Ascl1high/low cases, no abnormal expression STK11 or KRAS mutation was found, however, the tumor cells heteromorphism was obvious, along with significant necrosis, mitotic count > 40 / 2mm2, and Ki67 > 75%. Thus the 3 cases were classified as undefined type. In 43 LCNEC cases, 10 cases only had abnormal expression of p53 but no absence of Rb1. Of them, 4 cases had silence of STK11 and 1 case had KRAS mutation of exon c.34G > T (p.G12C). Additionally, Five p53+/- Rb1 + Ascl1high/low cases showed no abnormality of STK11 or KRAS. Combined with their tumor morphology and proliferation characteristics, they were classified as LCNEC-undefined. No p53wtRb1 + cases existed in the 43 typical LCNEC.

Table 3
Phenotype classification of LCNEC and undefined cases

Protein/gene				LCNEC	undefined	Phenotype
$p53^{+/-}$ $+Rb1^{-}$	$Ascl1^{high}$	$Stk11^{+}$	*KRAS*^{wt}	16	3	SCLC-Like $ASCL1^{high}$
	$Ascl1^{low}$			13	5	SCLC-Like $ASCL1^{low}$(LCNEC-Type II)
$Rb1^{-}$+$p53^{wt}$	$Ascl1^{low}$	$Stk11^{+}$	*KRAS*^{mut}	1	0	NSCLC-Like
	$Ascl1^{low/high}$		*KRAS*^{wt}	3	0	LCNEC-undefined
$p53^{+/-}$ $+Rb1^{+}$	$Ascl1^{high}$	$Stk11^{-}$	*KRAS*^{wt}	1	0	NSCLC-Like(LCNEC-Type I)
	$Ascl1^{low}$	$Stk11^{+}$	*KRAS*^{mut}	1	0	NSCLC-Like
		$Stk11^{-}$	*KRAS*^{wt}	3	0	
	$Ascl1^{low/high}$	$Stk11^{+}$	*KRAS*^{wt}	5	4	LCNEC-undefined#
$Rb1^{+}$+$p53^{wt}$	$Ascl1^{high}$	$Stk11^{+}$	*KRAS*^{mut}	0	1	$p53^{wt}Rb^{+}$ (High proliferation)
	$Ascl1^{low}$		*KRAS*^{wt}	0	4	
	$Ascl1^{high}$			0	2	

Note: # Here, LCNEC-undifined only refers to the five cases that met all diagnostic criteria of typical LCNEC and ki67 ≥ 60%,but can not be classified according to the study of Rekhtman N and George J et.al.

Immunohistochemical phenotype of p53wtRb1 + with high proliferation avtivity distinguished suspected WD-NET G3 in lung neuroendocrine tumors

In the 19 undefined lung neuroendocrine tumors, there were 8 p53+/-Rb1- cases fitted SCLC-Like LCNEC based on morphology. Another 4 p53+/-RB1 + cases had no STK11 loss or KRAS mutation, which were difficult to classify temporarily (Table 3). Importantly, There were 7 Rb1 + p53wt cases, one of which had a KRAS exon 2 c.34G > T (p.G12C) mutation. These p53wtRb1 + cases with high proliferative activity (3 of them have typical carcinoid morphological characteristics) are suspicious as WD-NET G3 subtype. We further analyzed the prognosis of 21 WD-NET, 38 LCNEC, 53 SCLC and 7 suspected WD-NET G3 cases. The survival analysis curve of WD-NET G3 was between WD-NET and PD-NEC. However, the difference did not meet a statistical significant level (Fig. 4D).

Low ASCL1 expression in p53+/-Rb1- predicted better outcomes in LCNEC

In this study, we analyzed the overall survival rate of LCNEC with different types (Fig. 4D-H). The results showed that there was no significant difference in the clinicopathological characteristics and prognosis among SCLC/NSCLC-Like LCNEC and undefined LCNEC. Among all LCNEC cases, ASCL1 high subgroup was more likely to have lymph node metastasis than ASCL1 low subgroup (Table 4), but there was no significant difference in prognosis (Fig. 4G). However, in SCLC-Like LCNEC, SCLC-Like ASCL1 low subtype showed better overall survival compared with SCLC-Like ASCL1 high subtype (Fig. 4G), along with lower risk of lymph node metastasis (Table 5). In addition, we conducted a bioinformatical validation to test the prognosis value of ASCL1 in LCNEC (Fig. 5A) and SCLC-Like LCNEC (Fig. 5B). There only existed one database for transcriptome analysis. The mRNA level of ASCL1 was not in consistence with LCNEC prognosis.

Table 4
Relationship between ASCL1 expression and clinicopathological features in all LCNEC cases

Clinicopathological features	Cases (n = 51)	LCNEC-Ascl 1^{high} (n = 25)	LCNEC-Ascl1 ^{low} (n = 26)	P value
Sex				0.967
Male	47	23(92%)	24(92%)	
Female	4	2(8%)	2(8%)	
Age (years)				0.473
≤ 60	28	15(60%)	13(50%)	
> 60	23	10(40%)	13(50%)	
Smoking				0.440
Yes	42	20(80%)	22(88%)	
No	9	5(20%)	4(12%)	
Tumor size				0.351
T1(d ≤ 3cm)	5	3(12%)	3(8%)	
T2(3cm < d ≤ 5cm)	15	8(32%)	7(27%)	
T3(5cm < d ≤ 7cm)	17	5(20%)	12(46%)	
T4(d ≥ 7cm)	6	4(16%)	2(8%)	
Tx	7	5(20%)	2(8%)	
LNM				0.003*
Yes	25	18(72%)	7(24%)	
No	22	5(20%)	17(59%)	
Not examined	4	2(8%)	2(8%)	
Location				0.147
Central	19	12(53%)	7(24%)	
Periphery	20	13(47%)	17(59%)	
Not described	2	0(0%)	2(8%)	

Table 5
Relationship between ASCL1 expression and clinicopathological features in LCNEC-SCLC-Like cases

Clinicopathological features	Cases (n = 37)	SCLC-Like Ascl1 ^{high} (n = 19)	SCLC-Like Ascl1^{low} (n = 18)	P value
Sex				0.954
Male	33	17(89%)	16(89%)	
Female	4	2(11%)	2(11%)	
Age (years)				0.898
≤ 60	14	7(39%)	7(39%)	
> 60	23	12(61%)	11(61%)	
Smoking				0.530
Yes	28	14(74%)	14(77%)	
No	9	5(26%)	4(23%)	
Tumor size				0.424
T1(d ≤ 3cm)	3	1(5%)	2(11%)	
T2(3cm < d ≤ 5cm)	14	9(47%)	5(29%)	
T3(5cm < d ≤ 7cm)	9	2(11%)	7(39%)	
T4(d ≥ 7cm)	5	4(21%)	2(6%)	
Tx	5	4(21%)	2(6%)	
LNM				0.002*
Yes	20	15(79%)	5(29%)	
No	14	3(16%)	11(61%)	
Not examined	3	1(5%)	2(11%)	
Location				0.200
Central	15	10(53%)	5(29%)	
Periphery	20	9(47%)	11(61%)	
Not described	2	0(0%)	2(11%)	

Discussion

Neuroendocrine tumors (NETs) are highly heterogeneous tumors with great differences in the embryo origin, biological behavior and clinicopathological characteristics [14]. In 2010, WHO revised the naming and classification of neuroendocrine tumors based on European Neuroendocrine Tumor Society (ENETs) classification system [15]. Dependent on the morphological differentiation degree, the NETs are divided into WD-NET and PD-NEC. According to the mitotic and Ki−67 index, the NETs are also divided into low grade (G1), medium grade (G2) and high grade (G3). G3 is also called NEC, including small cells tumors, large cells tumors, and mixed NEC.

According to 2010 WHO classification standard, G3 is defined as NETs with Ki−67 > 20%. But recently, a proportion of neuroendocrine tumors presenting a number of mitoses or a Ki−67 index higher than 20% and a wel-differentiated morphology have been identified, calling for a new category, well-differentiated NET grade 3 (WD-NET G3) [16,17,18]. For example, a prospective epidemiological study [19] by the American Society of Clinical Oncology (ASCO) showed that in 778 cases of non-small cell gastrointestinal and pancreatic neuroendocrine tumors (GEP-NET), 104 cases (13.5%) were G3, among which 72 cases (69%) were poorly differentiated, 21 cases (20%) were well differentiated, and 11 cases (10.5%) were adenocarcinoma. Despite morphological differences, in another study [20] enrolled 12 WD-NET G3 amd 16 LCNEC G3 cases, the positive rate of octreotide scanning was 88% and 50% respectively, the platinum response rate of WD-NET G3 group was 0% while LCNEC G3 group was 31%, along with median survival time of 41 vs 17 months. Sorbye et al. [21] reviewed 305 cases of gastrointestinal NEC (WHO G3). Patients with Ki−67 index less than 55% had poor efficacy (15% vs 42%) and longer survival time (14 months vs 10 months) compared with patients with Ki−67 index more than 55%. This study also suggested that WD-NET G3 should not be treated in the same as LCNEC G3. As described, the main localizations of WD-NET G3 are the pancreas, stomach, and colon. Whether WD-NET G3 exists in lung NETs remain unclear. In our study, p53wtRb1 + cases with high proliferation avtivity (mitoses > 10/HPF or Ki−67 index > 20%) were suspected as lung WD-NET G3 subtupe, which showed difference from LCNEC in prognosis.

LCNEC is a kind of highly heterogeneous tumor with high metastasis rate and low survival rate. The reported prognosis varied greatly and it lacks consensus on whether LCNEC should be treated like SCLC or NSCLC in clinical practice. According to the molecular genetic characteristics of LCNEC [11,12], it is divided into SCLC-Like（$TP53^{mut}Rb1^{mut}$）,NSCLC-Like（$TP53^{mut}Rb1^{mut}STK11^{mut}KRAS^{mut}$ $KEAP1^{mut}$）, and Carcinoid-Like（$MEN1^{mut}$）subtypes. George J et.al. [13] further defined $TP53^{mut}Rb1^{mut}ASCL1^{low}$ cases as LCNEC-Type II, $TP53^{mut}Rb1^{wt}ASCL1^{high}STK11^{mut}KRAS^{mut}$ cases as LCNEC-Type I. $TP53^{mut}Rb1^{wt}ASCL1^{low}STK11^{mut}KRAS^{mut}$ case has never been reported so far. In this study, we found ASCL1 was closely related to lymph node metastasis in LCNEC, suggesting ASCL1 may function as an oncogene regulating tumor migration and invasion. On the other hand, only in SCLC-Like LCNEC, low ASCL1 expression could predict better survival. This result indicates a synergistic effect between ASCL1 and TP53 and Rb1 might inhibit ASCL1 function as a tumor suppressor. Additionally, a bioinformatical analysis showed ASCL1 mRNA level was not related to prognosis in both LCNEC and SCLC-Like LCNEC

patients. Nonetheless, it is the proteins that serve as functional executors of cells. The classification at transcriptional level may have difficulty in guiding prediction of prognosis and precise treatment. Our study indicated IHC of ASCL1 may be more applicable in clinic.

Conclusion

Lung NETs, especially LCNEC, can be classified by a combined examination of p53, Rb1, STK11, ASCL1 and KRAS. In lung NETs, there may exist a WD-NET G3 subtype, which was previously diagnosed as LCNEC but showed carcinoid-like morphology, high proliferation activity, and is characterized by $Rb1^{+}$ $p53^{wt}$ in molecular phenotype. In lung LCNEC, $p53^{+/-}Rb1^{-}ASCL1^{low}$ may represent a novel phenotype with lower risk of lympha node metastasis and longer overall survival time. Above all, the investigation of different molecular types of lung NETs can not only help understand the pathogenesis or classify accurately, but is also advantage of evaluating the prognosis and selecting appropriate therapy.

List Of Abbreviations

AC Atypical carcinoid

Ascl1 Achaete-scute homolog 1

CgA Chromogranin A

DIPNECH Diffuse idiopathic pulmonary neuroendocrine cell hyperplasia

IARC International Agency for Research on Cancer

LCNEC Large cell neuroendocrine carcinoma

MEN1 Multiple endocrine neoplasia 1

NCAM/CD56 Neural cell adhesion membrane

NEN Neuroendocrine neoplasms

PanNEC Pancreatic neuroendocrine carcinoma

PD-NEC Poorly-differentiated neuroendocrine carcinoma

Rb1 Retinoblastoma protein 1

SCLC Small cell lung carcinoma

Stk11 Serine/threonine kinase 11

Syn Synaptophysin

TC Typical carcinoid

WD-NET Well-differentiated neuroendocrine tumor

WHO World Health Organization

Declarations

Ethics approval and consent to participate

This study was approved by the Ethical Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. All participants provided informed consent prior to this study.

Availability of data and material

We declare all data are available

Competing interests

The authors declare that they have no conflicts of interest.

Funding

This study was supported in part by grants from the National Natural Science Foundation of China (No. 81770263, 81974016 and 31271040).

Authors' contributions

Yuting Dong performed the experiments and analysed the results. Qian Zhang collected patient follow-up information and records. Guoping Wang, Zitian Huo and Yaqi Duan scored the IHC, Zitian Huo and Yaqi Duan conceived and designed the study. Zitian Huo wrote the paper. All authors have reviewed the manuscript.

Acknowledgements

None

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Figures

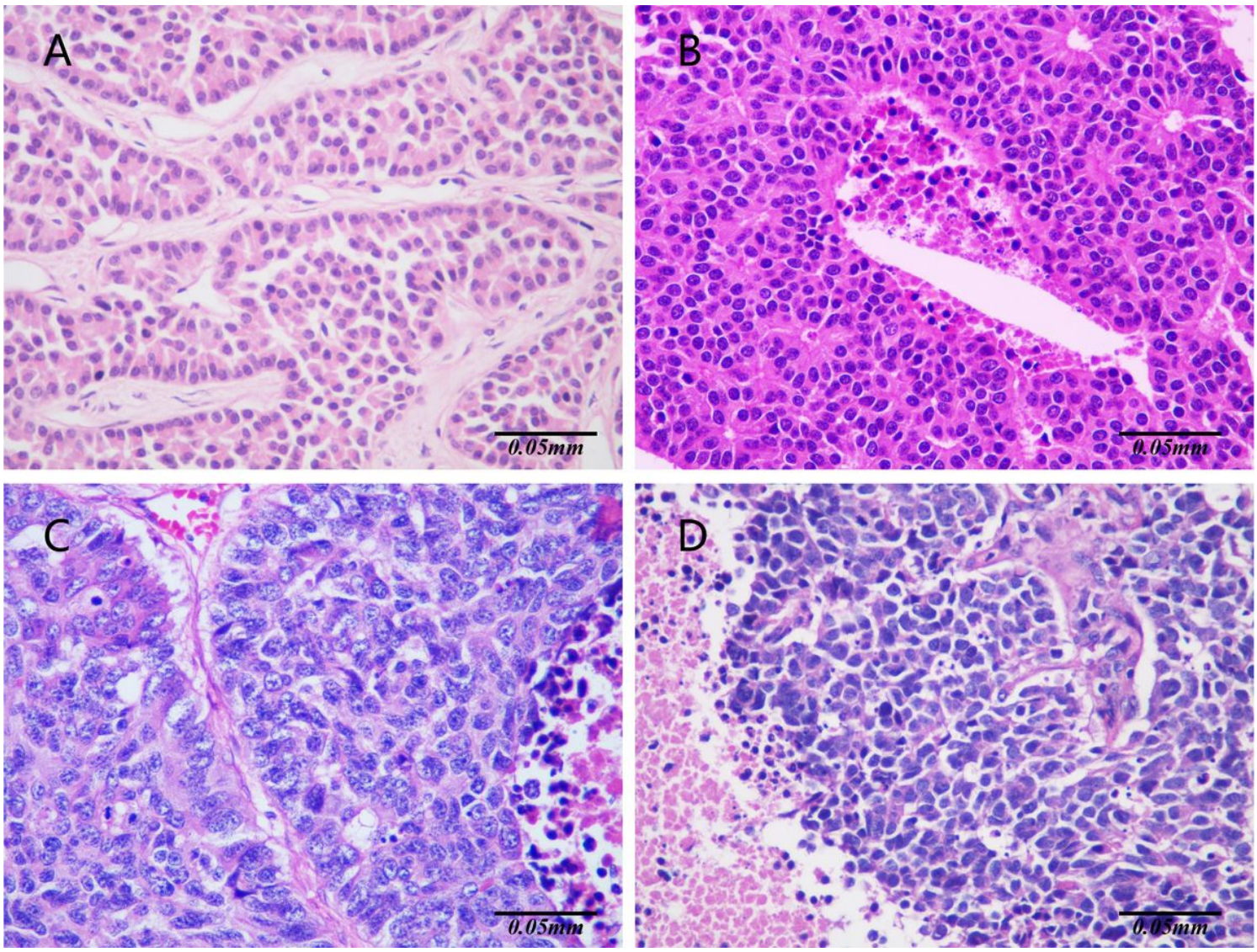

Figure 1

Representative HE image of AC (A), TC (B), LCNEC (C) and SCLC (D)

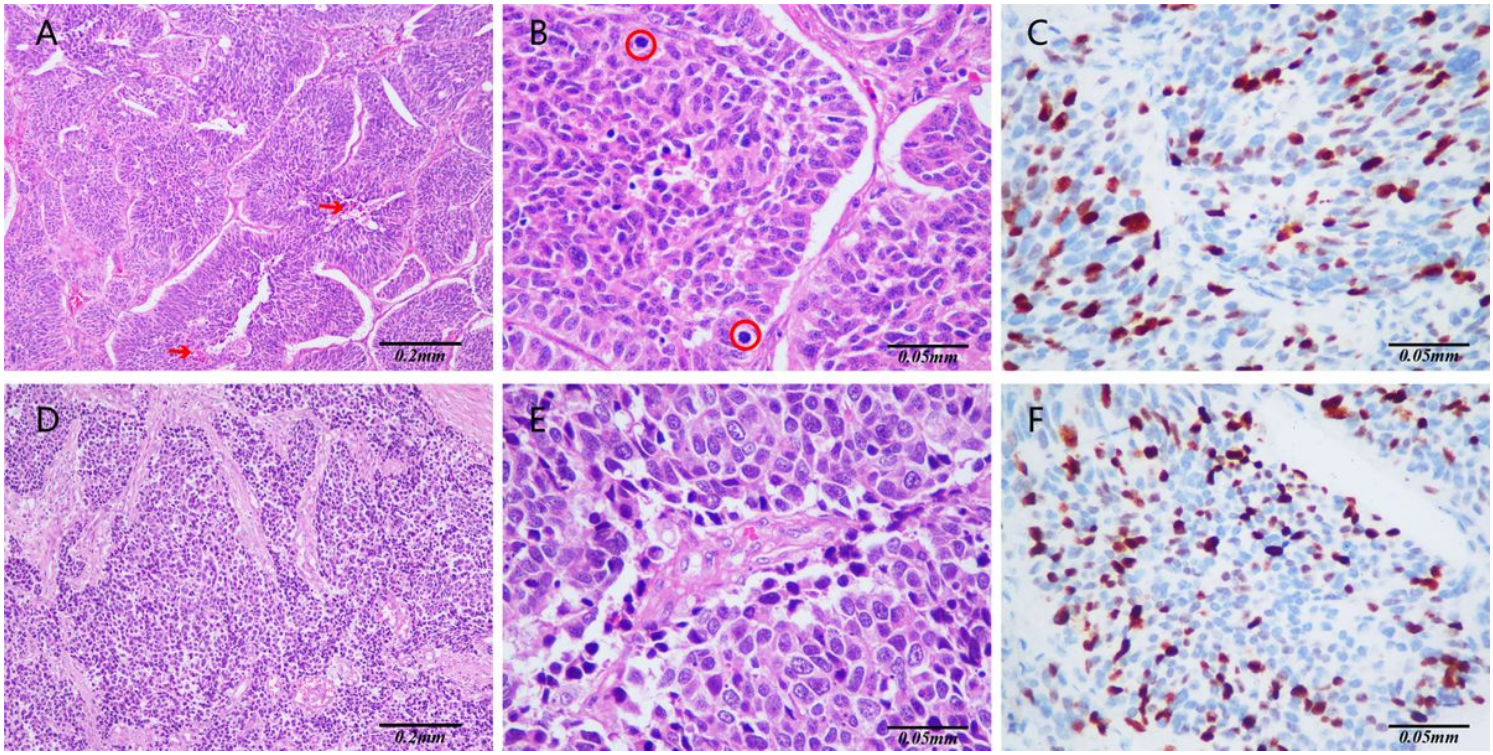

Figure 2

Pathological features of undefined lung neuroendocrine tumors. A. The tumor showed organoid arrangement, with focal necrosis (red arrow). (100x) B. Cell morphology is uniform, cytoplasm is slightly medium, mitosis is easy to see (red circle). (400x) C. Representative Ki67 LI for Carcinoid-Like undefined cases. (400x) D. Representative image of irregular infiltrative growth pattern and significant intratumoral fibrosis (100x) E. Organ like structure and vascular growth pattern of parenchyma. (400x) F. Representative Ki67 LI for undefined cases with morphology between WD-NET and PD-NEC (400x).

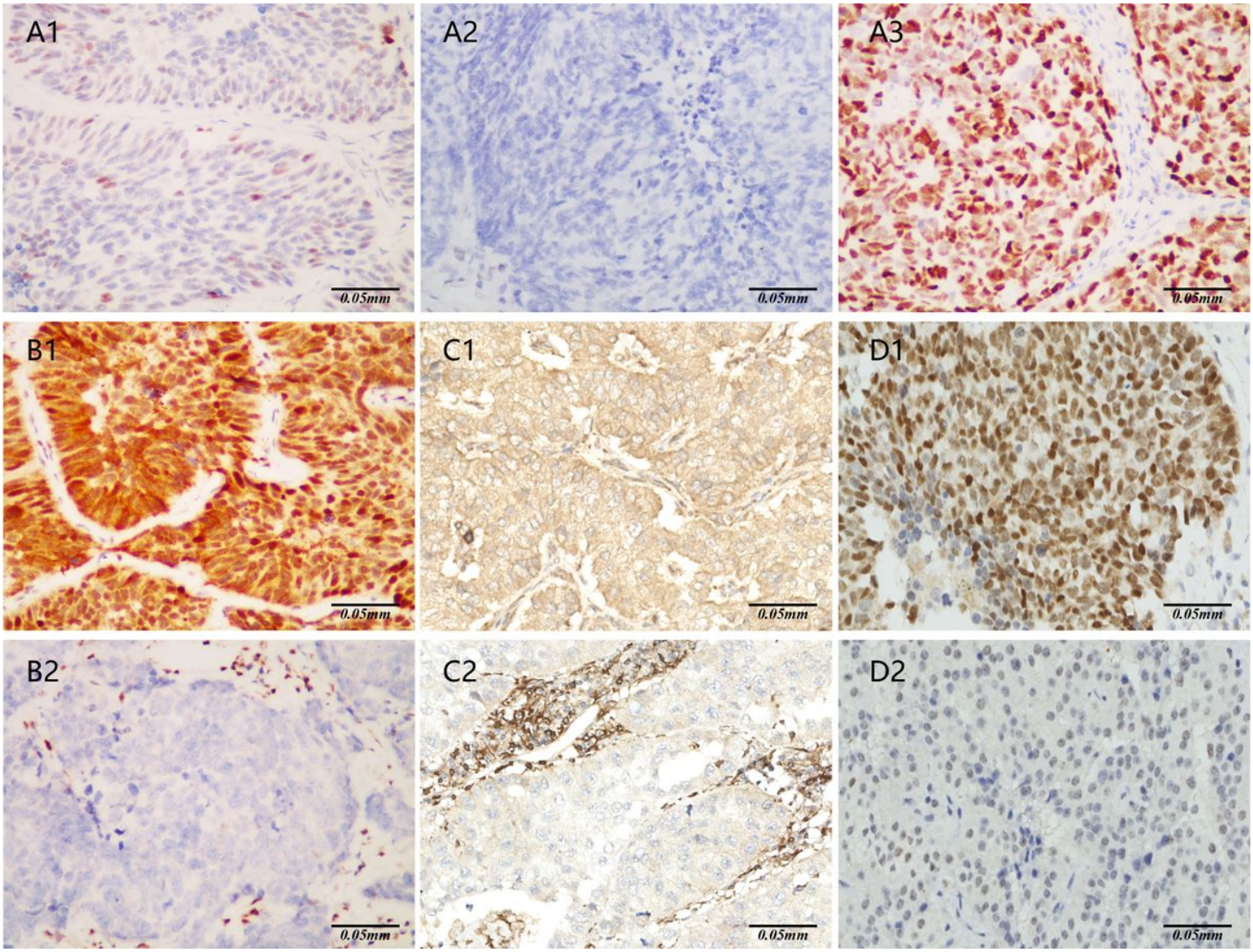

Figure 3

A1. The wild-type p53 protein was located in the nucleus, which could show the light or brownish yellow staining of scattered tumor cells (400x); A2. The tumor cells had no positive staining in the whole, suggesting that TP53 deletion mutation (400x); A3. The mutant TP53 showed diffuse and consistent brownish yellow staining (400x); B1. Rb1 protein was normally localized in the nucleus (400x); B2. when RB1 mutated, it was manifested as the loss of staining (400x); C1. STK11 protein was normally expressed in the cytoplasm (400x); C2. Representative image of absence of STK11 protein (400x); D1. Representative IHC image of high expression of ASCL1 (400x). D2. Representative IHC image of low expression of ASCL1 (400x).

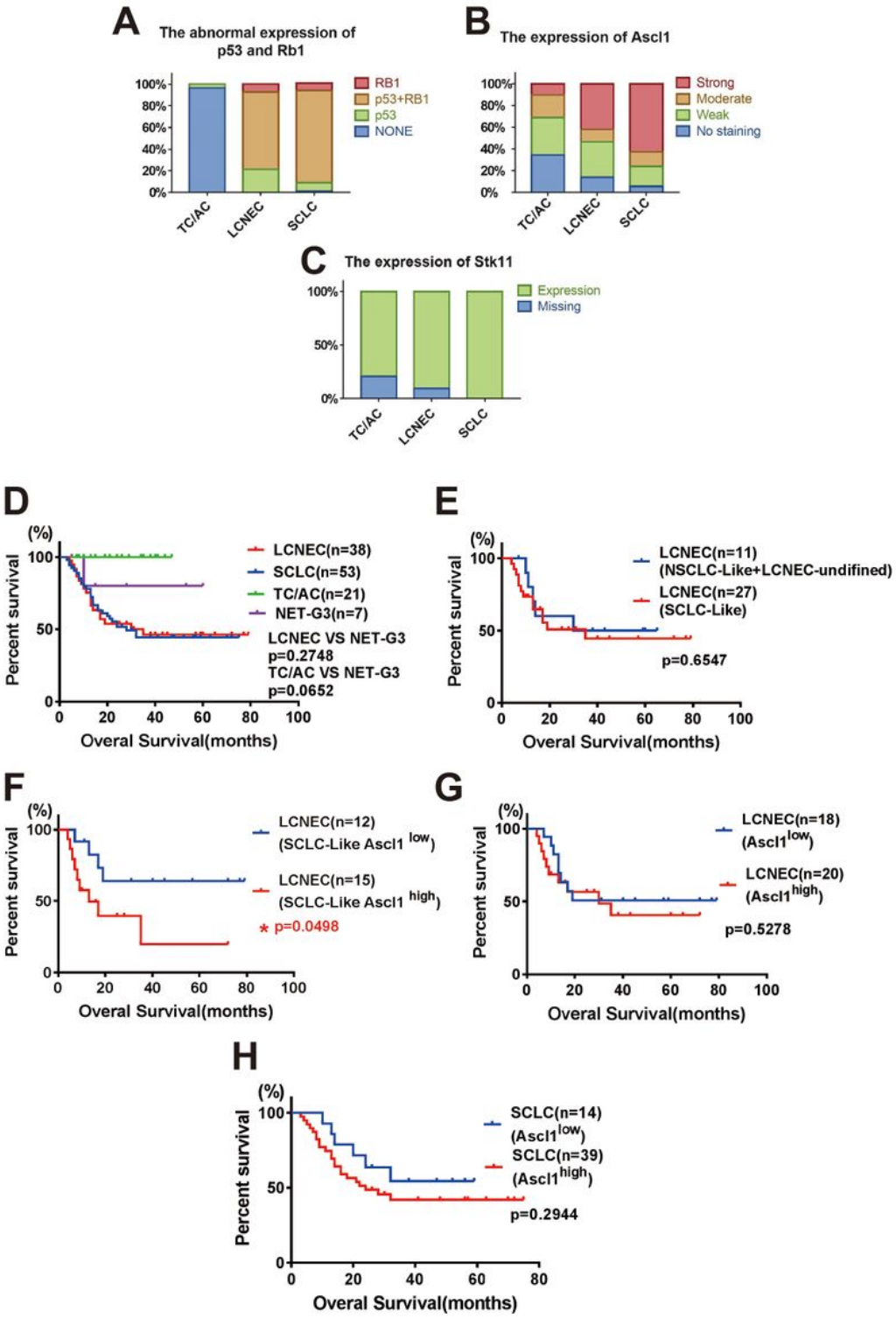

Figure 4

A. The p53 and Rb1 expression were significantly different inWD-NET (TC / AC) and PD-NEC (LCNEC / SCLC). B. The expression of ASCL1 protein in lung NENs. C. The expression of STK11 protein in lung NENs. D. Prognosis of high proliferative p53wtRb1+ neuroendocrine tumor, WD-NET (TC / AC) and PD-NEC (LCNEC / SCLC); E. Prognosis of SCLC-Like LCNEC, NSCLC-Like LCNEC and undefined LCNEC; F.

Prognosis of ASCL1 high and low subgroup in SCLC-Like LCNEC; G. Prognosis of ASCL1 high and low subgroup in all LCNEC; H. Prognosis of ASCL1 high and low subgroup in SCLC.

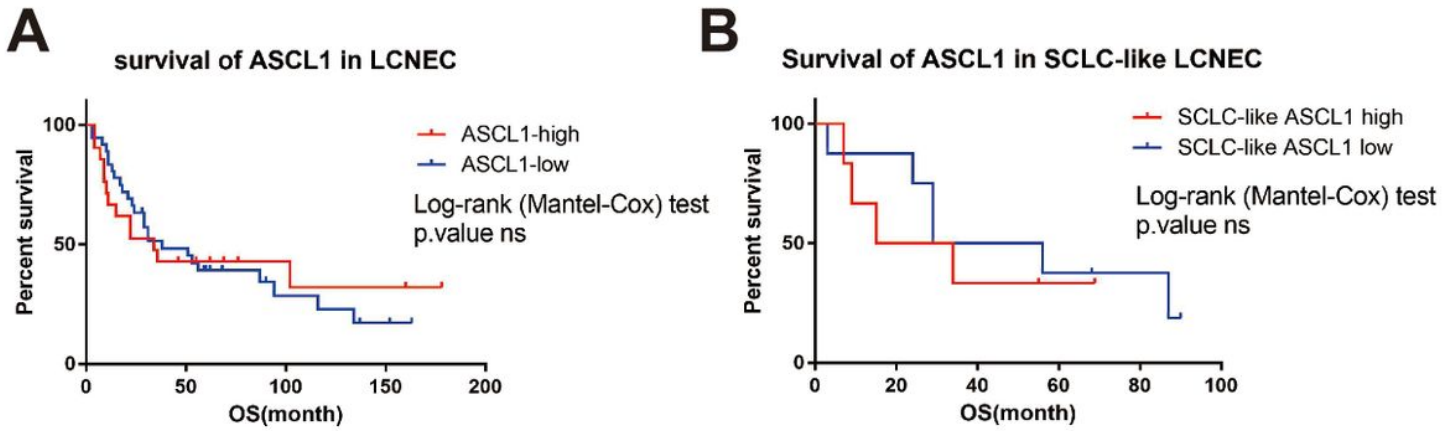

Figure 5

Bioinformatical analysis showed ASCL1 mRNA level was not related to prognosis in both LCNEC (A) and SCLC-Like LCNEC (B) patients.