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Original Research

Prognostic significance of YAP1 expression and its association with neuroendocrine markers in resected pulmonary large cell neuroendocrine carcinoma (LCNEC)

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ABSTRACT

Background: YAP1 (Yes-associated protein 1), an important effector of the Hippo pathway, acts as an oncogene and is overexpressed in various malignant tumors. However, the function and expression pattern of YAP1 in pulmonary large cell neuroendocrine carcinoma (LCNEC) have not been systematically established. This study aimed to explore the relationship between YAP1 expression and neuroendocrine differentiation markers and their prognostic significance in LCNEC.

Materials and methods: YAP1 protein and neuroendocrine markers (INSM1, NeuroD1 and DLL3) expression were examined by immunohistochemical (IHC) staining in 80 resected pulmonary LCNEC cases. The possible association between these markers and clinicopathological features was evaluated and survival analyses were performed.

Results: YAP1 was highly expressed in 25% LCNECs (20/80) , especially at a relatively higher T stage ($p = 0.015$). YAP1 expression was negatively correlated with INSM1 (χ^2=11.53, $p = 0.001$) and DLL3(χ^2=8.55, $p = 0.004$), but not with NeuroD1 ($p = 0.482$). For survival analyses, YAP1 expression was associated with worse disease-free survival (DFS) and overall survival (OS) (median DFS: 13 months vs. not reached (NR), $p = 0.0096$; median OS: not reached, NR vs. NR, $p = 0.038$), and was an unfavorable prognostic factor for DFS (HR:3.285; 95%CI: 1.526-7.071, $p = 0.002$) and OS (HR: 2.864, 95% CI: 0.932-8.796, $p = 0.066$).

Conclusions: YAP1 was found to be conversely correlated with neuroendocrine markers and a prognostic factor for worse survival in resected LCNEC patients, and mechanisms need to be further investigated.

Introduction

LCNEC is one of the lung neuroendocrine carcinomas, accounting for about 2%-3% of all lung cancers[1], which is closely related to smoking . In recent years, the incidence of LCNEC is slightly raising [2]. LCNEC patients had extremely poor outcomes with 5-year overall survival rates below 15–25% [3] and most of recurrences occurred within the first 3 years after surgery [4,5]. Clinically and histologically, LCNEC is deemed as a combination of non-small cell lung cancer (NSCLC) for a similarity of morphological features and small cell lung cancer for neuroendocrine expression [6]. According to the 5th World Health Organization-Thoracic tumors, LCNEC and SCLC are classified as high-grade neuroendocrine tumor with high invasiveness and poor prognosis [7].

YAP1 (yes-associated protein 1), a main downstream effector of the Hippo pathway, is a multifunctional intracellular connexin and transcriptional coactivator, which plays a significant role in signal transduction and gene transcriptional regulation in normal cells by regulating cell growth, cell apoptosis as well as organ growth [8,9]. Strong expression of *YAP1* has frequently been observed and recognized as a

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robust oncogene closely linked to the progression of several malignant tumors [10,11]. Ito et al. showed that the loss of *YAP1* has potential as a clinical marker for predicting up-regulating neuroendocrine features and lower chemo-sensitivity for high grade neuroendocrine carcinoma of the lung [12]. Still, *YAP1* has been reported as one of the key transcription factors for molecular subtypes [13], although it is not validated in studies like Gay's [14]. Our previous study found that expression of *YAP1* was significantly higher in combined SCLC than that of pure SCLC as well as an unfavorable prognostic factor for combined SCLC [15]. Genomic data indicated that LCENC could be divided into SCLC-like profile (*RB1/TP53* inactivation, *MYCL* amplification) and NSCLC-like profile (alteration in *STK11, KEAP1, KRAS,* and other *RAS* pathway genes) [16–18]. However, the transcriptome data showed contradictory results that NSCLC-like genomic subset was characterized by a typical feature of SCLC with *ASCL1*-high/*DLL3*-high/*Notch*-low, while SCLC-like genomic subset was associated with *ASCL1*-low/*DLL3*-low/-*Notch*-high [16]. Thus, we deemed that LCNEC as a unique entity which may distinct from either SCLC or NSCLC, in which *YAP1*'s role has been well investigated both in cell lines and pathological samples [12]. In the current study, we focused on LCNEC for exploring of YAP1 protein associated with neuroendocrine markers as well as prognosis by extracting archival resected tumors.

Methods

Patient identification and histologic reassessment

Eighty Archived surgical samples between December 2011 and March 2017 diagnosed as LCNEC in the department of pathology, Cancer Hospital, Chinese Academy of Medical Science with complete clinical and follow-up data were selected. The follow-up data was collected based on clinical outpatient records or telephone interview records. Disease-free survival (DFS) was defined as the time from the start of surgery to the observation of tumor recurrence or distant metastasis. Overall survival (OS) was defined as the time from the date of surgery to death or last follow-up (in the absence of death). The primary endpoint of this study was OS and the secondary endpoint was DFS. Tumor sections of all patients were subsequently reviewed by three pulmonary pathologists (Lin Yang, Li Liu and Xujie Sun) according to the 2021 WHO classification criteria of lung tumors. For the diagnosis of combined-LCNEC, there was at least 10% of large cell neuroendocrine carcinoma components to define combined LCNEC-SCLC. Since adenocarcinoma and squamous cell carcinoma were easy to identify, there was no percentage requirement. In addition to pathological identification, lymph node metastasis, pleural invasion and lymph-vascular invasion were recorded using hematoxylin and eosin (H&E) slide. Pathological staging was based on 8th edition of American Joint Committee on Cancer (AJCC/UICC).

The study was approved by the Ethics Committee of National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College (approval no.20/234-2430). Individual consent for this retrospective analysis was waived. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Tissue microarray and immunohistochemistry staining and evaluation

Tissue microarray (TAM) blocks were constructed from representative paraffin tissues selected by dedicated pathologist (Lin Yang), with diameter of 1.5mm (two cores/paraffin tissue). Consecutive tumor sections of 3-5 um were cut from tissue microarray to staining immunohistochemistry (IHC) and H&E. A rabbit monoclonal anti-YAP1 antibody (Abcam, Cat# ab52771, dilution 1:100), a rabbit monoclonal anti-DLL3 (Cell Signaling Technology, Cat# 71804, dilution 1:100) [19], a mouse monoclonal anti-NeuroD1 antibody (Abcam, Cat# ab60704, dilution 1:200) [20] and a mouse monoclonal anti-INSM1 antibody (Santa Cruz Biotechnology, Cat# sc-271408, dilution 1:500) were used for immunostaining. Positive control sections for YAP1, INSM1, NeuroD1 and DLL3 were from normal breast tissue, normal pancreatic tissue, ovarian tissue and glioma tissue, respectively. The IHC staining of YAP1, INSM1, NeuroD1 and DLL3 was performed on the fully automatic Roche immunohistochemical instruments (Roche Diagnostics, Shanghai, China) according to the manufacturer's instructions.

YAP1, INSM1 and NeuroD1 staining were located in the nucleus and cytoplasm, while DLL3 staining was located in the cell membrane (Fig. 1). H-score was applied to semi-quantitative expression intensity of these markers, which combined staining intensity (ranged 0-3) and percentage of positive cells. We then translated the continuous H-score into the 4 gradations: 0 (H-score ranged 0-9), 1+ (H-score ranged 10-49), 2+ (H-score ranged 50-149) and 3+ (H-score ranged 150-300). The expression of YAP1, INSM1, NeuroD1 and DLL3 was divided into low expression group (scores: 0 and 1+) and high expression group (scores: 2+ and 3+).

Statistical analysis

Descriptive analysis was used to describe the clinicopathological features. For continuous data, the mean ± standard deviation was calculated. For categorical data, the proportion was analyzed, and Chi-square test or Fisher's exact test was used to analyze the difference in categorical variables. The Kaplan-Meier curves were plotted for DFS and OS and compared by log-rank test with 95% confidence interval (CI). For multivariate survival analysis, the Cox proportional hazards regression model was applied to evaluate the independent prognostic factors. All tests were two-sided and p values < 0.05 was considered statistically significant. IBM SPSS statistic 25.0 and R software (version 4.2.0) were used in statistical analyses.

Results

Clinicopathological characteristics of selected patients

Totally, 80 patients with resected pulmonary LCNEC were retrospectively reviewed. The median age was 62.5 years (from 43 to 79 years) with gender ratio of 7.8:1 (Male/Female). As for pathological staging, I, II and III stage accounted for 35%, 20%, 25% respectively. As for histology subtypes, half of the patients were pure-LCNECs (P-LCNEC), and the other half were combined-LCNECs (C-LCNEC) which included 18 (22.5%) cases with combined adenocarcinoma, 14 (17.5%) with small cell lung cancer, 3 (3.75%) with squamous cell carcinoma, 3 (3.75%) with adenosquamous carcinoma, 2(2.5%) simultaneously with small cell lung cancer and adenocarcinoma. Pulmonary resection methods included: lobectomy in 71 cases, pneumonectomy in 3 cases, wedge resection in 3 cases, sleeve lobectomy in 1 case and two cases were unclear. Other detailed clinicopathological information (including TNM staging, treatment methods, pleural invasion, lymph-vascular invasion) was listed in Table 1. Until the end of follow-up time (December 9, 2019), 41 patients had recurrence and 19 patients deceased, 18 were lost. The median DFS was 41 months and the median OS was not reached. The estimated 5-year DFS and OS were 43.5% and 75%, respectively. The median follow-up time was 44 months, ranging from 0 to 95 months.

Expression of YAP1 and neuroendocrine-related markers

Firstly, all cases were classified as two groups (low expression group / high expression group) based on the score of YAP1 in large cell neuroendocrine component either in P-LCNEC or in C-LCNEC. Among 80 cases, 60 patients (75%) were in low expression groups and 20 patients (25%) were in high expression group. Then, we analyzed the association of YAP1 expression with clinicopathological features in LCENC. As shown in Table 1, high YAP1 expression was statistically

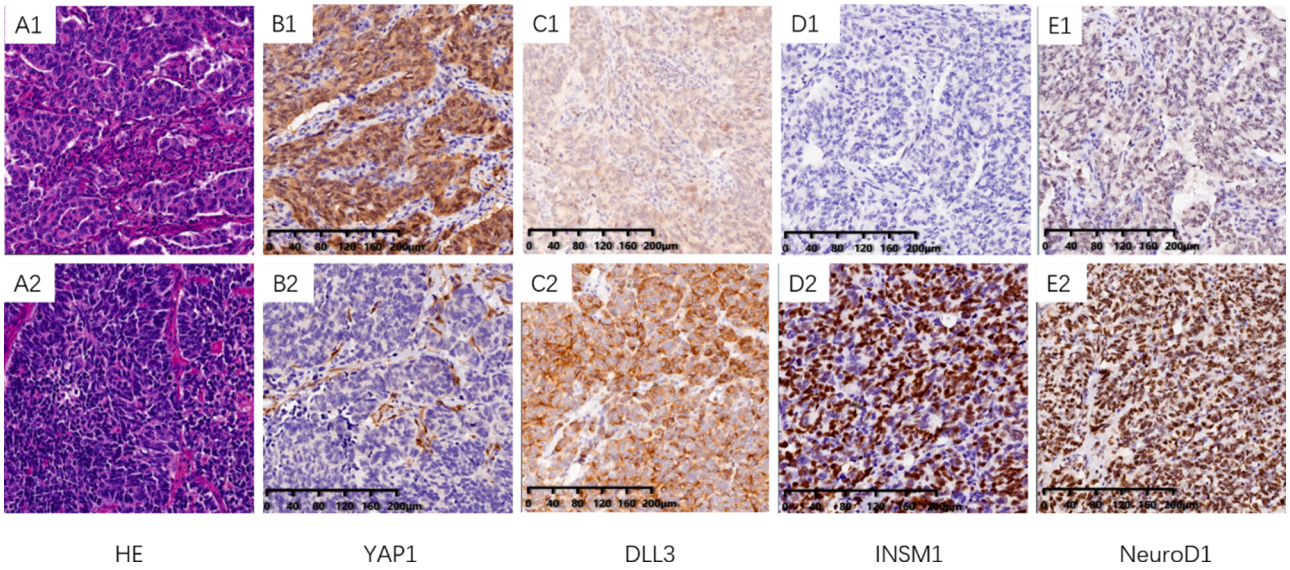

Fig.1. Representative sections of LCNEC stained with hematoxylin and eosin (H&E) and immunostained with YAP1, INSM1, NeuroD1 and DLL3 expression level. The five images in each line correspond to one patient. High YAP1 expression was associated with low expression of INSM1 and DLL3 (B1, C1, D1), vice versa (B2, C2, D2)

Table 1
Clinicopathological features in LCNEC patients and the correlation with YAP1 expression.

Clinicopathological features	Total No.(%) (N=80)	**YAP1**(%)		*p* value
		Low (n=60)	High (n=20)	
Age				
≤55 years	16(20)	9(15)	7(35)	0.102
>55years	64(80)	51(85)	13(65)	
Gender				
male	71(88.8)	52(86.7)	19(95)	0.437
female	9(11.2)	8(13.3)	1(5)	
Pathological TNM stage				
Early stage (I-II)	55(68.8)	43(71.7)	12(60)	0.406
Advanced stage (III)	25(31.2)	17(28.3)	8(40)	
pT stage				
T1	34(42.5)	28(46.7)	6(30.0)	**0.015** *
T2	27(33.75)	22(36.7)	5(25)	
T3	14(17.5)	8(13.3)	6(30.0)	
T4	5(6.25)	2(3.3)	3(15.0)	
pN stage				
N0	47(68.8)	36(60)	11(55)	0.800
N1	10(12.5)	8(13.3)	2(10)	
N2	21(26.2)	15(25)	6(30)	
N3	2(2.5)	1(1.7)	1(5)	
Histologic subtype				
Pure LCNEC	40(50)	30(50)	10(50)	1.000
Combined LCNEC	40(50)	30(50)	10(50)	
Pleural invasion				
Yes	44(55)	35(58.3)	9(45)	0.437
No	36(45)	25(41.7)	11(55)	
Lymph-vascular invasion				
Yes	20(25)	17(28.3)	3(15)	0.233
No	60(75)	43(71.7)	17(85)	
Treatment				
Surgery	30(37.5)	23(38.3)	7(35)	0.869
Surgery+chemotherapy	37(46.25)	27(45)	10(5)	
Surgery+radio-chemotherapy	10(12.5)	7(11.7)	3(15)	
Others/unknow#	3(3.75)	3(5)	0(0)	

* statistically significant.
# others/unknow include chemotherapy +surgery with/without chemotherapy, surgery + radiotherapy and surgery + unknow.

associated with higher pT stages($p = 0.015$). In the tissue array, there were five cases with obvious adenocarcinoma components, and we found that the expression of YAP1 in the adenocarcinoma component was significantly stronger than that in LCNEC component (Figure S1). Interestingly, the proportion of P-LCNEC with high YAP1 expression was comparable to that of C-LCNEC, suggesting no relationship between YAP1 expression and histological subtype. Also, there was no significant association between YAP1 expression and other clinicopathological variables.

According to neuroendocrine markers (INSM1, NeuroD1 and DLL3), we also stratified these cases into two groups (low expression group / high expression group) as previous criteria and investigated their relationships with clinicopathological characteristics, as shown in Table 2. The percentage of patients with age above 55 in INSM1 high expression group was higher than that in INSM1 low expression group (94.1% vs 69.6%, $p = 0.01$). And low INSM1 expression was significantly associated with higher pT stages ($p = 0.031$). Besides, no significant difference was observed.

Furthermore, the correlations between YAP1 and neuroendocrine-related markers we analyzed were shown in Table 3. High YAP1 expression was significantly associated with low expression of INSM1 ($p = 0.001$) and DLL3 ($p = 0.004$). However, the relationship between YAP1 and NeuroD1 was not significant.

Prognostic significance of YAP1 expression

According to Kaplan-Meier plotter analysis, patients with high expression of YAP1 had a shorter DFS and OS compared with those with low expression of YAP1 (median DFS: 13 months vs. not reached (NR), $p = 0.0096$; median OS: not reached, NR vs. NR, $p = 0.038$; Fig. 2). Univariate analysis revealed that DFS was associated with YAP1 expression (HR: 2.255, 95% CI: 1.189-4.277, $p = 0.013$) and lymph nodes metastasis (HR: 2.266, 95% CI: 1.221-4.207, $p = 0.01$); and OS was associated with YAP1 expression (HR: 2.634, 95% CI: 1.015–6.836, $p = 0.047$) (Fig. 3). In the multivariate analysis, high expression of YAP1 was an independent prognostic factor for poor DFS (HR: 3.285, 95% CI: 1.526-7.071, $p = 0.002$) and had a correlation with poor OS (HR: 2.864, 95% CI: 0.932-8.796, $p = 0.066$) (Fig. 4). Different from YAP1, INSM1, NeuroD1 and DLL3 were not statistically significant for survival (not show).

Table 2
Correlation between NE markers and clinicopathological features.

Clinicopathological features	**INSM1**(%)		P value	**DLL3**(%)		P value	**NeuroD1**(%)		*p* value
	Low (n=46)	High (n=34)		Low (n=55)	High (n=25)		Low (n=12)	High (n=68)	
Age									
≤55 years	14 (30.4)	2 (5.9)	**0.01***	11 (20)	5 (20)	1	4 (33.3)	12 (17.6)	0.245
>55years	32 (69.6)	32 (94.1)		44 (80)	20 (80)		8 (66.7)	56 (82.4)	
Gender									
male	42 (91.3)	29 (85.3)	0.484	49 (89.1)	22 (88)	1	11 (91.7)	60 (88.2)	1
female	4 (8.7)	5 (14.7)		6 (10.9)	3 (12)		1 (8.3)	8 (11.8)	
Pathological stage									
Early stage (I-II)	29 (63.0)	26 (76.5)	0.23	36 (65.5)	19 (76)	0.439	8 (66.7)	47 (69.1)	1
Advanced stage (III)	17 (37.0)	8 (23.5)		19 (34.5)	6 (24)		4 (33.3)	21 (30.9)	
pT stage									
T1	15 (32.6)	19 (55.9)	**0.031***	20 (36.4)	14 (56)	0.147	4 (33.3)	30 (44.1)	0.727
T2	15 (32.6)	12 (35.3)		18 (32.7)	9 (36)		4 (33.3)	23 (33.8)	
T3	11 (23.9)	3 (8.8)		12 (21.8)	2 (8)		3 (25)	11 (16.2)	
T4	5 (10.9)	0		5 (9.1)	0		1 (8.3)	4 (5.9)	
pN stage									
N0	27 (58.7)	20 (58.8)	0.576	32 (58.2)	15 (60)	0.623	7 (58.3)	40 (58.8)	0.931
N1	4 (8.7)	6 (17.6)		6 (10.9)	4 (16)		1 (8.3)	9 (13.2)	
N2	14 (30.4)	7 (20.6)		16 (29.1)	5 (20)		4 (33.3)	17 (25)	
N3	1 (2.2)	1 (2.9)		1 (1.8)	1 (4)		0	2 (2.9)	
Histologic subtype									
Pure LCNEC	21 (45.7)	19 (55.9)	0.498	27 (49.1)	13 (52)	1	4 (33.3)	36 (52.9)	0.348
Combined LCNEC	25 (54.3)	15 (44.1)		28 (50.9)	12 (48)		8 (66.7)	32 (47.1)	
Pleural invasion									
Yes	27 (58.7)	17 (50)	0.499	26 (47.3)	18 (72)	0.053	8 (66.7)	36 (52.9)	0.532
No	19 (41.3)	17 (50)		29 (52.7)	7 (28)		4 (33.3)	32 (47.1)	
Lymph-vascular invasion									
Yes	11 (23.9)	9 (26.5)	0.8	14 (25.5)	6 (24)	1	3 (25)	17 (25)	1
No	35 (76.1)	25 (73.5)		41 (74.5)	19 (76)		9 (75)	51 (75)	
Treatment									
Surgery	17 (37.0)	13 (38.2)	0.123	22 (40)	8 (32)	0.088	4 (33.3)	26 (38.2)	0.945
Surgery+chemotherapy	21 (45.6)	16 (47.1)		25 (45.5)	12 (48)		6 (50)	31 (45.6)	
Surgery+radio-chemotherapy	8 (17.4)	2 (5.9)		8 (14.5)	2 (8)		2 (16.7)	8 (11.8)	
Others/unknow[#]	0	3 (8.8)		0	3 (12)		0	3 (4.4)	

[*] statistically significant.
[#] others/unknow include chemotherapy +surgery with/without chemotherapy, surgery + radiotherapy and surgery + unknow.

Table 3
Correlation between YAP1 expression and neuroendocrine biomarkers.

Biomarker	YAP1(%)		*p*-value[a]	R [b]
	Low (n=60)	High (n=20)		
INSM1				
Low	28(46.7)	18(90)	**0.001***	-0.49
High	32(53.3)	2(10)		
DLL3				
Low	36(60)	19(95)	**0.004***	-0.47
High	24(40)	1(5)		
NeuroD1				
Low	8(13.3)	4(20)	0.482	-0.065
High	52(86.7)	16(80)		

[a] Chi-squared Test or Fisher's Exact Test.
[b] Pearson correlation coefficient.
[*] statistically significant.

Discussion

In this study, we explored the expression profile of YAP1 in pulmonary LCNEC and its impact on survival and neuroendocrine (NE) markers. The results of this study showed that YAP1 expression was negatively associated with expression of NE-related markers and acted as a predictor for unfavorable prognosis in LCNEC, especially for DFS.

Because of the increasing incidence of large cell neuroendocrine carcinoma and its aggressiveness plus poor prognosis, it has attached more attention than before. *YAP1* is an important nuclear effector of the Hippo signaling pathway, which is critical for regulating cell proliferation, apoptosis, stem/progenitor cell expansion and organ growth. *YAP1* plays an important role in the occurrence and development in various tumors as cutaneous squamous cell carcinoma, hepatocellular carcinoma and medulloblastoma etc. [9,21–23]. Based on distinct expression of *YAP* and *YAP*-responsive adhesion regulators, Pearson. et al has proposed a binary classification of cancer: YAP^{on} or YAP^{off}. For YAP^{on} tumors, the increased expression and activity of *YAP* will induce proliferative genes and promote malignant transformation of cells. On the contrary, *YAP* drove both adhesion and cytostasis in YAP^{off} cancers [24]. Therefore, *YAP1* had tumor- promoting and tumor- suppressive effects on YAP^{on} and YAP^{off} cancers respectively. SCLC belonged to YAP^{off} class while NSCLC was YAP^{on} cancer [24]. But there was no research on which category LCNEC falls into. Comprehensive next generation sequencing and transcriptome analysis indicated contradictory reciprocal subclassifications of LCNEC: one with mutations similar to SCLC but has a typical expression profile of NSCLC, the other harbors mutations that can be found in NSCLC but has expression characteristics like SCLC [16–18]. So, LCNEC is unique and heterogeneous and the role of YAP1 in LCENC is unknown and worth exploring. Moreover, in Kawai et al.'s research, gene clustering analysis was performed on 51 SCLC cell lines which found that the expression of neuroendocrine marker *INSM1* in the cells enriched *YAP1* gene was lower compared to the cells enriched *ASCL1, NEUROD1*, or *POU2F3* genes. Also, the results of RNA sequence analysis of 17 SCLC and NSCLC cell lines showed that it can be divided into two categories: *YAP* high group with NE-marker negative and *YAP1* low group with NE marker positive [25]. These all suggested the potential relationship between YAP1 and NE differentiation. Therefore, we selected 80 LCNEC to explore the relationship between YAP1 and NE differentiation and its prognostic significance and found that YAP1 was an independent prognosis predictor for worse DFS in

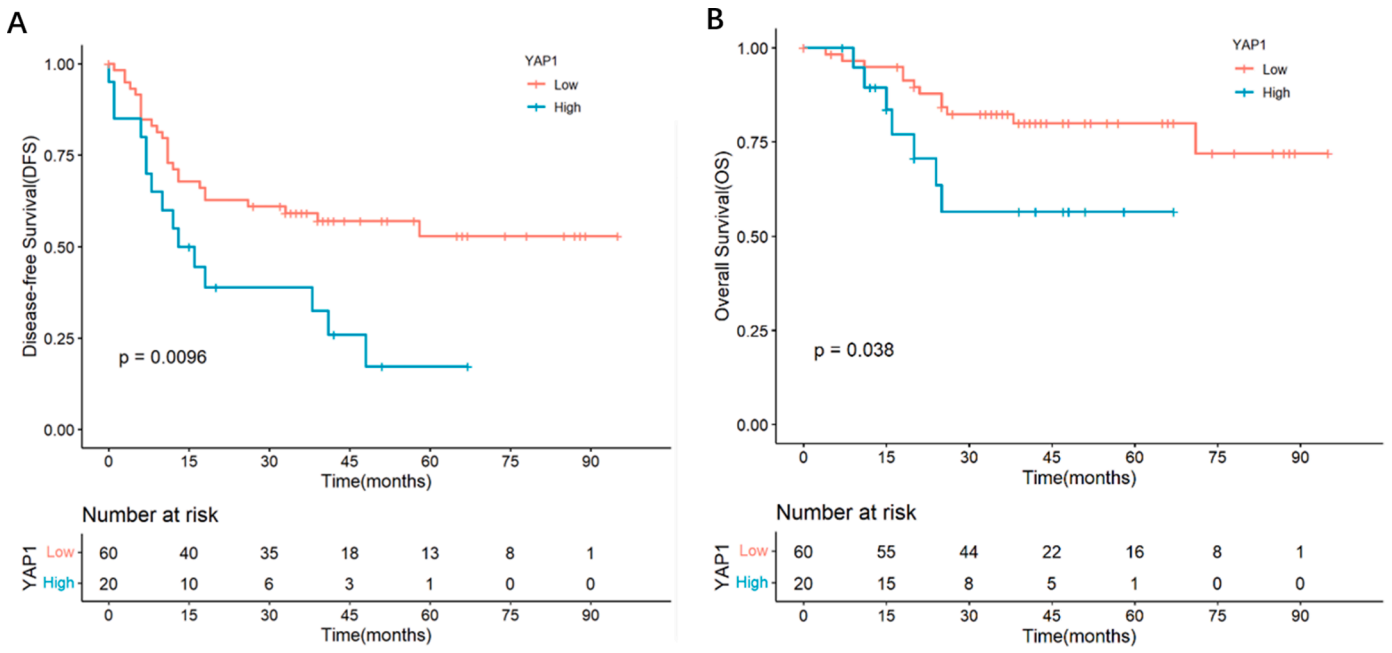

Fig. 2. Disease free survival (DFS) (A) and overall survival (OS) (B) of YAP1 high expression group are significant worse than that of YAP1 low expression group ($p = 0.0096$, $p = 0.038$, respectively).

LCNEC and negatively corelated with NE biomarkers which indicated LCNEC may be YAP^{on} cancer and YAP1 is resistant to NE differentiation. But the function of YAP1 and its mechanism requires further investigation and confirmation.

Neuroendocrine differentiation, an essential feature of neuroendocrine tumors, has been shown to be an important factor in tumor progression and prognosis [26]. In SCLC, researchers have found that the YAP1 subtype displayed low expression of neuroendocrine markers [13,27], and the loss of YAP1 correlated with the expression of neuroendocrine markers [12]. Yang et al. found that the miR-375/YAP axis is an important mediator of neuroendocrine differentiation in lung cancer [28]. Besides, studies have shown that the expression of the neuroendocrine marker *RAB3A* can be induced by knocking down *YAP1*[12]. These all suggested that YAP1 is involved in neuroendocrine differentiation of lung tumors. But for LCNEC, a high-grade neuroendocrine tumor of the lung, similar studies are rare. Insulinoma-associated protein 1 (*INSM1*), as a zinc finger transcriptional factor can involve in neuroendocrine differentiation [29] and as a promising marker of neuroendocrine lung neoplasms [30]. Delta-like ligand 3 (*DLL3*) expresses in a variety of neuroendocrine tumors, such as melanoma, small cell bladder cancer and neuroendocrine lung tumors [16,31,32], can participate in tumor neuroendocrine differentiation by inhibiting NOTCH pathway [33,34]. Moreover, Neurogenic differentiation factor 1 (*NEUROD1*) also plays an important role in the regulation of neuroendocrine differentiation [35]. Metovic et al. applied unsupervised gene cluster analysis on 48 LCNEC, found that LCNECs can be divided into two clusters according to the expression of neuroendocrine differentiation markers: one with overexpression of *ASCL1, DLL3 and NeuroD1* and the other with overexpression of *YAP1, POU2F3* and *Notch1*, but they both with high expression of *INSM1*. Besides, analysis on cases with both mixed neuroendocrine and non-neuroendocrine components displayed upregulation of *ASCL1, DLL3, INSM1* and *NeuroD1* in the neuroendocrine component [35]. Therefore, INSM1, DLL3 and NEUROD1 were selected as neuroendocrine markers in our study. We found that YAP1 was high expression in 25% LCNEC (20/80) and negatively correlated with neuroendocrine markers (INSM1, DLL3) expression. In terms of immunohistochemistry, our results are complementary and mutually validated with that of Kawai et al., in which examined the staining patterns of YAP1 and NE markers in 30 LCNEC cases and showed that YAP1-positive cases were weakly positive for ASCL1[25]. As for the cases, which contained mixture of YAP1-positive and YAP1-negative cells, they found YAP1-positive cell components were negative for ASCL1, and YAP1-negative cell components were positive for ASCL1[25]. We also found that the cells with high YAP1 expression were lower expression of INSM1 and DLL3. Above all, we speculated that the expression of YAP1 can reflect the neuroendocrine differentiation of LCNEC to a certain extent.

The expression level of YAP1 not only plays a role in neuroendocrine differentiation, but also has important implication for prognosis in LCNEC. In our research, high expression level of YAP1 was associated with advanced T stage and worse prognosis. Although there was a brief crossover at the beginning of the OS survival curve, its reason may be the effect of YAP1 on OS would be interfered by other factors, which has been confirmed that YAP1 was not an independent prognostic factor for OS in multivariate analysis. So not exactly the same as SCLC belonging to YAP^{off} cancer, whose progression is inhibited by YAP1, YAP1 expression is an unfavorable prognostic factor for LCNEC. M. et al. found that in non-small cell lung cancer with NE differentiation, patients with a high proportion of neuroendocrine tumor cells were responding better to paclitaxel-cisplatin treatment and were clinically less aggressive.[36] Therefore, we indicated that YAP1 may affect prognosis by participating in NE dedifferentiation as one mechanism. On the other hand, Hippo pathway is an important regulatory network for the occurrence and progression of tumors. YAP, the main effector molecule of the Hippo pathway, has been shown to be involved in actin dynamics and cell motility in recent years, which suggested that YAP1 may be related to epithelial-mesenchymal transition (EMT) and tumor metastasis [37,38]. In breast cancer, Shen et al. revealed the expression level of YAP was positively correlated with cell migration and invasion ability [39]. Pearson et al. found YAP1 is correlated with PC1+ genes which are adhesion and extracellular matrix (ECM) components [24]. And YAP1 can activate a transcriptional program involved in regulating the epithelial-mesenchymal transition (EMT) in a human KRAS-dependent colon cancer cell line [40]. So, the effect on tumor adhesion behavior may be another mechanism by which YAP1 affects prognosis. Additionally, YAP1 has a certain relationship with tumor sensitivity and drug resistance.

Previous study has shown that YAP1 positive cases have better

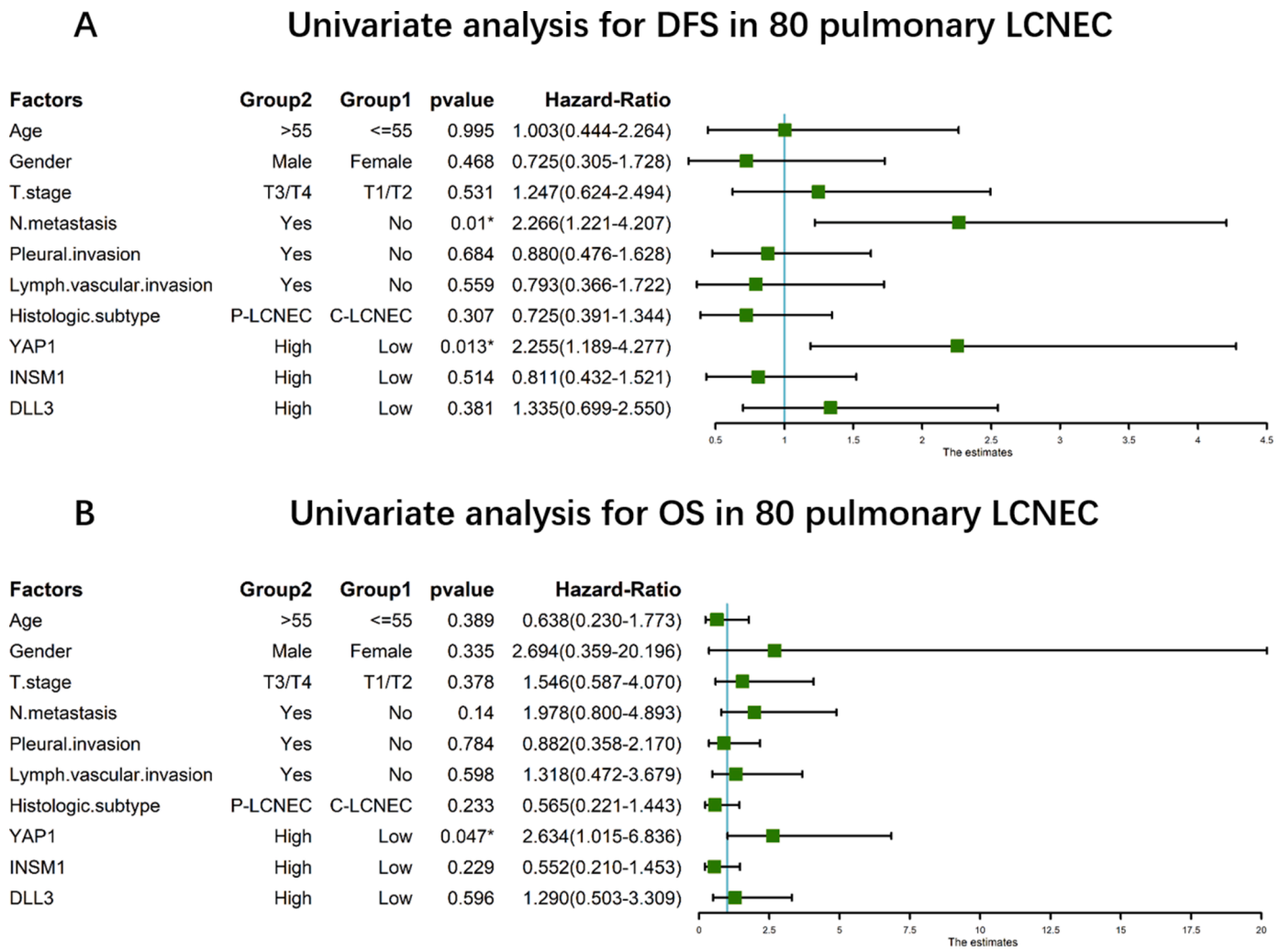

Fig. 3. Univariate analysis of disease-free survival (DFS) (A) and overall survival (OS) (B) in 80 pulmonary LCNEC by COX regression model. Group 1 was the reference group. YAP1 expression (HR: 2.255, 95% CI: 1.189-4.277, $p = 0.013$) and lymph nodes metastases (HR: 2.266, 95% CI: 1.221-4.207, $p = 0.01$) were significantly associated with DFS(A). And YAP1 expression (HR: 2.634, 95% CI: 1.015-6.836, $p = 0.047$) was significantly associated with OS(B)
*, statistically significant

chemosensitivity than YAP1 negative cases and loss of YAP1 has potential as a clinical marker for predicting chemosensitivity in high-grade neuroendocrine tumor [12]. However, in our study, there was no difference observed in overall survival between low expression group and high expression group of patients with adjuvant chemotherapy (not show). On the one hand, the reason lied in the retrospective study, from which the chemotherapy regimens of our patients fell into two rough categories: SCLC chemotherapy regimens and NSCLC chemotherapy regimens with lacking details of adjuvant chemotherapy. Moreover, for C-LCNEC, there was a few research on it and its treatment remains controversial [41,42]. The half of cases in our research was C-LCNEC, which was highly histopathological heterogeneous. Which also contributed to that there was no significant difference in survival when grouping based on treatment. Recent studies have shown that YAP1 signaling pathway was consistently related with occurrence of intrinsic or acquired resistance to chemotherapy in several tumors, which solidified by *in vitro* experiments [43], for silencing of *YAP1* was sufficient to restore the sensitivity of resistant cancer cells to chemotherapy [44]. In tumor cells of liver cancer patients treated with sorafenib, Castven et al. found that activation of *YAP*-related gene sets and decreased activity of the Hippo pathway were detected in resistant cell lines. Simultaneously inhibiting YAP activity can improve the therapeutic effect of liver cancer patients with drug resistance mechanism [45]. In breast cancer, YAP1 also can affect chemotherapy sensitivity through the HJURP/YAP1/NDRG1 axis [46]. However, there is still no research on the drug resistance mechanism of YAP1 in LCNEC, so the underlying mechanism of drug resistance induced by YAP1 still remains obscure. In summary, we thought that LCNEC may be molecularly classified according to expression level of YAP1 to guide individualized treatment and prognosis grouping. Further studies will be needed to determine the internal mechanism and validation.

In conclusion, YAP1 was found to be expressed in LCNEC patients with low level and inversely correlates with neuroendocrine differentiation and may act as an unfavorable prognostic factor for LCNEC. therefore, YAP1 is a promising potential therapeutic target and stratified marker and its internal mechanism is worthy to be further explored and validated.

Translational oncology

Dear editor:

We are submitting the enclosed manuscript entitled "**Prognostic significance of YAP1 expression and its association with neuroendocrine markers in resected pulmonary large cell neuroendocrine carcinoma (LCNEC)"** for your consideration as a research article in *Translational Oncology*. The work described has not been submitted

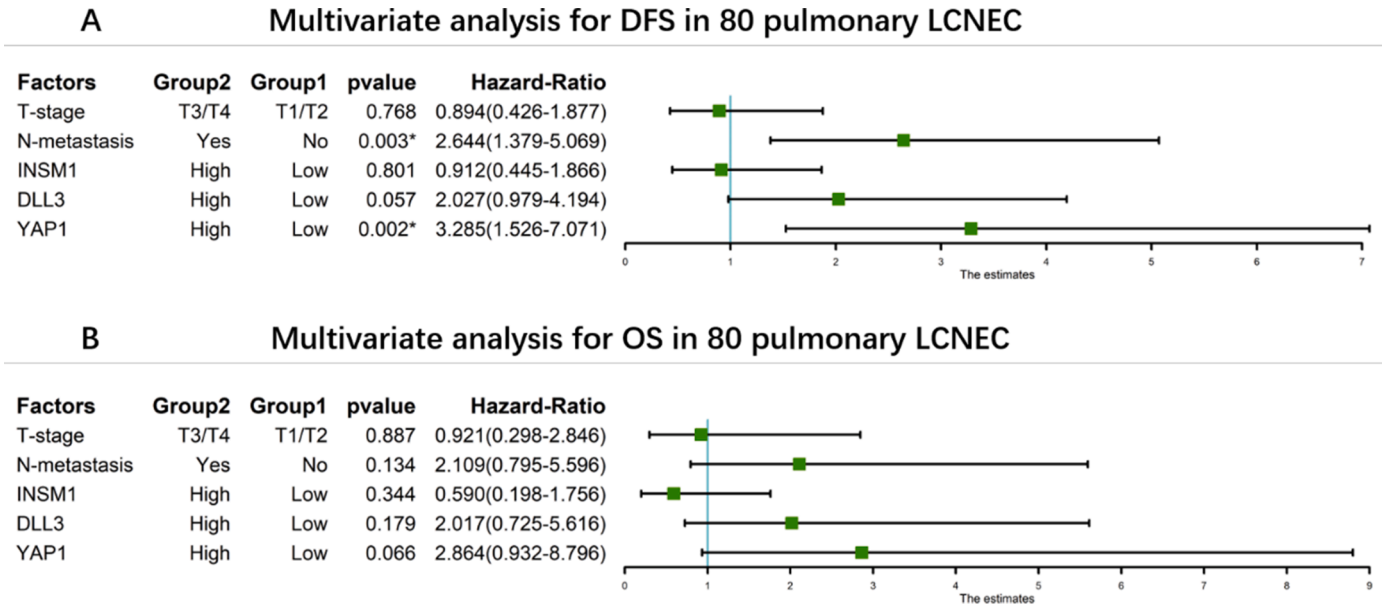

Fig. 4. Multivariate analysis of disease-free survival (DFS) (A) and overall survival (OS) (B) in 80 pulmonary LCNEC by COX regression model. Group 1 was the reference group. Multivariate analysis showed that YAP1 was an independent unfavorable prognostic factor for DFS (HR: 3.285, 95% CI: 1.526-7.071, $p = 0.002$) (A), but for OS there is no significant difference (B).
*, statistically significant

elsewhere for publication, in whole or in part, and all authors have contributed to read and approved the manuscript that is enclosed.

This manuscript addressed the influence of YAP1 (yes-associated protein 1) on the prognosis of LCNEC and its relationship with neuroendocrine markers (INSM1, NeuroD1 and DLL3 protein) and found that YAP1 is a prognostic predictor for worse survival in LCNEC and negatively correlated with neuroendocrine differentiation. Our research is very valuable, which showed that the neuroendocrine differentiation and prognosis of YAP1 in high expression group versus low expression group are significantly different by applying immunohistochemistry on 80 resected LCNEC samples. Therefore, we thought that the molecular subtypes and treatment stratification based on the expression of YAP1 and neuroendocrine markers at the immunohistochemical level is promising. Furthermore, our data from 80 resected LCNEC patient samples which is large and simple.

We deeply appreciate your consideration of our manuscript, and we are looking forward to receiving comments from the reviewers. If you have any queries, please don't hesitate to contact me at the address below.

Thank you and best regards.

Yours sincerely,

Lin Yang on behalf of all authors

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CRediT authorship contribution statement

Xujie Sun: Methodology, Writing – original draft. **Jinyao Zhang:** Formal analysis, Methodology, Writing – original draft. **Jiyan Dong:** Data curation, Methodology. **Li Liu:** Data curation, Writing – review & editing. **Xue Li:** Writing – review & editing. **Puyuan Xing:** Writing – review & editing, Supervision. **Jianming Ying:** Investigation, Supervision. **Yiqun Che:** Investigation, Supervision. **Junling Li:** Investigation, Supervision. **Lin Yang:** Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors state that they have no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101538.

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