Original Article

Clinicopathological and prognostic significance of NCALD protein expression in lung adenocarcinoma

Yi Chen[#], Xueren Feng[#], Hui Shen, Lijuan Zhang, Xiaoyong Li, Liliang Gao, Xiaohuo Zhang, Bin Wang

Department of Respiratory Medicine, Huzhou Hospital, Zhejiang University School of Medicine, Huzhou 313000, China

Contributions: (I) Conception and design: X Zhang, B Wang; (II) Administrative support: H Shen; (III) Provision of study materials or patients: Y Chen, X Feng; (IV) Collection and assembly of data: L Zhang; (V) Data analysis and interpretation: X Li; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Xiaohuo Zhang; Bin Wang. Department of Respiratory Medicine, Huzhou Hospital, Zhejiang University School of Medicine, Huzhou 313000, China. Email: zhangxiaohuo2834@163.com; 13757295077@139.com.

Background: Although there are great improvements in diagnostics and treatment of non-small cell lung cancer (NSCLC), it remains the leading cause of cancer-related death globally. NSCLC can be further categorized into two common subtypes, lung squamous cell carcinoma (LUSC) and lung adenocarcinoma (LUAD). The aims of this study were to evaluate the expression of neurocalcin delta (NCALD) in LUAD patients and to evaluate the relationship between NCALD expression and clinicopathological feature as well as overall survival (OS).

Methods: The expression level of NCALD protein was assessed by immunohistochemical analysis of tissue microarrays (TMAs) which contained 90 LUAD tissues and paired adjacent normal tissues. The relationship between NCALD expression level and clinicopathological characteristics as well as OS were investigated.

Results: The results showed that the expression level of NCALD protein in LUAD tissues was significantly down-regulated (P<0.01) and that the NCALD level was correlated with the tumor size (P<0.001), lymph node metastasis (P=0.03) and TNM stage (P=0.015). Both Kaplan-Meier survival analysis and Cox regression univariate analysis identified that NCALD expression level, lymph node metastasis, and clinical TNM stages were prognostic factors for patients' survival (P<0.05).

Conclusions: Overexpression of NCALD is a significant marker for a good prognosis in patients with LUAD.

Keywords: Neurocalcin delta (NCALD); lung adenocarcinoma (LUAD); prognosis

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Introduction

Lung cancer remains the leading cause of cancer-related death globally (1). In spite of tremendous efforts to improve the standard therapeutics, the prognosis of lung cancer is poor, with a 5-year survival rate as low as 15% (2,3). Among lung cancer, non-small cell lung cancer (NSCLC) is account for 85% of all cases and can be further categorized into two common subtypes, lung squamous cell carcinoma (LUSC) and lung adenocarcinoma (LUAD) (4,5). LUAD could be further classified into lepidic adenocarcinoma, acinar adenocarcinoma, papillary adenocarcinoma, micropapillary adenocarcinoma, solid adenocarcinoma and so on. Most patients with LUAD, the first time going to a doctor is usually started after cough or bloody sputum, which means most patients are diagnosed with LUAD in the advanced stage of the disease. The standard care of treatments for advanced stage LUAD patients are platinum-based doublet regimens and targeted cancer therapy. However, drug resistance and subsequent tumor progress are unavoidable.

Table 1 The expression level of NCALD in LUAD tissues and adjacent normal tissue

Protein	Histotype	Expression levels (number)							P
		0	1	2	3	4	5	6	
NCALD	N	1	1	17	39	18	12	2	<0.01
	T	30	8	25	19	7	1	0	

N, normal tissue; T, tumor tissue; NCALD, neurocalcin delta; LUAD, lung adenocarcinoma.

Therefore, searching novel diagnostic and prognostic marker of LUAD is essential for improving early diagnostic rate and patient outcomes.

NCALD, highly conserved across species, is a member of the visinin-like subfamily of EF hand calcium-binding proteins (6). It is reported that NCALD is abundant in axonal growth cones, cerebral neurons, spinal MNs and primarily involved in neuronal Ca^{2+} signaling (7). In fact, through a calcium dependent manner, NCALD could both interact with clathrin and regulates the activity of clathrin, which plays an important role in the process of endocytosis (8,9). Subsequent studies indicated that NCALD might be implicated in the pathogenesis of human cancer. A study conducted by Couvelard and colleagues documented that NCALD gene expression to be one of many genes which can distinguish between non-metastatic and metastatic pancreatic endocrine tumor tissues (10). What's more, Isaksson *et al.* presented a whole transcriptome profile in whole blood cell mRNA of ovarian cancer patients by the Affymetrix Human Gene 1.0 ST Array (11). They found that the expression level of NCALD mRNA was decreased in the group with poorly differentiated, having advanced stage and poor prognosis tumors. Besides, other research also has shown that down-regulation of NCALD in asbestos-related lung cancers (12). Together, these findings are implied NCALD a novel biomarker specific to these cancer patients. Indeed, in the study conducted by Shi *et al.* (13), they have showed that NCALD protein was declined in NSCLC samples and high NCALD protein expression was significantly associated with increased survival overall.

Nonetheless, the relationship between the expression level of NCALD and more detailed clinicopathological feature of LUAD remains unknown. In this study, we sought to determine the clinical relevance of NCALD protein expression in 90 primary operable lung adenocarcinoma cases. We investigated the association between NCALD expression level and clinicopathological characteristics as well as OS were investigated.

Methods

Patient and tissue specimen

Data from 90 patients who underwent surgical resection for lung adenocarcinoma at Huzhou Hospital, Zhejiang University School of Medicine between July 2004 and June 2009 were retrospectively analyzed. Patients with other types of malignancy or received local or systemic treatment before any operation were excluded from this study. All pathologic specimens were independently reviewed by at 2 pathologists. The clinicopathologic information of the patients with lung adenocarcinoma, including age, sex, tumor size, tumor differentiation, lymph node metastasis and TNM (AJCC 7th) were recorded and summarized in *Table 1*. Survival data were obtained from patients' medical records. The study protocol was approved by the Institutional Review Board of Huzhou Hospital and all of the participants signed an informed consent form.

Construction of tissue microarrays (TMAs)

Ninety cases of LUAD tissues and paired adjacent normal tissues were confirmed by reviewing hematoxylin and eosin (H&E) stained slides. One representative formalin-fixed paraffin-embedded archival block were selected for each case and used in the construction of tissues microarray. Using the TM-1 tissue microarray kit (Changzhou Ruipin Precision Instrument Co., Ltd., China), 2-μm thick tissue cores were extracted from individual paraffin blocks (donor blocks). They were subsequently re-arranged into recipient paraffin blocks (TMA blocks). The TMA blocks were incubated at 60 ℃ for about 30 min and cooled at normal temperature.

Immunohistochemistry (IHC)

Four μm thick tissue sections were obtained from TMA blocks and used for IHC. These sections were deparaffinized

in xylene for 20 min and then re-hydrated in a graded series of decreasing alcohol series. For staining with anti-NCALD antibodies, antigen retrieval was performed in a pressure cooker with an epitope retrieval solution (pH 6) for 20 min, followed by washing of the specimens in phosphate buffered saline (PBS). Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol at 37 ℃ for 30 min. Tissues were then stained with IHC using a primary antibody in a humidified chamber at 4 ℃ overnight. The primary antibodies were a polyclonal antibody against NCALD (1:50 dilution; Proteintech, Chicago, IL, USA). Subsequently, a secondary antibody was then applied for 30 min and the sections then were treated with Vectastain ABC reagent for 30 min. All the slices were visualized with the DAB chromogen for 10 min and counterstained with hematoxylin/eosin. For negative control the primary antibody was omitted. Every step was followed by washing in PBS.

Immunohistochemical assessments

To quantify NCALD protein expression, IHC staining was separately evaluated by 2 pathologists. Both the staining intensity and staining extensity were evaluated and scored in randomly selected five representative fields of vision. The percentage of positive cells was scored as 0 (≤9% positive), 1 (10–25% positive), 2 (26–50% positive), 3 (51–100% positive). The staining intensity (0= no staining, 1= mild staining, 2= moderate staining and 3= strong staining). The final semi-quantitative IHC scores were calculated by adding up the strongest intensity score and the total extensity score. Immunostaining results were considered as follows: low expression level of NCALD when the score was 0–2, high expression level of NCALD when the score was 3–6.

Statistical analysis

All statistical analyses were conducted using SPSS (Statistical Package for the Social Sciences) software 19.0 (IBM, Chicago, IL, USA) and differences were considered to be statistically significant at $P<0.05$. Using Pearson's χ^2 and the *t*-tests, associations between NCALD expression level and patient clinicopathological parameters were assessed. Overall survival (OS) was defined as the time from the date of histological diagnosis to the date of last contact or death from any cause. The correlation of different survival time with LUAD characteristics, clinical features and NCALD were evaluated by using the Kaplan-Meier method. Univariate or multivariate Cox regression analysis were performed to assess whether a factor was an independent predictor of prognosis of LUAD.

Results

Expression of NCALD in LUAD as determined by immunohistochemistry

We first examined the expression level of NCALD protein in LUAD specimens and paired adjacent normal tissue using a large TMA. The tissue staining was semi-quantitatively scored by staining intensity and staining extensity, which have already been introduced in Materials and Methods. Via immunohistochemistry, we found that NCALD is mainly located in alveolar epithelium derived cells cytoplasm and LUAD had a significantly lower expression of NCALD expression in tumor tissue than in normal tissue ($P<0.01$, *Figures 1* and *2A*). NCALD protein expression was detected in 89/90 normal tissue, including low expression levels in 19 tissue (21%) and high expression levels in 71 tissue (79%). Nonetheless, NCALD protein was weakly or not expressed in LUAD tissue (low expression 70% and high expression 30%, *Table 1*). Images of representative immunostaining are presented in *Figure 1*.

Relation between NCALD expression and clinicopathologic features

The clinicopathologic features of patients and the correlation with NCALD expression was analyzed in *Table 2*. In total, of the 90 patients with LUAD included in this study, 49 were men and 41 were women. The mean age at diagnosis was 63.5 years. Moreover, 40 patients with small tumor size (maximum diameter <4 cm) and 50 patients with large tumor size (maximum diameter ≥4 cm). Seventy cases had well or moderate differentiated tumor, 20 poorly differentiated. At the time of diagnosis, there were 49 patients showing signs of lymph node metastasis. Statistical analysis indicated that larger tumor diameter, which represent a higher tumor burden (*Figure 2B*), lymph node metastasis (*Figure 2C*), or more advanced tumors (*Figure 2D*) had lower NCALD protein expression. While, there was no significant association between NCALD expression and other clinical characteristics, such as differentiation, primary location or

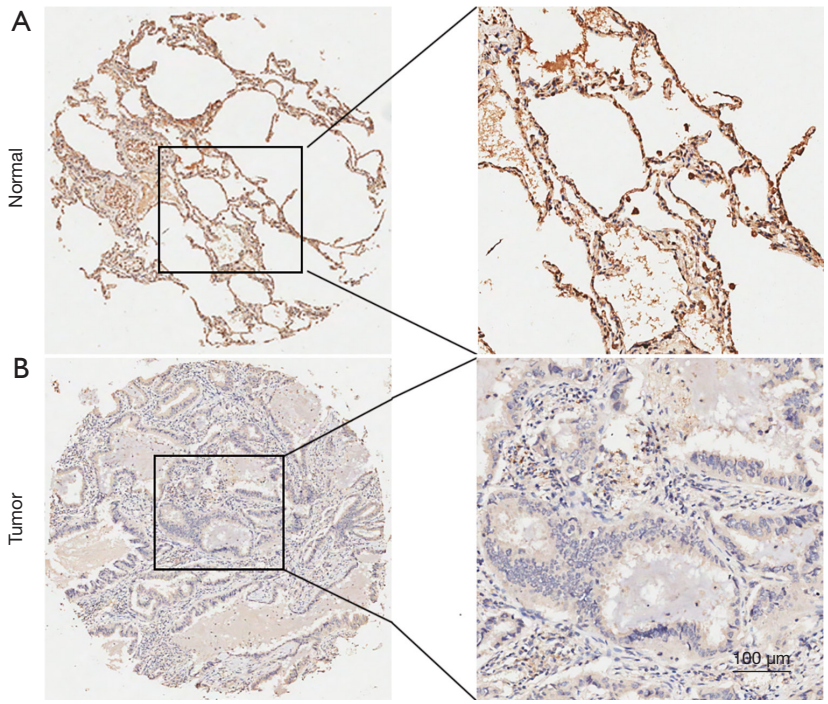

Figure 1 Representative photomicrographs of NCALD immunohistochemical expression adenocarcinomas of the lung. (A) Box-plot showing NACLD immunohistochemical expression of paired adjacent non-normal tissue. (B) Box-plot showing NACLD immunohistochemical expression of and LUAD tumors. NCALD, neurocalcin delta; LUAD, lung adenocarcinoma.

gender (P>0.05; *Table 2*).

Correlation between the NCALD protein expression level and prognosis of patients with LUAD

The Kaplan-Meier survival analysis indicated that low expression levels of NCALD protein were associated with poor prognosis of patients with LUAD (P<0.01, *Figure 3A*). What's more, both lymph node metastasis and clinical TNM stages were statistically related to the prognosis of patients with LAUD (P<0.01, *Figure 3B,C*). Whereas, there were no significant differences in tumor location, tumor size and sex (P>0.05, *Figure 3D,E,F*). What's more, well differentiation is related the good prognosis of patients with LUAD (*Figure 3G*).

In the Cox regression model analysis, univariate analysis revealed that NCALD expression, lymph node metastasis, and clinical TNM stages were prognostic factors for patients' survival (P<0.05, *Table 3*), which were consistent with the results of The Kaplan-Meier survival analysis. However, the multivariate Cox proportional hazards model demonstrated that there was no significant relation between NCALD expression and patients' survival (*Table 3*).

Discussion

It is well known that genetic abnormalities contribute to lung tumorigenesis. In previous studies had shown that mutations in EGFR, inactivation of p53 and activation of K-RAS critical for the pathogenesis of lung cancer, especially lung adenocarcinoma (14-16). However, the prognosis of lung cancer is still poor. Aimed to improve early diagnostic rate and patient outcomes, we explored novel diagnostic and prognostic marker of lung adenocarcinoma in the present study. Here, we found that the expression level of NCALD protein was frequently low in human lung adenocarcinoma and that low expression of NCALD predicts the poor prognosis of advanced LUAD patients, which reveals that NCALD might be a prognostic biomarker for LUAD patients.

NCALD is a predominantly cytosolic neuronal calcium sensor binding protein (17). Several lines of evidence

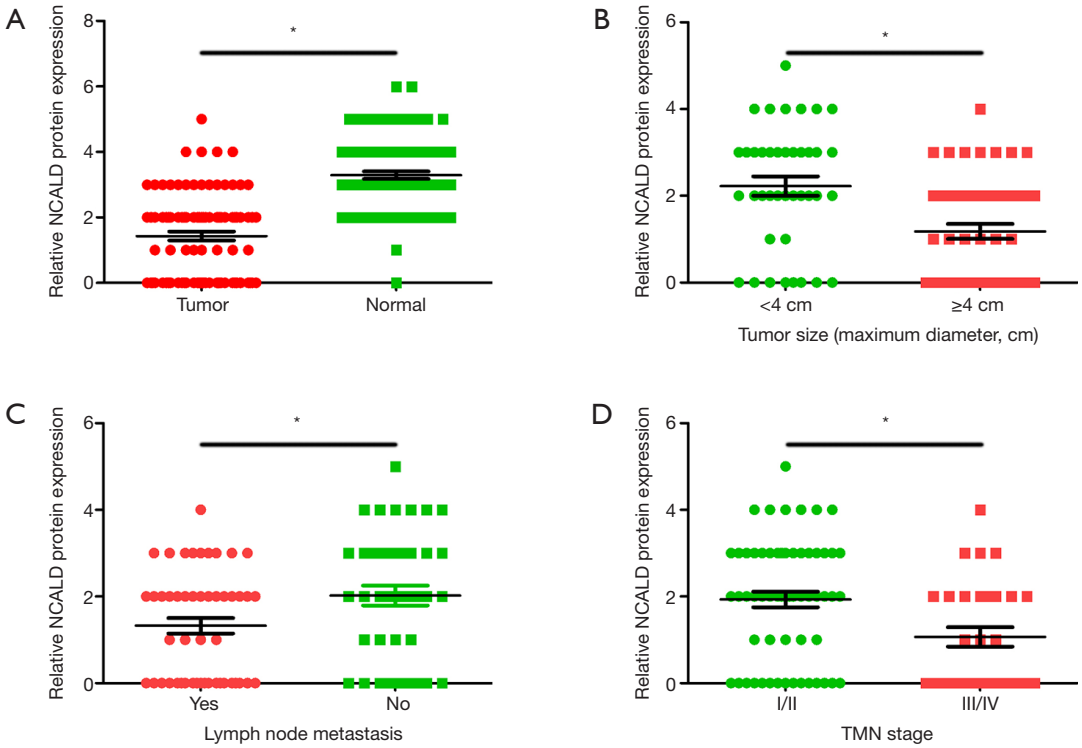

Figure 2 Relative NCALD expression in LUAD tissues and its clinical significance. (A) Immunohistochemical expression of NACLD in LUAD tumors (n=90) and paired adjacent normal tissue (n=90). NCALD expression was significantly lower in larger tumors (B), in patients with lymph node metastasis (C) and an advanced clinical stage (D). NCALD, neurocalcin delta; LUAD, lung adenocarcinoma. *, P<0.05.

show that calcium plays a crucial role in miscellaneous cellular processes, such as differentiation, migration and proliferation of normal and neoplastic cells (18). NCALD belongs to visinin-like protein (VILIP) superfamily, which comprises NCALD, hippocalcin, VILIP1, VILIP2 and VILIP3 (19,20). Although the biological functions of VILIP superfamily members are largely unknown, continuing advances in cancer demonstrate that VILIP superfamily already attracted interest. It is reported that VILIP1 acts as a tumor suppressor gene and suppressed cell proliferation and invasiveness by decreasing MMP-9 and RhoA activity as well as reducing the expression of αV and α5 integrins in human squamous cell carcinoma cells (21,22). Subsequently, Fu *et al.* revealed that VILIP1 was downregulated in 11 aggressive NSCLC cell lines due to abnormal promoter hypermethylation and histone deacetylation (23). Furthermore, VILIP3, also named as HPCAL1, was found to inhibit HCC (hepatocellular carcinoma) cell proliferation by interacting with P21 directly resulted in increasing the stabilization of P21 in an ERK1/2-MAPK dependent manner (24). Importantly, in 2016, Shi and colleagues demonstrated that the expression of NCALD was repressed in NSCLC because of the combination of lncRNA 00673 and epigenetic repressor LSD1 (13). Taken together, these findings provide novel insight into the relationship between VILIP superfamily and human cancer.

Therefore, in the present study, we hypothesized that low expression of NCALD would be related to a poor prognosis in patients with LUAD. To verify the assumption, we use tissue microarrays. We discovered that NCALD protein was downregulated in LUAD and the low expression of NCALD significantly associated with tumor size, lymph node metastasis and TNM stage. In addition, we use both Kaplan-Meier survival analysis and Cox regression model analysis to investigate whether the expression of NCALD was associated with survival in LUAD patients. The result showed that there was a statistically significant correlation between survival and NCALD expression in Kaplan-Meier survival analysis and Cox regression univariate analysis. However, there was no statistically significant correlation

Table 2 Association of NCALD protein levels with clinicopathological parameters of patients with LUAD

Variables	N of cases (%)	NCALD protein expression level		
		Low (n=63)	High (n=27)	P
Age (year, mean =63.5)				0.49
<63.5	45 (50.0)	30	15	
≥63.5	45 (50.0)	33	12	
Gender				0.746
Men	49 (54.4)	35	14	
Women	41 (45.6)	28	13	
Differentiation				0.268
Well, moderate	70 (77.8)	47	23	
Poor	20 (22.2)	16	4	
Tumor size (maximum diameter cm, mean =4.12)				<0.001
<4 cm	40 (44.4)	21	19	
≥4 cm	50 (55.6)	42	8	
Primary location				0.546
Left lung	39 (43.3)	26	13	
Right lung	51 (56.7)	37	14	
Lymph node metastasis				0.03
Positive	49 (54.4)	39	10	
Negative	41 (45.6)	24	17	
TMN stage				0.015
I/II	60 (66.7)	37	23	
III/IV	30 (33.3)	26	4	

NCALD, LUAD, neurocalcin delta; lung adenocarcinoma.

between survival and tumor size in univariate and multivariate analysis. It might be caused by the cut-off value of tumor size, which is the median of tumor size. What's more, an important shortage of this study was the lack of significant relationship between survival and TNM stage or NCALD expression in multivariate analyses due to the limitation of specimens. Hence, further studies of NCALD in LUAD cell lines will be needed to clarify its mechanisms after the preliminary results.

In summary, the expression of NCALD in patients with LUAD was found to be associated with a number of clinicopathologic factors, and importantly, was related to a good prognosis. If the functionality and molecular mechanisms of NCALD can be full identified, we might open avenues for the effect of NCALD on identification and treatment of LUAD.

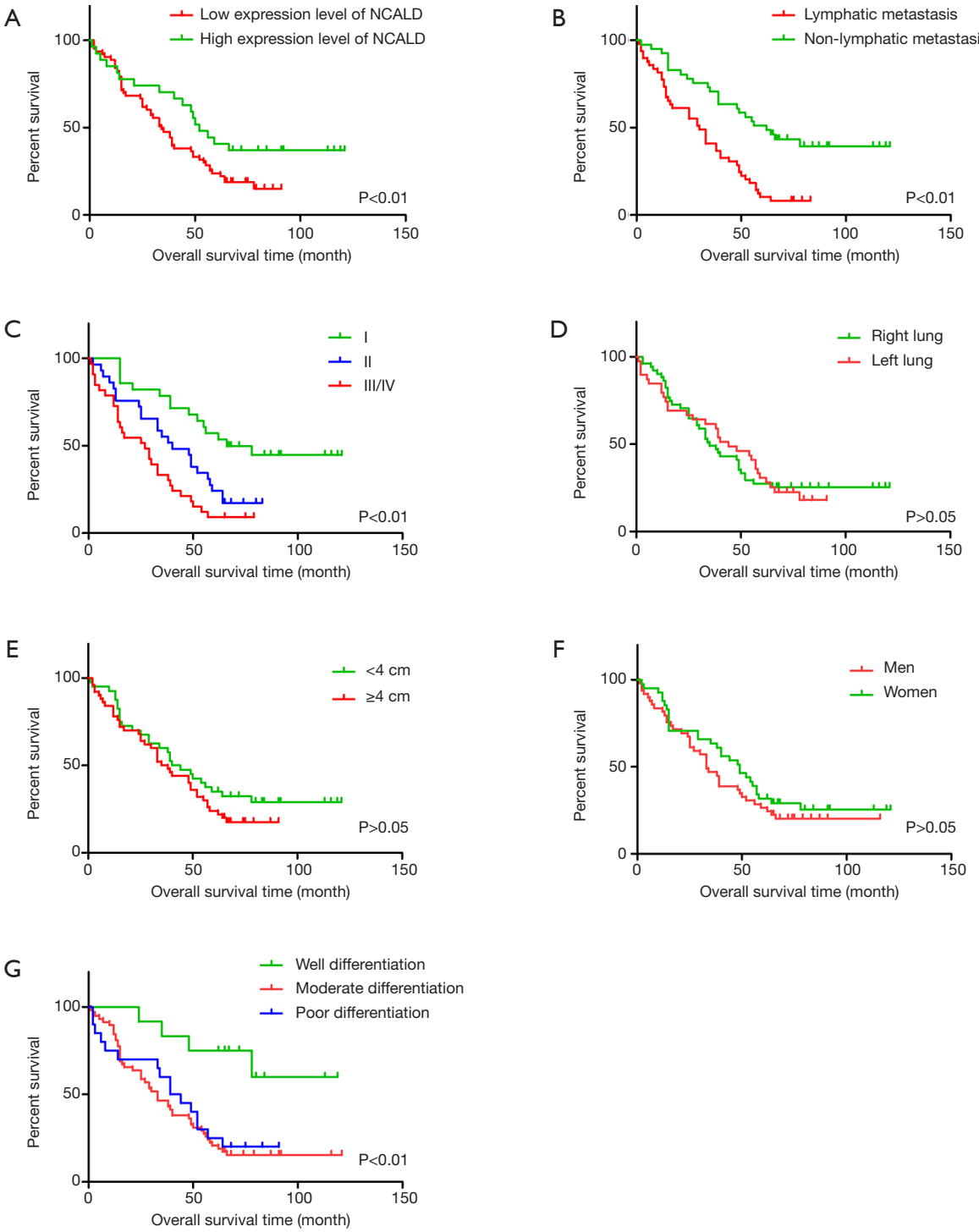

Figure 3 Clinicopathological parameters associated with overall survival in LUAD based on Kaplan-Meier survival curves. (A) High or low NCALD expression level and overall survival; (B) lymph node metastasis or non-lymph node metastasis and overall survival; (C) different TNM stage and overall survival; (D) primary location and overall survival; (E) tumor size and overall survival; (F) gender and overall survival; (G) differentiation and overall survival. NCALD, neurocalcin delta; LUAD, lung adenocarcinoma.

Table 3 Univariate and multivariate analysis of factors in patients with LUAD by Cox regression analysis

Parameters	Overall survival			
	Univariate analysis		Multivariate analysis	
	Exp (B) (95% CI)	P	Exp (B) (95% CI)	P
Gender (female *vs.* male)	0.816 (0.507–1.313)	0.403	1.143 (0.699–1.87)	0.595
Age (<63.5 *vs.* ≥63.5)	1.111 (0.693–1.781)	0.663	0.889 (0.539–1.464)	0.643
Differentiation (well/moderate *vs.* poor)	1.093 (0.624–1.913)	0.756	1.174 (0.65–2.12)	0.596
Tumor size (<4 *vs.* ≥4 cm)	1.363 (0.841–2.208)	0.209	0.941 (0.537–1.651)	0.833
Lymph node metastasis (absent *vs.* present)	2.758 (1.660–4.581)	<0.001	0.496 (0.273–0.902)	0.021
TMN stage (I/II *vs.* III/IV)	2.501 (1.521–4.111)	<0.001	0.581 (0.32–1.056)	0.075
NCALD expression (high *vs.* low)	1.744 (1.004–3.029)	0.048	1.23 (0.644–2.349)	0.53

CI, confidence interval; LUAD, lung adenocarcinoma; NCALD, neurocalcin delta.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.04.15). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study protocol was approved by the Institutional Review Board of Huzhou Hospital (No. 201805007), and all of the participants signed an informed consent form.

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