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Original Article

LAMC3 as a tumor suppressor gene associated with NID1 and NID2 in non-small cell lung carcinoma

Yuexia Huang[1*], Sanying Shen[1*], Jiwu Wang[2], Lan Luan[2], Xin Xing[2], Zhihong Xia[2], Wei Gao[2], Yiqun Zhou[2]

[1]Department of Respiration, Research Center for Clinical Medicine, Wuhan Fourth Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430033, China; [2]Department of Thoracic Surgery, Wuhan Fourth Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430033, China. [*]Equal contributors.

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Abstract: LAMC3 (Laminin gamma 3), a cellular adherence molecule with regulatory functions, has been proven to play a vital role in various biological processes, including tissue differentiation, wound healing, and carcinogenesis. To characterize the role and mechanism of LAMC3 in non-small cell lung cancer (NSCLC), we assessed the expression level of LAMC3 through the GEPIA2 database and immunohistochemical methods, and found that the expression level of LAMC3 in the tissues of patients with NSCLC was lower than that in adjacent lung tissues. In this study, clinicopathological features of patients and overall survival of patients were also analyzed. The statistical data showed that the expression of LAMC3 was not significantly correlated with the patient's gender, age, histology, node status and tumor status, but was significantly correlated with TNM stage (P=0.018) and differentiation (P=0.021). Moreover, the survival rate of patients in the LAMC3 low expression group was significantly lower than that of the high expression group (P=0.0015). PPI network and KEGG pathway analysis showed that LAMC3 associated up-regulated candidate genes included LAMA3, LAMA5, LAMB1, LAMB2, LAMB3, NID1, and NID2 in NSCLC. According to previous studies of the Nidogen protein family including NID1 (Nidogen-2) and NID2 (Nidogen-2), NID1 has been reported to promote lung metastasis of breast cancer, and its expression is associated with poor clinical outcomes. We further found that the LAMC3-positive tumor cells were not only positively correlated with the proportion of NID1-positive tumor cells (r=0.59, P=3.4e-120) but also positively correlated with NID2-positive tumor cells (r=0.48, P=3.1e-72). Taken together, our results from the current study indicate that LAMC3 offers considerable promise as a biomarker to predict the clinical prognosis and survival of NSCLC patients.

Keywords: Non-small cell lung cancer, LAMC3, prognosis, NID1, NID2

Introduction

Lung cancer is still the leading cause of cancer-related mortality, the number of newly diagnosed cancer is 11.6% and the number of all cancer deaths is 18.4% globally [1]. Non-small cell lung cancer (NSCLC) accounts for close to 85% of all lung cancers, and most patients with NSCLC are diagnosed at advanced stages of disease. In this stage, curative treatment such as surgical resection is not feasible [2-4]. Therefore, there is an urgent need to better understand the molecular biology of lung cancer, which may help to identify novel diagnostic and therapeutic targets.

Laminins are cell adhesion molecules found in the extracellular matrix, predominantly in basement membranes [5]. Laminin, which was initially isolated from a mouse sarcoma-like tumor, is a trimeric protein containing an α, a β, and a γ chain involving many biological processes, including tissue differentiation, wound healing, and tumorigenesis [6]. Recently studies have shown that LAMC3 (Laminin gamma 3) is not only significantly upregulated in the oral cavity and pharyngeal cancer [7], but it is also overexpressed in breast cancer [8]. However, the study of the role of LAMC3 expression in lung cancer progression has not been investigated.

The Nidogen protein family includes NID1 (Nidogen-2) and NID2 (Nidogen-2) [9]. NID1 has been reported to promote breast cancer lung metastasis, and its expression was associated with poor clinical outcomes. *In vitro* studies

revealed multiple prometastatic functions of NID1, such as enhancing cancer cell invasion and migration, improving vascular tube formation and promoting cell adhesion [10]. In addition, it has emerged that that NID2 methylation promoted lung cancer progression [11].

This study investigated the clinical significance of LAMC3 expression and revealed that its expression was not only positively associated with the proportion of NID1-positive tumor cells (r=0.59, P=3.4e-120) but also positively correlated with NID2-positive tumor cells (r=0.48, P=3.1e-72) in patients with NSCLC. The statistical results showed that LAMC3 might be a lung cancer-associated molecule that can be used as a candidate marker for investigating the clinical prognosis of NSCLC.

Materials and methods

Patients

This study included patients 18 years or older who underwent an operation and were diagnosed with American Joint Committee on Cancer (AJCC) stage I-III lung cancer between 2014 and 2019, and the patients had no other malignant tumors. Patients were excluded if they received postoperative standard first-line treatment or chemotherapeutic treatment after curative resection or had severe underlying disease. If no information on clinical treatment or survival was available, patients were also excluded. After screening, 69 NSCLC surgical specimens containing 25 adenocarcinomas, 30 squamous cell carcinomas and 14 specimens classified as "other" were investigated. Hospital ethics committee approval (KY2021-084-02) was obtained for the study, and informed consent was obtained from all study patients. All the patients were diagnosed and treated by the Partners for Human Research Committee at Wuhan Fourth Hospital, Tongji Medical College, Huazhong University of Science and Technology. All patient-related tissues were obtained from the pathology department of Wuhan Fourth Hospital, Tongji Medical College, Huazhong University of Science and Technology from 2014 to 2019, and were reviewed and approved by the human subjects study protocol. Patients with newly diagnosed lung cancer had not received therapy before sample collection. Tissue specimens were collected immediately after surgical removal. For histological examination, lung cancer tissue and surrounding non-tumorous tissue were fixed in 10% buffered formalin and were stored at -80°C. All or most of the diagnosis was confirmed histologically based on H&E stained sections. The age of all patients ranged from 26 to 77 years, with an average age of 62.12. The ratio of female to male was 34:35. The histological grades were classified according to the World Health Organization criteria. Of the 69 patients, 25 patients were classified as grade I, 24 were grade II and 20 were grade III. The follow-up time of the 69 patients ranged from 1.2 to 60.4 months. Formalin-fixed and paraffin-embedded tissue samples were prepared and reviewed by 3 expert pathologists. Clinical data such as patient diagnosis, staging, history and survival were obtained from the surgical pathology files of the National Cancer Institute "Regina Elena" databases. Survival data of all patients were integrated through personal interviews with their relatives.

Bioinformatics mining method

The GEPIA2 (Gene Expression Profiling Interactive Analysis 2) database (http://gepia2.cancer-pku.cn/index) could analyze LAMC3 expression profiles from the GTE (Genotype-Tissue Expression) project and the TCGA (the Cancer Genome Atlas) dataset. The LAMC3 expression level in different types of lung cancer could be obtained from Boxplot [12]. The log2FC cutoff was set as 1, and *p*-value was 0.01.

Immunohistochemistry

Sections were prepared before immunostaining. After dewaxing through graded ethanol series, the sections were soaked with 0.3% hydrogen peroxide to block endogenous peroxidase activity. The slices were treated with 10 mM citrate buffer (pH 6.0). After that, the sections were treated with 10 mM citrate buffer (pH 6.0) and heated to 121°C for 20 minutes in an autoclave to recover the antigen. To block any non-specific reactions, the sections were rinsed in phosphate-buffered saline (PBS; pH 7.2), and then the sections were blocked with 10% goat serum for 1 hour at room temperature. Finally, the sections were incubated with anti-human LAMC3 monoclonal antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) for 4 hours. Also, use a non-specific immunoglobulin IgG (diluted 1:100;

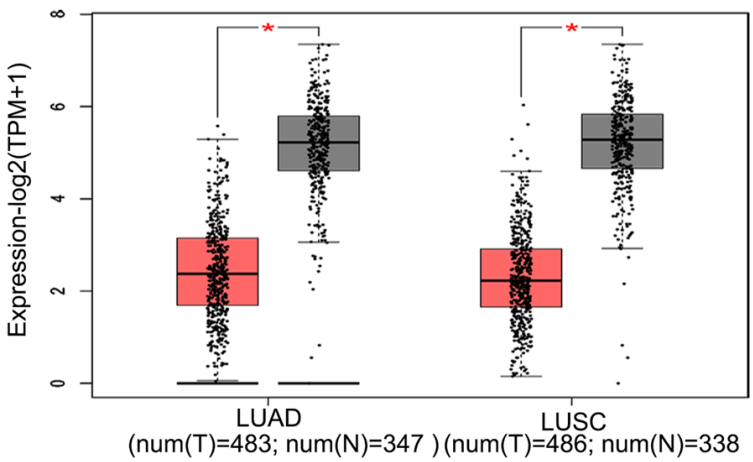

Figure 1. LAMC3 expression was analyzed by GEPIA2. Expression of LAMC3 was significantly lower in NSCLC tissue (red box) than in adjacent lung tissue grey box (*P < 0.01). GEPIA2: Gene Expression Profiling Interactive Analysis 2.

Santa Cruz Biotechnology) at the same concentration of the primary antibody was used for the parallel processing of Negative control slides. We treated all slides with the peroxidase-anti-peroxidase method (Dako, Hamburg, Germany), first counterstaining them with hematoxylin for 3 seconds, then rinsing the slides well with water for 10 minutes, dehydrating them, and cover-slip [13].

Immunohistochemical evaluation

Without knowing the clinical and pathological parameters of patients, a blind evaluation method was used for all immunostained sections. In order to evaluate LAMC3, stained sections were observed under the microscope. Five high-power fields were randomly selected in each specimen, and the cell staining was examined in these fields [14]. The following criteria were used for IHC staining scoring: The LAMC3 cytoplasmic staining intensity was scored as 0 (no staining), 1 (weak), 2 (marked), and the percentage scores were assigned as 1- 1-25%, 2- 26-50% and 3- 51-100%. We multipled the scores of each tumor sample to get a final score of 0 to 6, and then determined the total expression of LAMC3 as negative or low expression (-): score < 3, positive expression or high expression (+): score ≥3 [15]. To avoid possible technical errors, half of the samples were stained twice, and similar results were obtained in these samples.

PPI network construction and KEGG pathway analysis

STRING (https://string-db.org/cgi/input.pl) is a database that collects and integrates known and predicted protein-protein association data. The associations in the STRING database are specific and biologically meaningful, including direct (physical) interactions and indirect (functional) interactions.

Statistical analysis

Continuous variables (CV) were expressed as mean ± standard deviation (SD), and categorical variables were shown as frequency and constituent ratios. Differences of continuous variables were analyzed using independent sample t-test, and difference of categorical variables was detected using the chi-square test. P < 0.05 was regarded as statistically significant. Statistical analysis was performed with Stat-View 5.0 software, and the chi-square test was used to calculate the association between LAMC3 and clinicopathological variables. Kaplan-Meier method was used for survival estimates, and the log-rank test was also used for analysis. In addition, we used the STRING database to predict the protein interaction (PPI).

Results

Decreased expression of LAMC3 in NSCLC

By using the GEPIA2 database to determine the expression of LAMC3 in non-small cell lung cancer and normal tissues, the results showed that the LAMC3 expression in non-small cell lung cancer tissues (red box) is lower than that in normal tissues (grey box) (*P < .01, **Figure 1**).

Correlation between decreased expression of LAMC3 and malignancy of NSCLC

We further verified the prognostic value of LAMC3 expression by immunohistochemical method in 69 pairs of non-small cell lung cancer tissues and corresponding adjacent non-tumor tissues, and further determined whether the expression level of LAMC3 protein is relat-

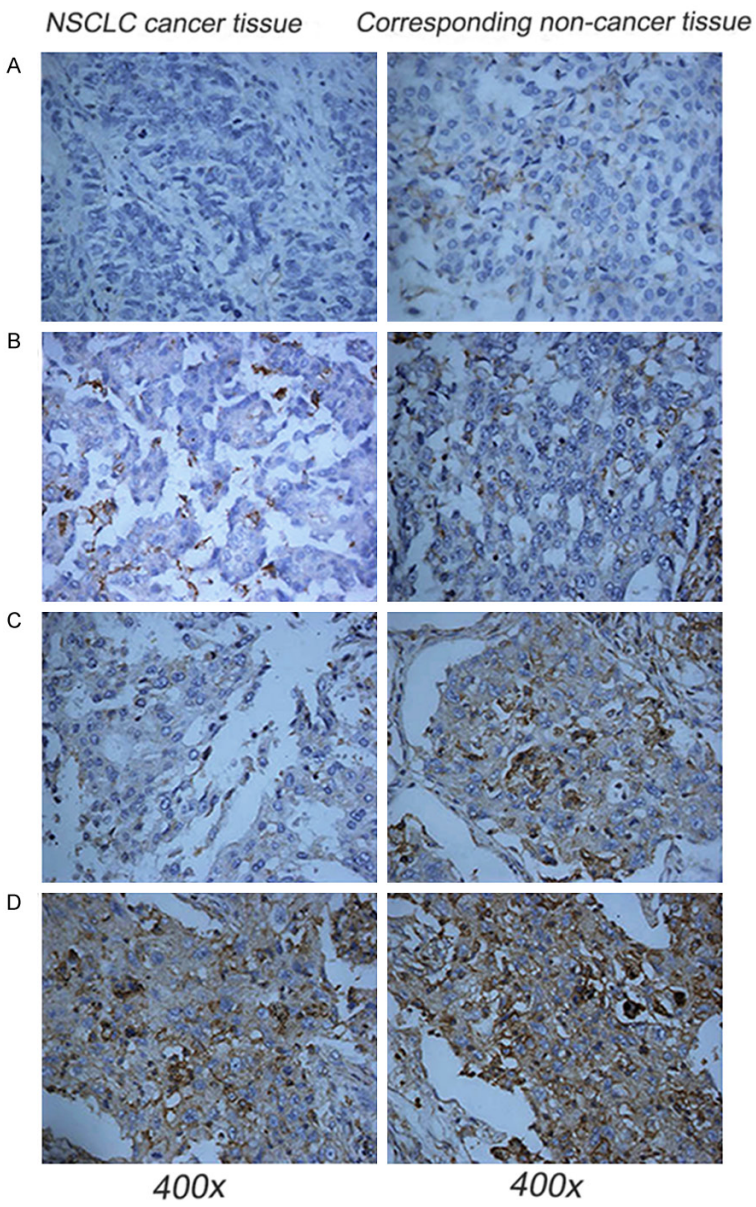

Figure 2. Different expression of LAMC3 between NSCLC tissue and their corresponding normal tissues. In IHC, the expression of LAMC3 mainly existed in the cytoplasm of NSCLC cells with different staining intensities (A-D). A: Negative LAMC3 intensity; B: Weak LAMC3 intensity; C: Moderate LAMC3 intensity; D: Strong LAMC3 intensity. Magnification: ×400.

ed to the histological characteristics of non-small cell lung cancer. Finally, 8 cases of invalid tissues were eliminated, including 61 cases of non-small cell lung cancer, with 35 cases from males and 34 cases from females. The age of the patients ranged from 26 to 77 years, with a median age of 60 years. In IHC, LAMC3 is mainly distributed in the cytoplasm of NSCLC cells. **Figure 2** shows the different staining intensities of LAMC3.

Correlation between LAMC3 expression and clinicopathological parameters in lung cancer

We summarized the clinicopathological data of patients in **Table 1**, and evaluated the correlation between LAMC3 expression and clinicopathological variables. For statistical analysis, the expression of LAMC3 in carcinoma specimens was divided into two groups according to the percentage of LAMC3 positive cells in the test results: high expression and low expression, and 30% was used as the cutoff value to represent the average value of LAMC3 expression. The analysis results showed that the expression of LAMC3 was not significantly correlated with the patient's gender, age, histology, node status and tumor status, but was significantly correlated with TNM stage (P=0.018) and differentiation (P=0.021). Moreover, the survival rate of patients in the LAMC3 low expression group was significantly lower than that of the high expression group (P= 0.0015, **Figure 3**).

PPI network and KEGG pathway analysis

We evaluated the PPI information about LAMC3 through the STRING database. The PPI network consists of 11 nodes and 48 edges, where each node represents all the proteins produced by a single protein-coding gene locus, and each edge represents the predicted functional association. The functional genes we predicted with LAMC3 mainly included LAMA1, LAMA3, LAMA5, LAMB1, LAMB2, LAMB3, NID1, NID2, NTN4 and DAG1 (**Figure 4A**). According to the enrichment analysis of PPI information and pathway data, LAMC3 was mainly enriched in the KEGG pathway, which is related to the interaction of

Table 1. The association between LAMC3 expression with clinicopathological variables in NSCLC

Parameters	Number of patients	LAMC3 (-)	LAMC3 (+)	P[a]
Age				0.151
< 60	30	12	18	
≥60	39	19	20	
Gender				0.285
Male	35	13	22	
Female	34	12	22	
Histologe				0.226
Adenocarcinoma	26	13	13	
Squamous cell carcinoma	30	14	16	
Other	13	8	5	
Differentiation				0.021
Well-Moderate	40	14	26	
Poor	29	8	21	
TNM stage				0.018
I	25	8	17	
II	24	7	17	
III	20	14	6	
Tumor status				0.520
I	22	8	14	
II+III	47	20	27	
Nodal status				0.387
N0	21	9	12	
N1+N2+N3	48	23	25	

[a]Statistical analyses were performed by the Pearson x^2 test. $P < 0.05$ was considered significant. LUAD: lung adenocarcinoma; LUSC: lung squamous cell carcinoma. LAMC3 expression and clinicopathological parameters in 69 NSCLC specimens.

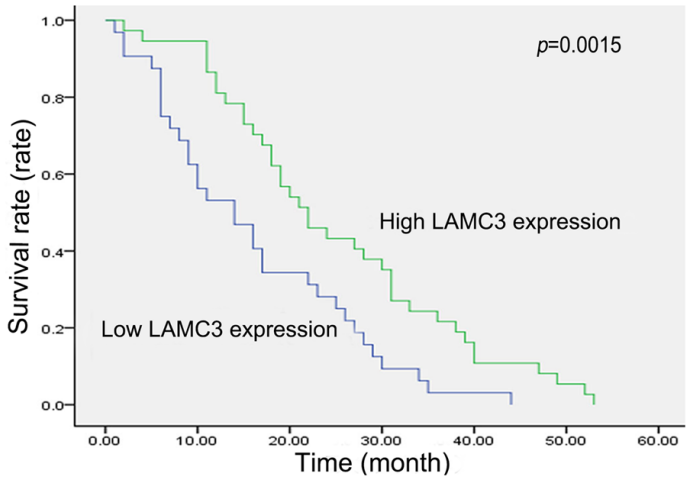

Figure 3. Kaplan-Meier survival curves indicated that the 5-year survival rate in NSCLC patients with low-expression LAMC3 was significantly lower than that of patients with high-expression LAMC3 group (*P*=0.0015).

ECM receptors and is related to Small cell lung cancer, Toxoplasmosis, and Amoebiasis, etc. There are candidate genes in these pathways that are all up-regulated in NSCLC, including LAMA3, LAMA5, LAMB1, LAMB2, LAMB3, NID1, NID2, and NTN4.

Correlation between LAMC3 expression and the associated molecules in NSCLC

The GEPIA2 database was used to determine the correlation between LAMC3 expression and the NID1 or NID2 expression in NSCLC. In this study, we found that the LAMC3-positive tumor cells were not only positively correlated with the proportion of NID1-positive tumor cells (*r*=0.59, *P*=3.4e-120; **Figure 4B**) but also positively correlated with NID2-positive tumor cells (*r*=0.48, *P*=3.1e-72; **Figure 4C**).

Discussion

Laminins, the important component of the extracellular matrix, are tissue-specific macromolecular glycoproteins composed of α, β, and γ subunits. Mammalian laminins are heterotrimeric proteins with 5 different α subunits (α1 to α5, encoded by LAMA1 to LAMA5), 4 different β chains (β1 to β4, encoded by LAMB1 to LAMB4) and 3 γ chains (γ1 to γ3, encoded by LAMC1 to LAMC3) [16]. The laminin family has common and unique important functions, which are endowed by the difference in the number, size, and organizational structure of the constitutive domains of laminin chains [17]. The role of laminin subunit expression in cancer progression has been vigorously studied. Taking the laminin subunit γ2 chain as an example, the LAMC2 gene encoding this subunit was an established prognostic marker for various types of cancer [18-20].

In this study, our research team found that LAMC3 had a significantly lowly expression in NSCLC, indicat-

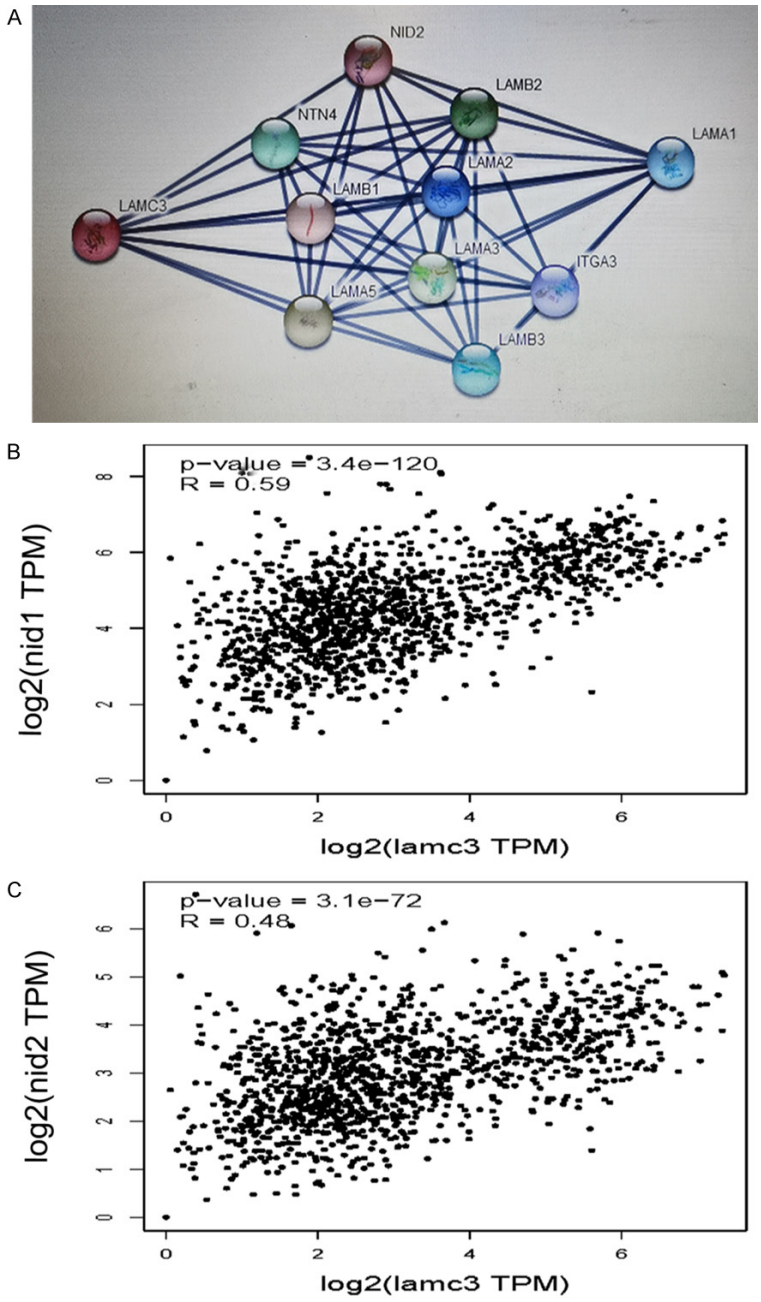

Figure 4. The PPI information showed that the predicted functional gene with LAMC3 mainly included LAMA1, LAMA3, LAMA5, LAMB1, LAMB2, LAMB3, NID1, NID2, NTN4 and DAG1 (A). LAMC3-positive tumor cells was not only positively correlated with the proportion of NID1-positive tumor cells (B) but also positively correlated with NID2-positive tumor cells (C).

ing that the LAMC3 may be involved in the carcinogenesis of NSCLC. According to reports, LAMC3 is lowly expressed in serous intraepithelial carcinoma [21-23], hence we expect LAMC3 to become an early diagnostic marker for NSCLC. The IHC data also revealed that LAMC3 staining in most NSCLC tissues showed a moderate to strong cytoplasmic immune response, and the LAMC3 expression was significantly correlated with TNM differentiation and staging. We further found that, compared with patients with low expression, the high expression of LAMC3 in NSCLC tissue was significantly related to the poor survival rate of patients.

Through enrichment analysis of PPI information and pathway data, it can be concluded that LAMC3 is mainly enriched in KEGG pathways related to MET activates PTK2 signaling, and ECM-receptor interaction, etc. Candidate genes in these pathways include LAMA3, LAMA5, LAMB1, LAMB2, LAMB3, NID1, and NID2. The candidate genes mentioned above were significantly up-regulated in NSCLC, which is worth noting.

The basement membrane is a highly specialized thin, pliable layer of extracellular matrix. The basement membrane underlines cells and separates them from and connects them to their interstitial matrix, preventing malignant tumor cells from directly invading other tissues [24]. In order to seed at new sites, cancer cells must spread across the basement membrane barrier, and then expand by recruiting the basement membrane with vascular supply. Therefore, the basement membrane is closely related to tumor invasion and metastasis [25]. The main components of the basement membrane include collagen IV, laminins, perlecan and nidogens. The nidogen family in humans consists of two members, nidogen-1 (NID1) and nidogen-2 (NID2). One possible mechanism by which NID2 may de-

crease tumorigenesis could be its hypermethylation. In this study, we found that the LAMC3 expression was not only positively correlated with NID1 expression but also positively correlated with NID2 expression. Combined with the above findings we hypothesized that LAMC3 might also play important roles in the regulation of NID1 or NID2 expression, although the precise cellular and molecular mechanism underlying the association between them needs to be further studied [26].

The current research has several limitations. First, there are few reports of LAMC3 in tumors, and its exact carcinogenic mechanism is still unclear. Secondly, the carcinogenic mechanism of NID1 or NID2 is still in the preliminary research stage, and the relationship between NID1 or NID2 and other tumor molecules is rarely reported. Based on the above reasons, this experimental study has certain limitations. However, according to the results of this study, it may prove that LAMC3 could have an anti-tumor effects in addition to its role in immunological checkpoints. For example, anti-LAMC3 antibody therapy may directly kill tumors. Although the tumor tissue can be entirely removed via surgery in patients with early NSCLC, many patients experience recurrence shortly after surgery because of micrometastasis. The results of this study also showed that some patients with early NSCLC also exhibit LAMC3 expression. Therefore, an anti-LAMC3 antibody could be used to fight micrometastasis in early NSCLC patients. LAMC3 expression was significantly elevated in stage III patients in contrast to stage I patients, suggesting that an anti-LAMC3 antibody might be useful as a postoperative adjuvant therapy or even as a preoperative neoadjuvant therapy. Matjaz Rokavec reported that a p53/miR-192/215/NID1 axis participated in the epithelial-mesenchymal transition in colorectal cancer cells [27]. In this study, we found that the LAMC3 expression was not only positively correlated with NID1 expression but also positively correlated with NID2 expression. However, how LAMC3 protein coordinates p53/miR-192/215/NID1 signaling pathway and what implications the LAMC3-regulated p53/miR-192/215/NID1 pathway has in lung cancer are yet to be investigated in future study.

In summary, our results indicated that down-regulated expression of LAMC3 in NSCLC is significantly related to the lower survival rate of patients and LAMC3 expression level is an independent risk factor affecting the prognosis of patients. Correspondingly, the abnormal expression of LAMC3 may be related to the occurrence of NSCLC and can be used to predict unfavorable clinical behavior. Therefore, LAMC3 has potential as a therapeutic target, which would represent a major breakthrough in the treatment of advanced NSCLC.

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Disclosure of conflict of interest

None.

Address correspondence to: Yuexia Huang, Wuhan Fourth Hospital, Tongji Medical College, Huazhong University of Science and Technology, 76 liberation Avenue, Wuhan 430000, Hubei Province, China. Tel: +86-0712-68831594; Fax: +86-0712-68831594; E-mail: 940224239@qq.com

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