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RESEARCH

Overexpression of NEDD9 is Associated with Altered Expression of E-Cadherin, β-Catenin and N-Cadherin and Predictive of Poor Prognosis in non-Small Cell Lung Cancer

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Abstract Neural precursor cell expressed, developmentally down-regulated 9 (NEDD9) is overexpressed in multiple tumor types, where it is thought to regulate tumor cell metastasis and act as a trigger of the epithelial-mesenchymal transition (EMT). Loss of E-cadherin/β-catenin and upregulation of N-cadherin are hallmarks of the EMT. The expression and correlation of NEDD9 with E-cadherin, β-catenin and N-cadherin in lung cancer are poorly characterized. We examined NEDD9, E-cadherin, β-catenin and N-cadherin protein expression in 105 cases of non-small cell lung carcinoma (NSCLC), including 43 cases of squamous cell carcinoma and 62 cases of lung adenocarcinoma, and the corresponding normal lung tissues using immunohistochemistry. NEDD9 was overexpressed in 56.2 % (59/105) of the NSCLC samples compared to normal lung tissue. Overexpression of NEDD9 correlated with abnormal expression of E-cadherin, β-catenin and N-cadherin ($P<0.001$, $P=0.008$ and $P=0.027$, respectively). Additionally, overexpression of NEDD9 correlated positively with lymph node metastasis in NSCLC (Chi-square test; $P=0.015$). The mean overall survival of NSCLC patients overexpressing NEDD9 (39.10±6.49 months) was markedly shorter than patients with normal NEDD9 expression (56.67±7.44 months; Log-Rank, $P=0.001$). Moreover, for patients with adenocarcinoma or squarmous cell carcinoma, the survival is also dramatically poorer upon overexpression of NEDD9. In multivariate analysis, overexpression of NEDD9 ($P=0.013$) and TNM stage ($P=0.001$) were significant independent prognostic factors for overall survival in NSCLC. In conclusion, overexpression of NEDD9 correlates with altered expression of EMT markers, increased lymph node metastasis and poorer survival in lung cancer.

Keywords Non-small cell lung cancer · NEDD9 · Epithelial-mesenchymal transition · Lymphatic metastasis · Prognosis

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Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide [1], and the high mortality of this disease is largely attributable to the difficulty of early diagnosis and the high frequency of metastases at the time of diagnosis [2]. Neural precursor cell expressed, developmentally down-regulated 9 (NEDD9), also known as Crk-associated substrate lymphocyte type (Cas-L) or human enhancer of filamentation-1 (HEF1), is an focal adhesion scaffold protein which has been associated with metastasis of solid tumors [3–6]. Overexpression of NEDD9 is frequently observed in glioblastoma [7], melanoma [8, 9], colorectal carcinoma [10, 11] and breast carcinoma [12] and is associated with metastasis in these tumor types. However, a three-

fold downregulation of NEDD9 has been associated with a metastasis signature in breast cancer [13]. Although conflicting results have been reported in the literature, overexpression of NEDD9 is thought to promote invasion and metastasis in multiple types of cancer. A recent study indicated that NEDD9 may be upregulated by inactivation of the tumor suppressor STK11/LKB1 in lung adenocarcinoma [14]; however, the precise function of NEDD9 in lung cancer remains largely unknown. Therefore, the role of NEDD9 in lung cancer invasion and metastasis requires further elucidation.

Tumor invasion often involves the epithelial-mesenchymal transition (EMT), during which cells lose the lateral attachments to their neighbors and become more motile. The hallmarks of the EMT include downregulation of E-cadherin/β-catenin and upregulation of N-cadherin, leading to a mesenchymal-cell-like phenotype and destabilization of adherent junctions [15]. In previous studies, NEDD9 has been proven to be a positive regulator of the EMT, and negative regulator of the membrane localization of E-cadherin/catenin complexes in aggressive breast cancer [12, 16]. NEDD9 can also promote neurite-like extensions in epithelial cells [17]. Taken together, these studies indicate that NEDD9 may play an important role in EMT-induced invasion and metastasis. However, it is still unclear whether expression of NEDD9 correlates with the EMT in lung cancer, and the relationship between NEDD9 protein expression and the clinicopathological features of lung cancer require further investigation.

In the present study, we used immunohistochemistry to examine the protein expression levels of NEDD9, E-cadherin, β-catenin and N-cadherin in 105 samples of non-small cell lung carcinoma (NSCLC). We demonstrate that upregulation of NEDD9 was related to downregulation of E-cadherin and β-catenin, upregulation of N-cadherin and lymph node metastasis in lung cancer. More importantly, overexpression of NEDD9 correlated significantly with poor prognosis in NSCLC.

Materials and Methods

Materials

We selected tissues from 105 cases of NSCLC which had been collected along with the corresponding normal lung tissues, from patients diagnosed at the First Affiliated Hospital of China Medical University (Shenyang, China) between October 2004 and July 2006. The samples were obtained from 63 male and 42 female patients with an average age of 60.4 years. According to the 2004 World Health Organization (WHO) lung tumor histological classification criteria [18], the samples were classified as squamous cell lung carcinoma (43 cases) or lung adenocarcinoma (62 cases). Thirty-six cases were highly differentiated and 69 cases were moderately or poorly differentiated. Lymph node metastases were present in 64 cases and absent in 41 cases. Tumor staging was performed according to the seventh edition of the International Union against Cancer (UICC) TNM for Lung Cancer [19], and there were 46 cases of stage I–II disease and 59 cases of stage IIIa–IIIb disease. None of the patients had received radiotherapy or chemotherapy before surgery. Patient survival was defined as the time from the day of surgery to the end of follow-up or day of death due to recurrence or metastasis. The samples were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin for pathological analysis and confirmation of the diagnosis.

Immunohistochemistry and Evaluation of Immunostaining

The surgically excised tumor specimens were fixed in 10 % neutral formalin, embedded in paraffin and 4 μm thick serial sections were prepared. The normal bronchial epithelium present in the tumor slides was used as an internal positive control. Immunostaining was performed using a streptavidin-peroxidase (S-P) method. The sections were incubated with a NEDD9 monoclonal antibody (ab18056, 1:400; Abcam, Cambridge, MA, USA), E-cadherin monoclonal antibody (sc-8426, 1:150; Santa Cruz Biotechnology, Santa Cruz, CA, USA), N-cadherin polyclonal antibody (ab18203, 1:200; Abcam) or β-catenin monoclonal antibody (610154, 1:200; BD Transduction Laboratories, Lexington, KY, USA) at 4 °C overnight. Biotinylated goat anti-mouse serum IgG (1:500; Santa Cruz Biotechnology) was used as the secondary antibody. After washing, the sections were incubated with streptavidin–biotin conjugated with horseradish peroxidase (Ultrasensitive, MaiXin, Fuzhou, China), developed using 3,3-diaminobenzidine tetrahydrochloride (MaiXin, Fuzhou, China), lightly counterstained with hematoxylin and dehydrated in alcohol before mounting.

Two investigators, who were blinded to the clinical data, examined all the of tumor slides. Five random fields of view were examined per slide, and 100 cells were observed per view at 400x magnification.

The cytoplasmic NEDD9 labeling score was defined by multiplying the percentage of positive cells by the staining intensity [9]. The percentage of positive cells was scored as: 0, 0–25 %; 1+, 26–50 %; 2+, 51–75 % or 3+, 76–100 %. The staining intensity was scored as: 0, negative; 1, weak; 2, moderate or 3, strong. The normal bronchial epithelium was averagely scored 3 (weakly expressed in 76–100 % of cells). Tumor NEDD9 immunoreactivity greater than 3 was defined as NEDD9 overexpression; tumor NEDD9 immunoreactivity equal to or less than 3 was defined as normal NEDD9 expression.

The E-cadherin/β-catenin scores were determined by the percentage of membranous positive cells per slide, as described in our previous studies [20, 21]. Briefly, if the cell membrane of more than 90 % of the tumor cells were positively stained for E-cadherin or β-catenin, the case was defined as normal membranous expression. If fewer than 90 % of the tumor cells were stained, the case was defined as abnormal expression.

N-cadherin staining was scored based on both the intensity and extent of protein expression on the membrane and/or in the cytoplasm, as described previously [22]. The case was defined as normal expression if N-cadherin was absent or only weakly expressed in less than 10 % of the tumor cells. The case was defined as abnormal expression if N-cadherin was moderately or strongly expressed and/or the percentage of positive cells was more than 10 %.

Statistical Analysis

All statistical analyses were performed using SPSS for Windows 17.0 (Chicago, IL, USA). The immunohistochemistry results were analyzed using the Chi-square test and Spearman correlation test. Survival curves were generated using the Kaplan-Meier method and the log-rank test was used for survival analysis. The Cox regression model was used to test the prognostic value. All clinicopathological parameters were entered into the Cox model and tested by univariate analysis using the enter method, and multivariate analysis using the forward stepwise logistic regression method. *P*-values less than 0.05 were considered statistically significant.

Results

Expression Patterns of NEDD9, E-Cadherin, β-Catenin and N-Cadherin in NSCLC

In all samples, NEDD9 was weakly expressed in the cytoplasm of more than 90 % of the normal bronchial epithelial cells. The average immunostaining score for NEDD9 in normal bronchial epithelium was equal to or less than 3 (Fig. 1a). E-cadherin and β-catenin were strongly expressed on the cell membrane of more than 90 % of the normal bronchial epithelial cells in all samples (Fig. 1b, c). N-cadherin was negative or only weakly expressed on the

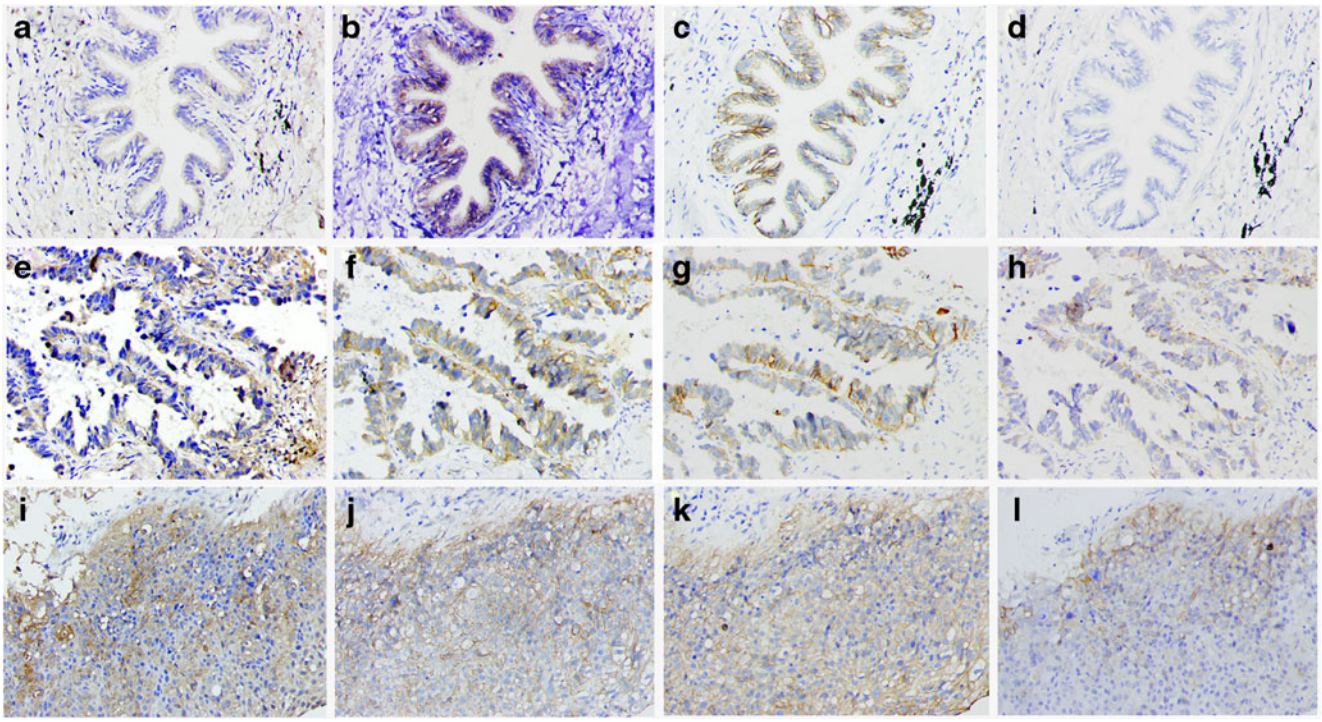

Fig. 1 Immunohistochemical analysis of NEDD9, β-catenin, E-cadherin and N-cadherin in non-small cell lung carcinoma. The cytoplasm of normal bronchial epithelium was weakly immunoreactive for NEDD9 (**a**); the cell membrane of normal bronchial epithelium was strongly immunoreactive for β-catenin (**b**) and E-cadherin (**c**); and N-cadherin was absent or weakly stained at the membrane and/or cytoplasm of normal bronchial epithelium (**d**). Moderately or strong NEDD9 immunoreactivity was observed in lung adenocarcinoma (**e**) and squamous cell carcinoma (**i**). Negative or weak β-catenin immunoreactivity was observed at the cell membrane in lung adenocarcinoma (**f**) and squamous cell carcinoma (**j**). Negative or weak E-cadherin immunoreactivity was observed at the cell membrane in lung adenocarcinoma (**g**) and squamous cell carcinoma (**k**). Strong N-cadherin immunoreactivity was observed at the membrane and/or in the cytoplasm of lung adenocarcinoma (**h**) and squamous cell carcinoma (**l**); magnification: 200x

membrane and/or cytoplasm in less than 10 % of the normal bronchial epithelial cells (Fig. 1d).

The intensity of NEDD9 expression in NSCLC cells was significantly higher than normal bronchial epithelial cells. In total, 56.2 % (59/105) of the tumor tissues had an NEDD9 immunostaining score greater than 3 (Fig. 1e, i). Membranous E-cadherin and β-catenin expression were lower in NSCLC cells than normal bronchial epithelium, with less than 90 % of the tumor cells positive for E-cadherin or β-catenin. Membranous E-cadherin and β-catenin were loss in 66.7 % (70/105) and 82.9 % (87/105) of the NSCLC tumors, respectively (Fig. 1f, g, j, k). Abnormal N-cadherin expression was observed in 44.8 % (47/105) of the NSCLC tumors, as the staining intensity was higher than normal bronchial epithelium and/or the percentage of positive cells was greater than 10 % (Fig. 1h, l).

Overexpression of NEDD9 Correlates with Abnormal Expression of E-Cadherin, β-Catenin and N-Cadherin in NSCLC

The correlations between NEDD9 expression and expression of E-cadherin, β-catenin and N-cadherin in 105 cases of NSCLC are shown in Table 1. Overexpression of NEDD9 correlated with abnormal expression of E-cadherin, β-catenin and N-cadherin ($P<0.001$, $P=0.008$ and $P=0.027$, respectively).

Relationship Between NEDD9 and the Clinicopathological Features of NSCLC

Overexpression of NEDD9 correlated positively with lymph node metastasis in 105 cases of NSCLC; however, there was no significant association between NEDD9 and sex, age, histological type, differentiation or TNM stage in NSCLC (Table 2). The Log-Rank test revealed that the survival time of NSCLC patients overexpressing NEDD9 (39.10±6.49 months) was markedly shorter that patients with normal NEDD9 expression (56.67±7.44 months; $P=0.001$, Fig. 2a). Furthermore, the survival time of lung adenocarcinoma patients (35.04±9.17 months, Fig. 2b) and lung squamous cell carcinoma patients (42.48±8.09 months, Fig. 2c) overexpressing NEDD9 was also significantly shorter than patients with normal NEDD9 expression (53.20±9.74 for lung adenocarcinoma and 60.69±9.30 for lung squamous cell carcinoma). Univariate analysis indicated that the TNM stage ($P<0.001$), lymph node metastasis ($P=0.006$), overexpression of NEDD9 ($P<0.001$) and loss of membranous E-cadherin expression ($P=0.013$) were associated with poorer prognosis in NSCLC (Table 3). In multivariate survival analysis, only overexpression of NEDD9 ($P=0.013$) and TNM stage ($P=0.001$) were significantly associated with clinical outcome. Therefore, overexpression of NEDD9 and the TNM stage can be considered as independent and effective predictors of prognosis in NSCLC.

Table 1 Correlation of NEDD9 protein expression with abnormal expression of E-cadherin, β-catenin and N-cadherin in 105 cases of non-small cell lung carcinoma

		NEDD9 expression		χ^2	*P* value
		Normal	Increased		
E-cadherin expression	Normal	29	6	32.518	0.000
	Abnormal	17	53		
β-catenin expression	Normal	13	5	7.124	0.008
	Abnormal	33	54		
N-cadherin expression	Normal	31	27	4.890	0.027
	Abnormal	15	32		

Table 2 Correlation of NEDD9 overexpression with clinicopathological features in 105 cases of non-small cell lung carcinoma

Clinicopathological feature	Number of patients	No. overexpressing NEDD9	χ^2	*P* value
Gender				
Male	63	35	0.026	0.872
Female	42	24		
Age (years)				
<60	47	29	1.050	0.306
≥60	58	30		
Histological type				
Squamous cell carcinoma	43	27	1.289	0.256
Adenocarcinoma	62	32		
Lymph node metastasis				
Negative	41	17	5.926	0.015
Positive	64	42		
Differentiation				
Well	36	18	0.853	0.356
Moderate or poor	69	41		
TNM classification				
I–II	46	23	1.274	0.259
III_a–III_b	59	36		

Discussion

In recent years, it has been demonstrated that NEDD9 is required for invasion and metastasis in multiple types of cancer [7, 8, 11, 12]. The expression of NEDD9 is tissue-specific. High levels of NEDD9 mRNA and protein expression are observed in the lung, kidney, fetal brain and tissues rich in immature lymphoid cells [6]. In the present study, we investigated the expression of NEDD9, E-cadherin, β-

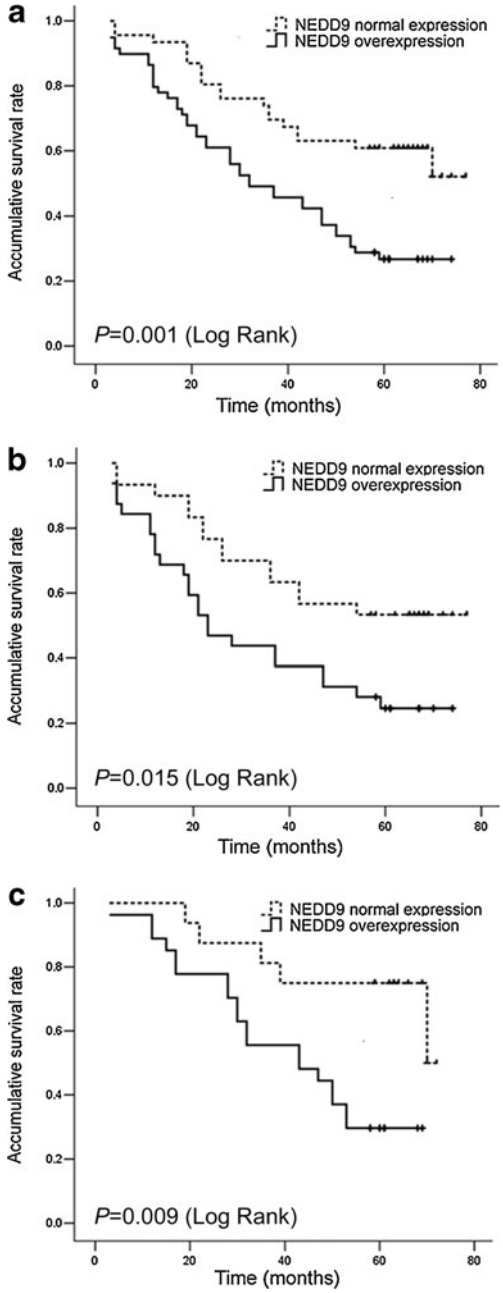

Fig. 2 Kaplan-Meier survival curves for 105 non-small cell lung carcinoma patients stratified by expression of NEDD9. **a** NEDD9 overexpression (score more than 3) positively correlated with the overall survival (P=0.001); **b** NEDD9 overexpression positively correlated with the survival of lung adenocarcinoma patients (P=0.015); **c** NEDD9 overexpression positively correlated with the survival of lung squamous celll carcinoma patients (P=0.009)

catenin and N-cadherin 105 cases of NSCLC and paired adjacent normal lung tissues.

The expression pattern of NEDD9 observed in normal lung tissues in this study was consistent with previous studies in other tissues [6, 16]. In all patients, NEDD9 was weakly expressed in the cytoplasm of more than 90 % of the normal bronchial epithelial cells. In contrast, NEDD9

Table 3 Summary of univariate and multivariate Cox regression analysis of the association between clinicopathological features with overall survival in 105 cases of non-small cell lung carcinoma

Factor	Regression coefficient	Wald chi-square test	P value	Risk ratio
Univariate analysis				
Gender	0.238	0.711	0.399	1.269
Age	0.491	2.424	0.119	1.634
Differentiation	−0.031	0.010	0.921	0.969
TNM classification	1.763	20.222	0.000	5.827
Histological type	0.177	0.302	0.583	1.194
Lymph node metastasis	−1.077	7.442	0.006	0.341
NEDD9	1.275	12.672	0.000	3.580
E-cadherin	−1.011	6.157	0.013	0.364
N-cadherin	0.551	3.304	0.069	1.735
β-catenin	0.473	1.179	0.278	1.605
Multivariate analysis				
NEDD9	0.700	6.124	0.013	2.013
TNM classification	0.987	11.383	0.001	2.682

protein expression was upregulated in 56.2 % (59/105) of the NSCLC lung cancer samples, accompanied by a loss of membranous E-cadherin and β-catenin expression and increased membranous/cytoplasmic N-cadherin expression, which are hallmarks of the EMT [15]. Previous reports have demonstrated that overexpression of NEDD9 negatively regulates E-cadherin membrane expression and promotes degradation of E-cadherin in breast cancer [16], and also positively mediates the canonical Wnt/β-catenin signaling pathway in colorectal cancer [10]. Furthermore, a recent study indicated that overexpression of NEDD9 triggered a series of events in aggressive breast cancer, including induction of the EMT [12]. Taken together, these studies and our data suggest that overexpression of NEDD9 may play a critical role in EMT-induced invasion and metastasis in NSCLC.

To investigate whether overexpression of NEDD9 was associated with invasion and metastasis in lung cancer, we analyzed the correlation between NEDD9 and the clinicopathological parameters of NSCLC. In agreement with earlier studies in other tumor types [3–5], this study indicated that overexpression of NEDD9 correlated positively with lymph node metastasis in NSCLC, but not the sex or age of the patient or the histological type, differentiation or TNM stage of the tumor. Moreover, we also examined the relationship between NEDD9 protein expression and prognosis. Overexpression of NEDD9 correlated significantly with poorer survival in NSCLC patients. Meanwhile, we also found that overexpression of NEDD9 indicated adverse clinical outcome in both lung adenocarcinoma and lung squamous cell carcinoma. Thus, NEDD9 may be a useful prognosis marker in both types of NSCLCs. Furthermore,

the Cox regression model also demonstrated that overexpression of NEDD9 was an independent factor associated with the prognosis of NSCLC patients. Previous research has demonstrated that NEDD9, as a scaffolding protein, mainly regulates the dynamics of focal adhesion formation and disassembly in tumor cells, and does not affect cancer cell differentiation or proliferation [4, 23]. Consistent with these observations, the data presented in this study suggests that NEDD9 plays a role in lung cancer invasion and metastasis, as overexpression of NEDD9 positively correlated with lymph node metastasis and poorer prognosis in NSCLC. It should be noted that this study examined the expression of NEDD9 in a relatively small sample of lung cancer patients; therefore, larger-scale studies are required to further elucidate and confirm the function of NEDD9 in NSCLC.

In conclusion, NEDD9 is overexpressed in the tumors of some patients with NSCLC. Overexpression of NEDD9 correlated with altered expression of EMT markers, including downregulation of membranous E-cadherin and β-catenin expression and upregulation of membranous/cytoplasmic N-cadherin expression. Furthermore, overexpression of NEDD9 was linked to lymph node metastasis in NSCLC and importantly, NSCLC patients overexpressing NEDD9 had a poorer prognosis, suggesting that NEDD9 plays a role in the invasion and metastasis of lung cancer.

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