PATHOLOGICA 2017;109:133-139

ORIGINAL ARTICLE

PRR11 immunoreactivity is a weak prognostic factor in non-mucinous invasive adenocarcinoma of the lung

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Key words

Lung cancer • Adenocarcinoma • PRR11 • Immunohistochemistry • Prognostic factor

Summary

Introduction. Proline-rich protein 11 (PRR11) functions in the progression of cell cycle, and silencing the PRR11 gene in lung cancer cells results in the inhibition of cellular proliferation, cell cycle progression, cell migration, invasion and colony formation. PRR11 may therefore be a therapeutic target in lung cancer.

Materials and methods. Microarrays of surgical specimens of non-mucinous invasive adenocarcinoma of the lung, from 346 subjects that were not given preoperative therapy, were autoimmunostained with PRR11 and, except for trace and pseudo-positivity, assessed as "positive" at any proportion and intensity.

Results. PRR11 immunoreactivity demonstrated a tendency to associate with an aggressive phenotype (tumor size, vascular invasion, and adjuvant therapy) and some effect on overall survival (Hazard ratio 1.51).

Conclusions. PRR11 may be a weak prognostic indicator of overall survival of patients with non-mucinous invasive adenocarcinoma of the lung.

Introduction

Recently, molecular target drugs for various human cancers have been vigorously developed, and many relevant clinical trials have been conducted globally. Epidermal growth factor receptor inhibitors such as Gefitinib, Erlotinib and Afatinib are now available for treatment of lung cancer in Japan, as are anaplastic lymphoma kinase inhibitors such as Crizotinib and Alectinib. High-level response to these drugs is attractive to patients with advanced lung cancer of specific gene mutation or rearrangement. It is striking that Novolumab, an anti-programmed cell death protein 1 monoclonal antibody, is one of the immune checkpoint inhibitors for not only melanoma but also lung cancer. The development and application of individualized therapy to lung cancer is expected to gain momentum.

Proline-rich protein 11 (PRR11) is known as a novel gene product implicated in cell cycle progression [1]; its silencing in lung cancer cells results in the inhibition of cellular proliferation, cell cycle progression, cell migration, invasion and colony formation *in vitro*. PRR11 may thus serve as a potential indicator in the diagnosis and/or treatment of human lung cancer [1-3].

Here, we retrospectively assessed the prognostic significance of PRR11 immunoreactivity with the use of resected specimens of non-mucinous invasive adenocarcinoma from patients given no preoperative therapy.

Materials and methods

Subjects and patient selection

Because the pilot test with the use of paraffin-embedded sections revealed that almost all cases of primary squamous cell carcinoma of the lung were to various degrees immunoreactive to PRR11, this study was focused on primary adenocarcinoma of the lung, other than special-type adenocarcinoma and adenocarcinoma in situ.

The subjects were 346 consecutive Asian patients who had undergone surgical resection for lung adenocarci-

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noma but had not undergone any preoperative therapy at our hospitals. The subjects were generally followed up for more than five years after surgery, and clinical information was retrospectively obtained from their medical records. Informed consent was obtained from each patient. The age and the smoking history of the patients were recorded at the time of surgery. The level of serum carcinoembryonic antigen was measured just before surgery. The interval to recurrence spanned the period from the date of surgery to the date of the identification of recurrence. The interval to death spanned the period from the date of surgery to the date of death.

Histopathology

All resected specimens were inflated until maximally expanded with 10% neutral buffered formalin (Masked Form A18 (PH), Japan Tanner, Osaka, Japan), through the respective bronchi by gravity drainage or by direct injection with a syringe attached to a fine needle. The specimens were then immediately fixed at room temperature for one to three days and subsequently subjected to a routine histopathological workup and paraffin embedding with a vacuum infiltration tissue processor (Tissue-Tek® VIP™, Sakura Finetek Japan, Tokyo, Japan). Sections (5 microns thick) stained with hematoxylin and eosin (H&E) were covered with glass by using Malinol mounting medium (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) and examined under a light microscope (BX51, Olympus, Tokyo, Japan). The cancers were graded according to the World Health Organization classification of lung tumors [4], the AJCC/UICC TNM classification [5,6], and the IASLC staging manual in thoracic oncology [7]. Lymphatic and vascular invasions were diagnosed by H&E, podoplanin staining, and Elastica van Gieson staining or CD31 or CD34 staining.

Preparation of tissue microarray (TMA) sections

The most representative histopathological region of the tumor was selected by a board-certified pathologist, and TMA paraffin blocks (4mm core) were made from the 346 specimens with a tissue microarrayer (Azumaya Medical Instruments, Tokyo, Japan). The tumors were graded into "conventional invasive adenocarcinoma" or "poorly differentiated ADC" (> 10% solid component in a core) in the TMA H&E sections.

Immunohistochemistry (IHC)

Immunohistochemical staining of TMA sections (5 microns thick) was carried out with a fully automated IHC stainer, Leica BOND-MAX™ (Leica Microsystems K.K., Tokyo, Japan). Bond Epitope Retrieval Solution 1 was used as a buffer for heat-induced epitope retrieval for 20 minutes. PRR11 staining was carried out with a 1:100 dilution of anti-PRR11 rabbit polyclonal antibody (HPA023923, Sigma-Aldrich Inc., St. Louis, MO). The immunoreaction was visualized through Bond Polymer Refine Detection. Hematoxylin was used for counterstaining. The sections were covered with glass using Malinol mounting medium.

Assessment of PRR11 immunostaining

In TMA cores, positive immunoreactivity of any proportion or intensity, except trace and pseudo-positivity, was evaluated on the basis of membranous and/or cytoplasmic staining patterns (Fig. 1).

Statistical analysis

Statistical analyses were carried out with the use of software packages of IBM® SPSS® Statistics Base 20.0, IBM® SPSS® Advanced Statistics 20.0, IBM® SPSS® Regression 20.0, and IBM® SPSS® Exact Tests 20.0 (IBM Japan, Tokyo, Japan).

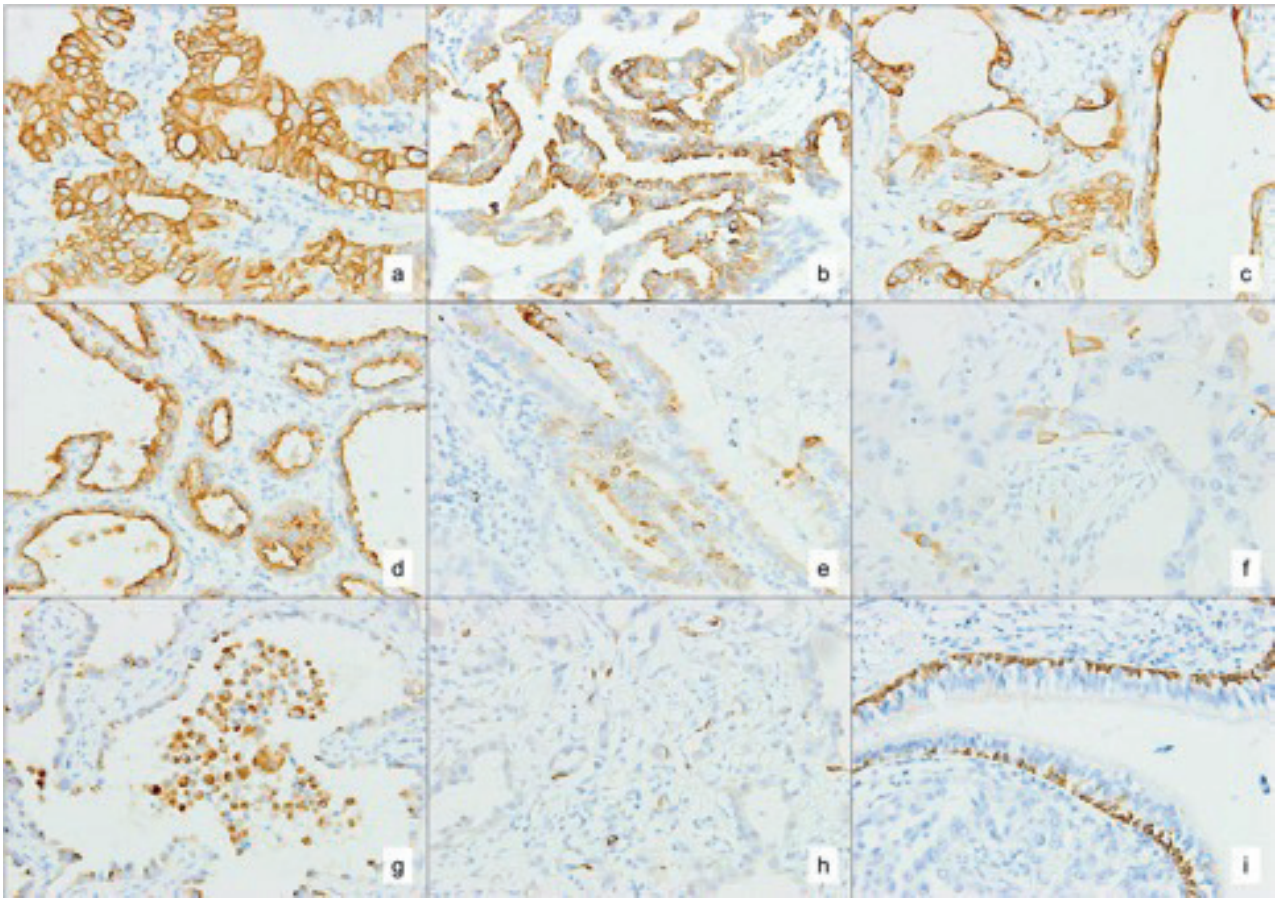
The difference of distribution in a 2 ´ 2 contingency table was analyzed by Fisher's exact test when two variables were independent. The difference of median and distribution between two independent groups was analyzed by the Mann-Whitney U test (two-sided). The correlation among clinicopathological factors was analyzed by the rank test for Spearman's correlation coefficient. Survival was estimated by the product limit method of Kaplan and Meier, and differences in survival were determined by the log-rank test (Mantel-Cox). A multivariate survival analysis was conducted by the direct entry method of Cox's proportional hazards regression model. In the two survival analyses, zero time was the date of surgical resection, and censoring was cancer death. $P < 0.05$ was considered statistically significant.

Results

Clinicopathological data of PRR11 immunostaining showed that the large diameter of whole size was significantly larger in PRR11+ than in PRR11- ($P = 0.03$) (Tab. I); however, no statistically significant differences were observed among the clinicopathological factors. No differences of PRR11 immunopositivity were identified from microscopic observation, among proliferating patterns or in tumor differentiation. No statistically significant correlation was noted between PRR11 and gender ($P = 0.37$), age ($P = 0.20$), Brinkman index ($P = 0.42$), serum carcinoembryonic antigen level ($P = 0.24$), site of occurrence ($P = 0.88$), affected lobe of occurrence ($P = 0.87$), treatment ($P = 0.34$), histopathological subtype ($P = 0.37$), large diameter of whole size ($P = 0.07$), lymph node metastasis ($P = 0.96$), thoracic cavity dissemination and/or malignant pleural effusion ($P = 0.62$), intrapulmonary metastasis ($P = 0.14$), pleural invasion ($P = 0.37$), lymphatic invasion ($P = 0.78$), vascular invasion ($P = 0.06$), adjuvant therapy ($P = 0.07$), recurrence ($P = 0.42$), survival ($P = 0.06$).

Univariate analyses of 5-year overall survival and 5-year disease-free survival, based on clinicopathological factors showed no statistically significant differences, regardless of decades-long separation of age, adjuvant chemotherapy or radiotherapy (Tab. II, Fig. 1, and Fig.

Fig. 1. PRR11 immunoreactivity of non-mucinous invasive adenocarcinoma of the lung. (a) to (d) strongly immunopositive to PRR11 with membranous and/or cytoplasmic pattern, (e) and (f) weakly immunoreactive to PRR11, (g) adenocarcinoma cells and alveolar macrophages sometimes stained by PRR11 with cytoplasmic granular pattern but considered immunonegative. (h) and (i) bronchoepithelial basal cells immunoreactive to PRR11 as an internal control.



2). PRR11 immunoreactivity might be a weak prognostic factor (Hazard ratio 1.49, $P = 0.05$). Cox's multivariate analysis of overall survival based on clinicopathological factors except age, location of occurrence, and adjuvant therapy, also revealed no statistically significant differences and that PRR11 immunoreactivity might be a weak prognostic factor (Hazard ratio 1.51, $P = 0.06$) (Tab. III).

Discussion

PRR11 is thought to play a role in cell cycle progression because silencing it in human cell lines through small interfering RNA causes S-phase and mild G2/M arrest with growth retardation, as well as reduced colony formation, cell migration, invasion and inhibition of tumor formation in nude mice [1 3 8]. The PRR11 gene composed of 10 exons is mapped to chromosome 17q22 [9], where the spindle- and kinetochore-associated protein 2 (SKA2) gene lies in a head-to-head orientation with the PRR11 gene, only 548 base pairs apart, on the opposite strand [2]. The gene pair PRR11 and SKA2 shares a bidirectional promoter that nuclear transcription factor Y binds to and directly transactivates. The major transcript of PRR11 is a deduced 360-amino acid protein with a calculated molecular mass of 40 kDa, and has an N-terminal bipartite nuclear localization signal, followed by a proline-rich region, a central zinc finger domain, and a second proline-rich region [1]. In the present study, quantitative RT-PCR experiments revealed that PRR11 was ubiquitously expressed in normal human tissues; the highest expression was found in the thymus and the ovary, whereas it was modest in the lung, and such. According to human data on protein localization, respiratory epithelia, urothelium and a subset of cells in the male genital tract show strong staining [10]. In this study, a majority of normal tissues displayed weak to moderate cytoplasmic positivity with additional membranous staining in some cases.

From the clinicopathological point of view, PRR11 protein expression is associated with aggressive phenotypes such as tumor invasion, lymph node metastasis, and advanced TNM stage, and reduced survival in cases of gastric cancer [11], hilar cholangiocarcinoma [12], and breast cancer [13]. With several coordinating genes,

Tab. I. PRR11 immunoreactivity and clinicopathological features of patients with non-mucinous invasive adenocarcinoma of the lung.

	PRR11-	PRR11+	*P*
Total number of subjects	216	130	-
Gender			
Men	126	85	0.21
Women	90	45	
Age [Median ± 2SD years]	67 ± 20	66 ± 18	0.47
Smoking history			
Yes	133	75	0.50
No	83	55	
Brinkman index (Cigarettes per day × years smoked) [Median ± 2SD]	375 ± 1398	400 ± 1204	0.95
Serum CEA level [Median ± 2SD ng/ml]	3.3 ± 25.9	3.6 ± 77.1	0.56
Location of ocurrence			
Right			
Uppper lobe	71	44	0.62
Middle lobe	20	7	
Lower lobe	42	28	
Left			
Upper lobe	50	35	
Lower lobe	33	16	
Treatment			
Partial resection/ segmentectomy	39	18	0.66
Lobectomy/ Sleeve lobectomy	175	111	
Bilobectomy	1	1	
Unilateral pneumonectomy	1	0	
Histolopathological subtype			
Conventional invasive adenocarcinoma	164	93	0.38
Poorly differentiated adenocarcinoma (Solid component >10 %)	52	37	
Large diameter of whole size [Median ± 2SD mm]	25 ± 26	30 ± 31	0.03
Lymph node metastasis			
None	159	96	0.87
In ipsilateral peribronchial, hilar and/or intrapulmonary lymph node(s)	32	17	
In ipsilateral mediastinal and/or subcarinal lymph node(s)	24	17	
In contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s)	1	0	
Thoracic cavity dissemination and/or malignant pleural effusion			
Yes	5	2	0.72
No	211	128	
Intrapulmonary metastasis			
None	212	124	0.12
In the same lobe	3	6	
In the different ipsilateral lobe	1	0	
In the contralateral lung	0	0	
Pleural invasion			
No pleural invasion and invasion beneath the elastic layer	156	88	0.50
Invasion beyond the elastic layer	26	23	
Invasion to the pleural surface	19	12	
Invasion into any component of the parietal pleura	15	7	
Lymphatic invasion			
Yes	88	55	0.82
No	128	75	
Vascular invasion			
Yes	82	63	0.06
No	134	67	
Adjuvant therapy			
Yes	78	60	0.07
No	138	70	

	PRR11-	PRR11+	*P*
Recurrence			
Yes	77	52	0.42
No	139	78	
Interval between surgery and recurrence [Median ± 2SD months]	13 ± 37	15 ± 28	0.41
Survival			
Alive	142	77	0.25
Dead	74	53	
Cause of death			
Cancer death	50	43	0.16
Death by non-cancerous disease	22	8	
Not available	2	2	
Interval between surgery and cancer death [Median ± 2SD months]	31 ± 40	30 ± 42	0.81
SD, standard deviation; CEA, carcinoembryonic antigen			

Tab. II. Univariate analyses of five-year overall and disease-free survival by PRR11 immunoreactivity.

	5-year overall survival rate	*P*	5-year disease-free survival	*P*
PRR11 immunostaining				
Positive	0.72	0.05	0.60	0.42
Negative	0.78		0.65	
Gender				
Men	0.71	< 0.01	0.57	< 0.01
Women	0.83		0.72	
Age				
≤ 80 years old	0.76	0.24	0.63	0.54
> 80 yeas old	0.61		0.57	
Brinkman index				
≤ 1000	0.79	< 0.01	0.67	< 0.01
> 1000	0.64		0.48	
Serum CEA level				
≤ 8 ng/ml	0.80	< 0.01	0.68	< 0.01
> 8 ng/ml	0.57		0.38	
Site of ocurrence				
Right	0.73	0.38	0.62	0.96
Left	0.80		0.64	
Affected lobe of ocurrence				
Upper/ midddle lobe	0.79	0.10	0.64	0.47
Lower lobe	0.70		0.62	
Histolopathological subtype				
Invasive adenocarcinoma	0.81	< 0.01	0.70	< 0.01
Poorly differentiated adenocarcinoma (Solid component > 10 %)	0.61		0.43	
Large diameter				
≤ 3 cm	0.85	< 0.01	0.75	< 0.01
> 3 cm	0.60		0.42	
Lymph node metastasis				
Yes	0.52	< 0.01	0.31	< 0.01
No	0.84		0.74	
Thoracic cavity dissemination and/or malignant pleural effusion				
Yes	0.14	< 0.01	0.00	< 0.01
No	0.77		0.64	
Intrapulmonary metastasis				
Yes	0.40	< 0.01	0.00	< 0.01
No	0.77		0.65	
Pleural invasion				
Yes	0.52	< 0.01	0.38	< 0.01

	5-year overall survival rate	*P*	5-year disease-free survival	*P*
No	0.86		0.73	
Lymphatic invasion				
Yes	0.56	< 0.01	0.39	< 0.01
No	0.89		0.79	
Vascular invasion				
Yes	0.60	< 0.01	0.42	< 0.01
No	0.87		0.78	
Adjuvant therapy				
Yes	0.74	0.68	0.59	0.31
No	0.77		0.65	
CEA, carcinoembryonic antigen				

Fig. 2. Overall survival distribution by PRR11 immunoreactivity. A weakly significant difference is observed by the log-rank test in terms of PRR11 immunoreactivity ($P = 0.05$).

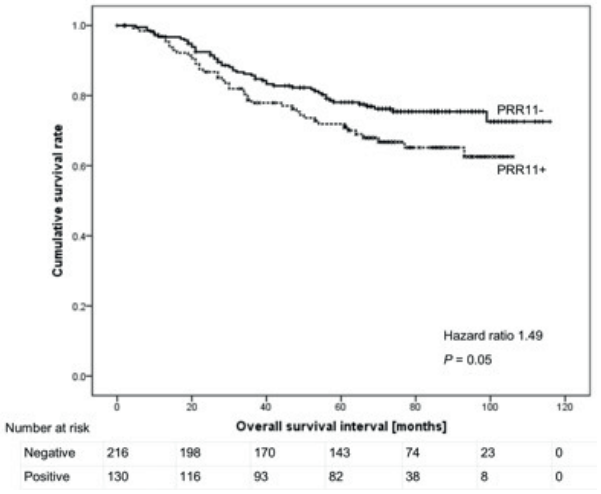
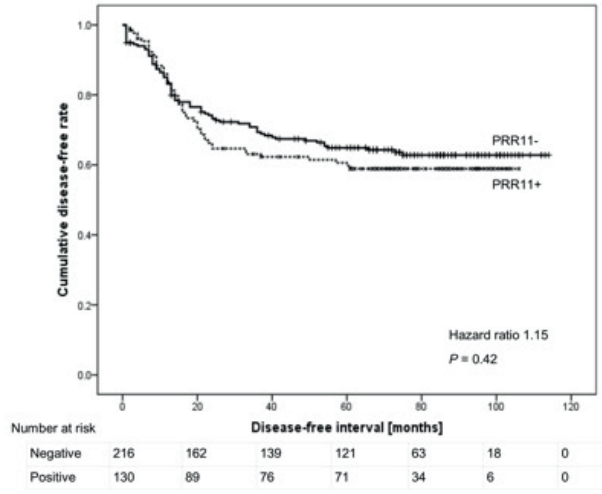


Fig. 3. Disease-free survival distribution by PRR11 immunoreactivity. No statistically significant difference is observed by log-rank test in terms of PRR11 immunoreactivity ($P = 0.42$).



Tab. III. Cox's multivariate analysis of prognostic factors by the direct entry method for non-mucinous invasive adenocarcinoma of the lung.

Factors	Unfavorable	Favorable	Hazard ratio	95% CI	*P*
PRR11 immunostaining	Positive	Negative	1.51	0.98-2.33	0.06
Gender	Men	Women	1.05	0.62-1.77	0.85
Brinkman index	> 1000	≤ 1000	1.77	1.05-2.96	0.03
Serum CEA level	> 8 ng/ml	≤ 8 ng/ml	1.05	0.64-1.70	0.86
Histopathological subtype	Poorly differentiated ADC	Invasive ADC	1.26	0.79-2.01	0.33
Large diameter	> 3 cm	≤ 3 cm	1.94	1.22-3.07	0.01
Lymph node metastasis	Yes	No	1.52	0.93-2.48	0.10
Thoracic cavity dissemination and/or malignant pleural effusion	Yes	No	3.93	1.48-10.44	0.01
Intrapulmonary metastasis	Yes	No	1.56	0.67-3.65	0.30
Pleural invasion	Yes	No	1.77	1.10-2.83	0.02
Lymphatic invasion	Yes	No	2.61	1.47-4.65	< 0.01
Vascular invasion	Yes	No	1.18	0.69-2.00	0.55

CEA, carcinoemkbryonic antigen; ADC, adenocarcinoma; CI, confidence interval

PRR11 in cancers may also function as an oncogene in the development, progression, and promotion of cancer cell invasion through cellular proliferation, cell migration, colony formation, tumor growth, and/or regulation of epithelial-to-mesenchymal transition [11-13].

PRR11 is also likely to play a critical role in both cell cycle progression and tumorigenesis in human lung cancer with other genes involved in cell cycle, tumorigenesis, and metastasis [1-3]. The PRR11-SKA2 bidirectional transcription unit is essential for the accelerated proliferation and motility of lung cancer cells [2]. Statistically significant differences have been observed in the analysis of overall survival through high to low expression of PRR11 and PRR11/SKA2 [1 2]. PRR11 may serve as a potential indicator in the diagnosis and/or treatment of human lung cancer. Therefore, we first evaluated the clinicopathological significance of PRR11 expression in primary lung cancer cases encountered in our hospitals.

Surprisingly, the significance of squamous cell carcinoma of the lung could not be analyzed, despite our following the same anti-PRR11 antibody and immunostaining method used in a previous study [1]. In our study, however, PRR11 immunoreactivity tended to associate with an aggressive phenotype (tumor size ($P = 0.03$), vascular invasion ($P = 0.06$), adjuvant therapy ($P = 0.07$)) and overall survival ($P = 0.05$) in non-mucinous invasive adenocarcinoma of the lung (Tabs. I, II), although there is little statistical difference in our results, particularly as compared with gastric cancer [11] and hilar cholangiocarcinoma [12]. PRR11 also has some effect on overall survival (Hazard ratio 1.51, $P = 0.06$) (Tab. III).

Conclusions

Our results suggest that PRR11 is a weak prognostic factor of overall survival of patients with non-mucinous invasive adenocarcinoma of the lung. Further studies are needed involving a larger number of lung adenocarcinoma cases.

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