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Original Article

Immunohistochemical screening combined FISH for accurate detection ros1 positive lung adenocarcinoma

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Abstract: Objective: ROS1 is a new driver gene of non-small cell lung cancer and a target of crizotinib. Right detection of ROS1 rearrangement rapidly as well as accurately is pivotal ROS1-targeted therapy. We analyze ROS1 immunohistochemistry (IHC) to detect ROS1 rearrangement in a large cohort of patients with primary lung adenocarcinoma (ACA) and compared with ROS1 fluorescent in situ hybridization (FISH), to explore the potential of combination of IHC screening and FISH as an optimal practice. Methods: We detected TMAs containing 332 primary lung ACA by ROS1 IHC (D4D6 clone) and FISH. Some discrepant and equivocal cases were further analyzed by RT-PCR. The EGFR mutation and ALK rearrangement were detected by nested PCR and FISH, respectively. Results: Of the 332 patients were evaluated as IHC score 3+ (n = 13), score 2+ (n = 5), score 1+ (n = 2), and score 0 (n = 312), respectively. FISH positive was 10 of 13 IHC 3+ and 2 of 5 IHC 2+ cases, whereas FISH negative was 3 of 13 IHC 3+, 3 of 5 IHC 2+ and all 314 IHC 1+/0 cases. One case of three IHC 3+ cases reported as FISH "negative" was actually ROS1 positive detected by ROS1 RT-PCR confirmed. Based on the final classification, ROS1 IHC 3+/2+ was 100% sensitive and 98.43% specific. However, FISH was 92.31% sensitive and 100% specific. In the 332 cases, the frequency of ROS1 rearrangement is 3.92%, 8.4% cases had ALK fusion, 34.9% cases had EGFR mutation and only one case had a concurrent ROS1 positive. Conclusion: IHC as the initial screening with FISH is an optimal approach to identify ROS1 positive patients to targeted therapy for this rare but clinically important subset of lung ACA.

Keywords: ROS1, lung adenocarcinoma, FISH, IHC, RT-PCR

Introduction

Lung ACA incidence is increasing and ranks a high mortality in malignant tumor around of the world [1]. Although the treatment has improved with radiation and chemotherapy, the survival of patients with lung adenocarcinoma remains poor [2]. Along with the molecular biology development and lucubrate study of tumor pathogenesis, a number of oncogenic drivers of non-small-cell lung cancer (NSCLC) have been found, include EGFR, ALK, RET, BRAF, PI3KCA, Her-2 and MET [3, 4]. The patients with NSCLC expressing oncogenic drivers have shown to be notable effective to small molecular tyrosine kinase inhibitors [5-7]. Recently, ROS1 rearrangement has become a new molecular subtype in NSCLC, and serve as an response to the targeted inhibitor crizotinib. Accurate determination of ROS1 status was the key to successful treatment of lung adenocarcinoma. In this study, we applied IHC and FISH to detect ROS1 rearrangement in a cohort of 332 cases in patients with primary lung adenocarcinoma, and some discrepant and equivocal cases were further detected by RT-PCR. EGFR mutational and ALK fusion was determined in all cases by nest PCR and ALK FISH, respectively.

Materials and methods

Clinical information and tissue microarrays

This study selected 332 patients who had received curative surgery at the Gansu Provincial Hospital and Lanzhou General Hospital of Lanzhou Military Area Command between January 2009 and January 2016. All the tumor samples were fixed in 10% neutral buffered formalin for 24-48 h and embedded in paraffin and routinely diagnosed as primary lung primary adenocarcinoma. All patients had not a previous history of anti-tumor therapy. Their basic characteristics date included age, gender, smoking status, metastases (TNM) stage. Non-smokers were defined as patients who had

smoked <100 cigarettes in their lifetime. Pathological diagnosis and staging was carried out according to the 2014 World Health Organization (WHO) classification and the tumor-node-metastasis staging system of the 7th edition of the American Joint Committee for Cancer (AJCC) staging system [8]. The histological subtypes of the adenocarcinomas were the predominant pattern was determined as lepidic, acinar, papillary, solid and invasive mucinous adenocarcinoma. All formalin-fixed paraffin-embedded (FFPE) tissue sections were reviewed by pathologists for confirmation of histology and assessment of tumor content.

The representative areas which contained more than 75% tumor cells were marked on individual paraffin blocks. Two cores of the tissue, each with a diameter of 2 mm, were patched from the representative region and were used for tissue microarrays (TMA). Serial sections of the TMAs were cut, and hematoxylin and eosin staining, FISH, and IHC were performed.

Reagents and instrument

The rabbit monoclonal anti-ROS1 antibody (D4-D6) was purchased from Cell Signaling Technology, Danvers, MA, USA. D4D6 antibody was 1:200 dilution. The ROS1 and ALK dual color break-apart rearrangement probe was purchased from ZytoVision GmbH, Bremerhaven, Germany. AmoyDx ROS1 Fusion Gene Detection Kit was purchased from Amoy Diagnostics, Xiamen, China. Benchmark XT stainer was purchased Roche Diagnostics, Switzerland.

ROS1 immunohistochemistry

ROS1 IHC was performed on 4 μm-thick FFPE TMA on benchmark XT stainer. The slides were deparaffinized and pretreated with CC1 for 60 min at 42°C. Then, the slides were washed with reaction buffer and incubated with the rabbit monoclonal anti-ROS1 antibody (D4D6), and the UltraView Universal DAB kit were used according to the manufacturers' instructions, followed by counterstaining with hematoxylin. The slides were then washed with mild detergent and dehydrated in a series of 70% to 100% gradient alcohol baths, cleared in a xylene bath, and cover slipped. The interpretation of IHC results was based on the a four-tier scoring system: 0, 1+, 2+ and 3+, IHC score 0 for completely no staining; score 1+ for faint, focal cytoplasmic staining; Score 2+ for moderate, cytoplasmic staining (also can partly present strong staining) in most of tumor cells, at least more than 50% tumor cells; score 3+ for strong, granular cytoplasmic staining; staining in most of tumor cells, at least more than 50% tumor cells.

ROS1 fluorescence in situ hybridization

FISH was performed on 5-μm-thick TMA slides of FFPE TMA using the break-apart probe specific to the ROS1 locus according to the manufacturer's instructions. Tumor cells, the nuclei of which had one or more FISH signals of each color, were counted. The rearrangement positive cells were defined as red and green signals by ≥2 signal diameters of deleted 5' ROS1 green signal observed in tumor cell nuclei, those with split signals or isolated green signals. ROS1-rearranged were defined as tumors harboring at least 50 tumor cells were scored for the presence of split signals. The specimens were considered as ROS1-rearranged if >15% of the cells showed split signals or single 3' signals.

RNA extraction and reverse-transcriptase polymerase chain reaction

Some discrepant and equivocal cases were then analyzed by RT-PCR. Total RNA was extracted from 10 sections of 5 μm-thick FFPE tissue using a RNeasy FFPE kit (Qiagen, Hilden, Germany). The ROS1 rearrangements were tested by RT-PCR using an AmoyDx ROS1 Fusion Gene Detection Kit according to the manufactures' instructions.

EGFR mutation and ALK rearranged status

EGFR mutation data of all patients were investigated. The mutation analyses of EGFR (exons 18, 19, 20, and 21) were tested using nested PCR. Genomic DNA was extracted from the FFPE tissue. ALK FISH was carried out FFPE tumors by using a break-apart to ALK.

Statistics

X^2 or Fisher's exact test was used to examine the association between the ROS1 rearrangements and the clinicopathological features. All of the statistical tests were performed using the SPSS 20.0 software (SPSS Inc. Chicago, IL). The *P* values were 2 tailed for all of the tests. Statistical significance was defined as $P<0.05$.

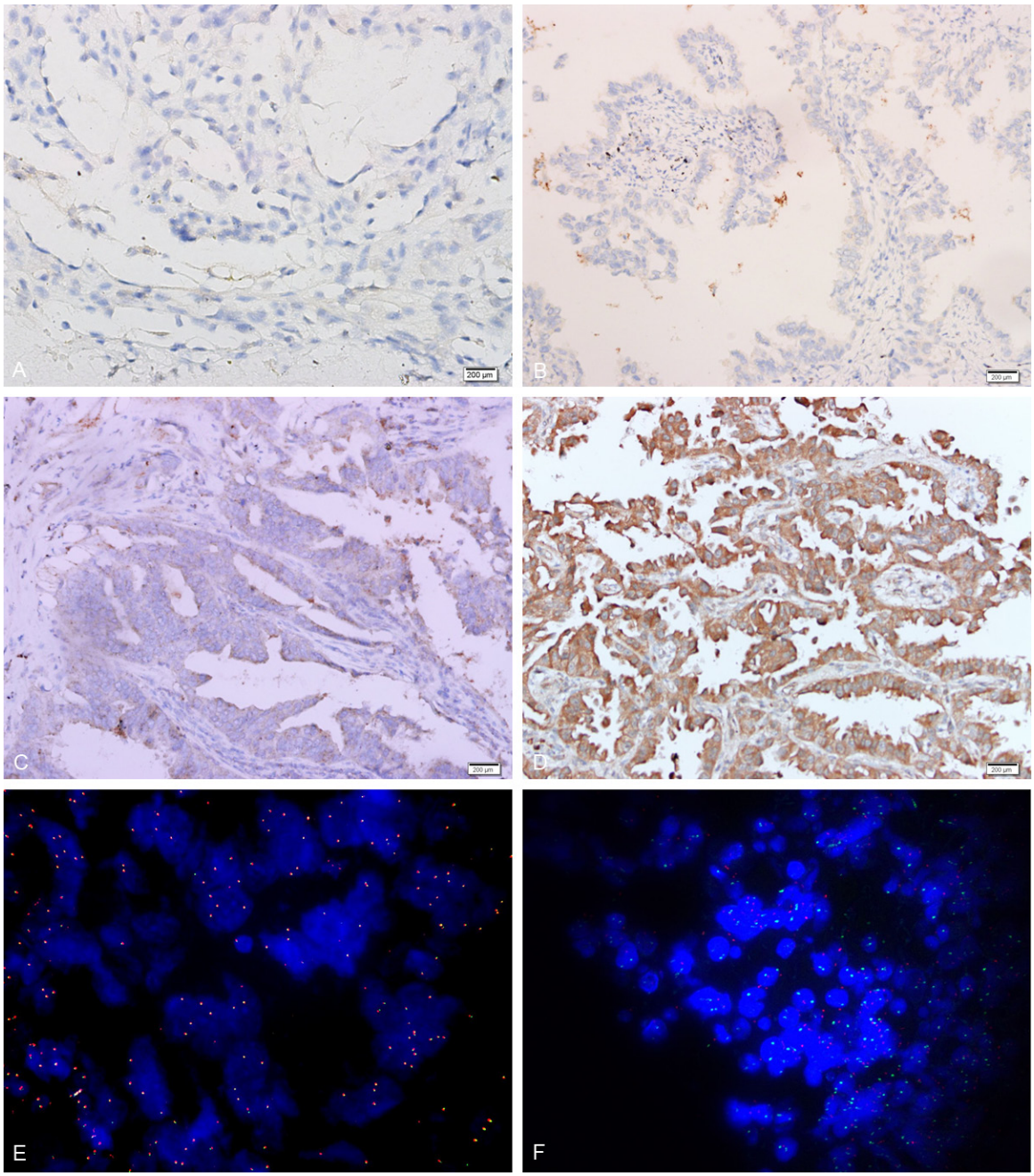

Figure 1. Representative images of ROS1 IHC and ROS1 FISH results. A: IHC score 0 for completely absent staining (×200); B: IHC score 1+ for weak, focal cytoplasmic staining in scattered tumor cells (×200); C: IHC score 2+ for moderate cytoplasmic staining (×200); D: IHC score 3+ for strong, diffuse (3+) staining in most tumor cells (×200); E: FISH-negative case with IHC3+ showing intact two fused signals per nucleus (×1000); F: FISH-positive case with IHC2+ showed split signals and/or deleted green signals (×1000).

Results

ROS1 IHC

The membrane and cytoplasm of tumor cells were stained with ROS1. Of 332 lung ACAs were detected ROS1 protein by IHC, ROS1 IHC was negative in 312 cases (**Figure 1A**). 20 cases expressed ROS1 protein (6.0%, 20/332), In total of 20 cases with ROS1 protein expression ranged from weak to strong, including weak expression (1+) in 2 cases (0.06%) (**Figure**

Table 1. Correlation of ROS1 IHC and FISH

	ROS1	FISH		Total
		Positive	Negative	
IHC	0	0	312	312
	1+	0	2	2
	2+	2	3	5
	3+	10	3	13
Total		12	320	332

Table 2. Six discrepant cases with ROS IHC 2+/3+ and FISH negative result

Case	IHC Score	FISH	RT-PCR	EGFR mutation	ALK-fusion
1	3+	-	+	WT	Negative
2	3+	-	-	WT	Negative
3	3+	-	-	WT	Negative
4	2+	-	-	WT	Negative
5	2+	-	-	WT	Negative
6	2+	-	-	WT	Negative

1B); moderate expression (2+) in 5 cases (1.5%) (**Figure 1C**) and strong ROS1 expression (3+) was identified in 13 cases (3.92%) (**Figure 1D**).

ROS1 FISH

FISH testing was undergone on the 332 lung ADCs, of which 13 cases (21.7%) were demonstrated ROS1 rearrangement (FISH-positive) and 319 cases were FISH-negative. Of FISH-positive cases, 12 cases observed split signal pattern and one cases showed unbalanced rearrangement, characterized by a loss of the red probe.

ROS1 FISH and IHC correlation

Thirteen cases with IHC positive showed an ROS1 rearrangement by FISH. Among ten cases with IHC 3+ were FISH positive, while three cases IHC 3+ were reported as FISH negative (**Figure 1E**). Of five cases with IHC2+, two cases showed were FISH positive (**Figure 1F**) and three cases were FISH negative. All the patients with score 1+/0 were FISH negative (**Table 1**). If the FISH test results were as the standard, the sensitivity and specificity of IHC for detection of ROS1 rearrangement were 100% and 99.06% respectively, when IHC staining score 3+ is considered to be positive. If IHC 3+/2+ were regarded as ROS1 positive and IHC 1+/0 as ROS1 negative, the sensitivity and specificity of ROS1 IHC with staining score of 2+/3+ were 100% and 98.12%, respectively.

Further examination of discrepant cases and the IHC 2+ cases

Three cases had discrepant ROS1 IHC 3+ and FISH "negative" results. Two cases were ROS1 negative by RT-PCR. The other case had ROS1 rearrangement detected below the 15% cutoff value (12%); however, it showed positive by RT-PCR. The 5 IHC with 2+ cases was further assessed by ROS1 RT-PCR, and 2 FISH positive cases were reported as ROS1 positive by RT-PCR, 3 FISH negative cases were ROS1 RT-PCR negative (**Table 2**). Considering the above results, we determined 13 patients to contain ROS1 rearrangements in 332 patients, of which 11 cases showed IHC 3+, 2 cases showed IHC 2+. All the 11 cases with IHC3+ cases were regarded as ROS1 positive. Among 5 cases with IHC 2+, 2 cases were ROS1 positive and 3 cases were ROS1 negative, respectively. So ground on final ROS1 status classify, the sensitivity and specificity of ROS1 IHC 3+/2+ was 100% sensitive and 98.43% specificity, However, FISH were 92.31% and 100%, respectively.

Clinicopathological features of ROS1-positive patients

Thirteen cases were confirmed with ROS1 rearrangement by two methodologies. There was no significant difference in age, sex, smoking and pathological stage between ROS1-positive and ROS1-negative cases (**Table 3**). The ROS1 rearrangement cases included eight women and five men with mean age of 54 years (range, 41-73 years). Non-smokers ROS1 fusion gene positive rate 4.63% (7/151) higher than that of smokers 3.3% (6/181), The predominant histological subtype of ROS1-positive patients was solid in 6/14 of cases and papillary in 5/14. In this study, ALK arrangement results were available for 28 patients by FISH. None of the ALK fusion positive cases had a concurrent ROS1 positive. 116 cases were detected EGFR mutation by nested PCR. EGFR mutation was presented at a frequency of 34.9% (116/332); one of thirteen ROS1 positive cases had a concurrent EGFR mutation.

Table 3. Clinical characteristics of patients with ROS1-positive ACAs

Variables	Total (n = 332)	ROS1-rearrangment		*P*
		(+) (n = 13)	(-) (n = 319)	
Age (year)				
≤60	157 (47.28%)	7 (2.11%)	150 (45.18%)	0.629
>60	175 (52.72%)	6 (1.81%)	169 (50.90%)	
Sex				
Male	177 (53.31%)	5 (1.51%)	172 (51.81%)	0.274
Female	155 (46.69%)	8 (2.41%)	147 (44.28%)	
Smoking				
Never	151 (45.48%)	7 (2.11%)	144 (43.37%)	0.537
Ever	181 (54.52%)	6 (1.81%)	175 (52.71%)	
Stage				
I+II	187 (56.33%)	5 (1.51%)	182 (54.82%)	0.185
III+IV	145 (43.67%)	8 (2.41%)	137 (41.27%)	
EGFR				
Mutation (+)	116 (34.94%)	1 (0.30%)	115 (34.64%)	0.071
Mutation (-)	216 (65.06%)	12 (3.61%)	204 (61.45%)	
ALK				
Fusion (+)	28 (15.96%)	0 (0%)	28 (15.96%)	0.039
Fusion (-)	304 (21.08%)	13 (3.92%)	291 (87.65%)	

Discussion

ROS1 (also known as c-Ros oncogene 1), a receptor tyrosine kinase, has high homology with ALK in its protein kinase domain [3]. ROS1 chromosomal rearrangements were first described in glioblastomas [9, 10]. Recently, ROS1 rearrangement has emerged in a variety of tumor cell [11-13]. In 2007, Rikova et al [14] discovered ROS1 rearrangement as potential oncogenic drivers in primary NSCLC. Studies have described ROS1 rearrangements frequency approximately 1-3% of lung adenocarcinomas [15-17]. Preclinical data suggest that the patients of NSCLC with ROS1-rearranged respond to ALK inhibitors [18, 19]. Very recently, the clinical trial [20] showed a prominent inhibition of this molecular subclass by crizotinib. In March 2016, the US food and drug administration (FDA) approved crizotinib to treat patients with advanced (metastatic) NSCLC whose tumors have an ROS1 gene alteration, crizotinib is the first and only FDA approved treatment to patients with ROS1 or ALK positive NSCLC [21]. A subset of patients with lung ACA must be selected who will benefit from molecular therapy. Therefore, these date underscore accurately detecting ROS1 status of lung adenocarcinoma is the key to successful targeted therapy.

So ROS1 detected should be routinely used. However, a generally accepted gold standard for ROS1 has not been established. Currently, there are the three primary methods of detecting ROS1 rearrangements, including RT-PCR, FISH and IHC. Each of these individual methods has both advantages and disadvantages. RT-PCR is a fast and sensitive method for testing of ROS1 rearrangement, but it can't detect new fusion of unknown type. FISH is the mainstay for detection of ROS1 rearrangements [22, 23], and FISH testing is expensive, time-consuming and requires specialized equipment and expertise. IHC is a cheap and widely used testing for fusion proteins; moreover, there has been preferred antibody (D4D6) for identification of ROS1 fusion proteins. D4D6 is a new ROS1 antibody clone, and has shown excellent sensitivity and specificity based on small number and mostly tissue microarray samples studies. Several recent studies [16, 24] had demonstrated that D4D6 accurately identify ROS1rearranged lung ACA compared to ROS1 FISH, the D4D6 antibody had a sensitivity and specificity of 95 to 100%. The current results suggest that IHC be a reliable screening tool to identification for ROS1 positive in lung ACA.

In this study, we examined a cohort of 332 lung adenocarcinoma cases by IHC and FISH, and compared the FISH results to ROS1 IHC. We adopted IHC assay with anti-ROS1 (D4D6) Rabbit monoclonal antibody for detection of the ROS1 rearrangement. Of the 13 cases identified as ROS1 IHC scores 3+, 10 cases demonstrated containing a ROS1 rearrangement by FISH. We then detected three discrepant cases, one of three cases had ROS1 rearrangement detected below the 15% cutoff value for positive (12%) by FISH, but it showed positive by RT-PCR. Sholl et al [25] tested 56 lung adenocarcinoma tissue by IHC and FISH, among 8 cases with IHC score 3+, 7 cases is FISH positive, IHC 3+ coincidence rate is as high as 87.5% (7/8) with FISH, if only 3+ (diffusely) expressing tumors are considered positive,

ROS1 IHC is 87.5% sensitive and 98% specific for the presence of a ROS1 translocation by FISH. Recently Yoshida et al [24] tested ROS1 gene rearrangement of lung ACA harboring ROS1 translocations by ROS1 antibodies and ROS1 FISH, ROS1 IHC had 94% sensitivity and 98% specificity. These above results support that the patients with IHC 3+ was higher probability of ROS1 rearrangement.

In our study, among 5 cases with IHC score 2+, two cases was positive by FISH testing, three negative cases were further examined by RT-PCR, no case was showed RT-PCR positive. Therefore, cases which were known as ROS1 IHC 2+ showed variable FISH/RT-PCR results, but there are only 0.1% (3/332) cases with IHC 2+ in our cohort. Case with IHC 2+ needs further FISH test. If FISH was failed or negative, additional RT-PCR or sequencing assay was required for final determination.

Our study observed good consistency between cases assigned ROS1 IHC scores 3+ and ROS1 positivity, as well as ROS1 IHC 1+/0 and ROS1 negativity. In our study, the cases which present the staining in inflammatory cells, reactive pneumocyte hyperplasia, mesenchymal tissue etc. Showing faint granular cytoplasmic staining was recorded as false staining in IHC 0 group, and all were confirmed negative by FISH. The result hinted that IHC had excellent consistency between two kinds of detection methods.

In this study, we describe the clinical features of ROS1-rearranged tumors. We identified 13 cases with ROS1 rearrangement from 332 lung adenocarcinoma patients. Patients with ROS1-altered lung ACA showed a trend toward disease presentation at a younger age (mean age was 54 years). Most of cases with ROS1 rearrangement were non-smoking patients with advanced Stage, although statistical comparisons in this study are limited by the small number of ROS1-altered tumors. Similar to prior reports [26, 27], the above results show that the majority of ROS1-rearranged tumors occurred in younger female patients with never-smoking history, and higher grade cancer, The dominant histological feature was papillary and solid growth pattern. Sholl et al [25] showed all ROS1 translocations occurred exclusive with three major recurrent oncogenic mutations, such as EGFR or KRAS mutation or ALK rearrangement in western people. In our study, one case with an ROS1 rearrangement had EGFR mutation, Rimkunas et al [16] reported 2 patients of ROS1-rearranged has been associated with EGFR mutations in a set of lung cancers from a Chinese population. The significance of dual alterations in different populations is unclear, and remains to be determined.

In conclusion, the ROS1 IHC using antibody D4D6 was a high sensitivity and specificity detection ROS1 gene fusion; IHC screening should be the first step in ROS1 testing operation, which can maximize the detection percentage of ROS1 positive case. The ROS1 IHC followed by auxiliary FISH is a reliable, economical approach to screen ROS1 positive lung adenocarcinoma. Some ROS1 IHC-positive but FISH-negative lung adenocarcinomas did harbor the translocation events as confirmed by RT-PCR.

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Disclosure of conflict of interest

None.

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