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Impact of Ki-67 and E-cadherin expression on lymphovascular invasion in upper urinary tract urothelial carcinoma

Uticaj ekspresije Ki-67 i E-kaderina na limfovaskalarnu invaziju kod urotelnog karcinoma gornjeg urotrakta

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Abstract

Bacground/Aim. Upper urinary tract urothelial carcinoma (UUT-UC) constitutes 5% of malignant neoplasms arising from transitional epithelium, but is more invasive than bladder cancer. Lzmphovascular invasion (LVI) is associated with biologically aggressive carcinoma and with occult metastases. The aim of this study was to investigate the correlation between LVI and immunohistochemical expression of two frequently routinely applied immunohistochemical biomarkers, Ki-67 and E-cadherin, in UUT-UC. **Methods.** The specimens from 106 patients with UUT-UC who had undergone nephroureterectomy were analyzed for pathologic parameters and LVI, while Ki-67 and E-cadherin expression were assessed by immunohistochemistry. **Results.** Ki-67 was overexpressed in 38% of the cases, while 45% of tumors demonstrated aberrant E-cadherin staining. The presence of LVI was significantly associated with tumor stage, grade, non-papillary growth, nodular invasion pattern, high Ki-67 labeling index and altered E-cadherin expression. Analyzing logistic regression models, we have shown that tumor properties such as stage, grade, growth and invasion pattern ($p < 0.001$), as well as the expression of Ki-67 and E-cadherin ($p < 0.001$) significantly predicted the presence of LVI. In the first model, only solid tumor architecture ($p < 0.05$) and nodular invasion pattern ($p < 0.05$) were significant predictors of LVI. In the second model, Ki-67 expression was found to improve the prediction of LVI ($p < 0.05$). **Conslusion.** Our results suggest that Ki-67 overexpression is an independent predictor of LVI in UUT-UC, indicating the progression of the disease. E-cadherin staining adds no valuable information to LVI probability assessment. This emphasizes the importance of Ki-67 staining of UUT-UC sections in routine pathological practice. Patients with Ki-67 overexpression, especially in solid tumors with nodular invasion, should be monitored more closely after surgery.

Key words:
urologic neoplasms; lymphatic metastasis; ki-67 antigen; cadherins; immunohistochemistry; predictive value of tests.

Apstrakt

Uvod/Cilj. Urotelni karcinom gornjeg dela urinarnog trakta (UUT-UC) čini 5% malignih neoplazmi koje potiču iz tranzicionalnog epitela, ali je invazivniji nego karcinom mokraćne bešike. Limfovaskularna invazija (LVI) je pokazatelj biološki agresivnog karcinoma, kao i okultnih metastaza. Cilj istraživanja bilo je ispitivanje povezanosti LVI i imunohistohemijske ekspresije dva često rutinski primenjivana biomarkera, Ki-67 i E-kaderina, u UUT-UC. **Metode.** Patohistološka analiza i određivanje prisustva LVI urađeni su na uzorcima UUT-UC dobijenih od 106 bolesnika podvrgnutih nefroureterektomiji. Ekspresija Ki-67 i E-kaderina procenjivana je imunohistohemijskom metodom. **Rezultati.** Prekomerna ekspresija Ki-67 zabeležena je kod 38% bolesnika, dok je 45% tumora pokazalo izmenjenu ekspresiju E-kaderina. Prisustvo LVI bilo je značajno udruženo sa stadijumom, gradusom tumora, nepapilarnim načinom rasta, nodularnim tipom invazije, visokim Ki-67 proliferativnim indeksom i aberantnom ekspresijom E-kaderina. Analizom logističkih modela regresije utvrđeno je da karakteristike tumora poput stadijuma, gradusa, načina rasta i invazije ($p < 0,001$), kao i ekspresija Ki-67 i E-kaderina ($p < 0,001$), značajno predviđaju prisustvo LVI. U prvom modelu samo solidna arhitektura tumora i nodularni način invazije ($p < 0,05$) predstavljali su značajne prediktore LVI. Drugi model utvrdio je da povećana ekspresija Ki-67 povećava verovatnoću za LVI ($p < 0.05$). **Zaključak.** Rezultati istraživanja ukazuju na to da prekomerna ekspresija Ki-67

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predstavlja nezavisni prediktor LVI u UUT-UC. Ekspresija E-kaderina ne doprinosi značajno proceni verovatnoće prisustva LVI. To naglašava značaj određivanja Ki-67 proliferativnog indeksa u ovom tumoru u rutinskoj patološkoj praksi. Bolesnike sa prekomernom ekspresijom Ki-67, posebno kod tumora sa solidnim rastom i nodularnim tipom invazije, trebalo bi pažljivije pratiti nakon hirurškog lečenja.

Ključne reči:
urološke neoplazme; neoplazme, limfne metastaze; ki-67 antigen; kaderini; imunohistohemija; testovi, prognostička vrednost.

Introduction

Upper urinary tract urothelial carcinoma (UUT-UC) constitutes only 5% of malignant neoplasms arising from transitional epithelium, but is more invasive and worse differentiated than bladder cancer [1]. Therefore, there is a strong need to acquire as precise as possible assessment of disease progression and tumor invasiveness in every individual case.

In UUT-UC, lymphovascular invasion (LVI) is associated with established features of biologically aggressive carcinoma, such as advanced stage, high tumor grade, metastases to lymph nodes, sessile tumor architecture, tumor necrosis, and concomitant carcinoma *in situ* [2, 3]. LVI may be associated with occult metastasis, and thus identify patients who are at increased risk of cancer recurrence and mortality despite apparently effective radical nephroureterectomy [2]. UUT-UC patients with LVI detected in primary tumor require to be followed-up more closely [4] and may be selected for postoperative adjuvant chemotherapy [3].

Abnormal cell proliferation, which results from deregulation of the cell cycle, is fundamental in tumorigenesis. Previous studies have demonstrated that cell proliferation, as detected by Ki-67 staining, is significantly associated with differentiation, tumor stage, tumor recurrence and prognosis in patients with UUT-UC [4, 5]. However, the expression of metastatic phenotype requires activation of additional effector genes or suppression of local inhibitors over and above those required for uncontrolled growth alone [6]. LVI, as a critical step in the systemic dissemination of cancer cells [7], may be tightly linked to inactivation of molecules involved in intercellular adhesion. Decreased expression of E-cadherin, the invasion suppressor, even in a limited fraction of neoplastic cells, is sufficient to allow the onset of invasion [8].

In urothelial carcinoma, the loss of membranous expression of E-cadherin has been unanimously attributed to an aggressive neoplastic phenotype. Besides the correlation between decreasing E-cadherin staining and the depth of invasion and higher grade in urothelial carcinoma [9–11], recent studies has indicated that immunohistochemical determination of E-cadherin expression may be a useful diagnostic aid and prognostic factor for UUT-UC [6, 9, 12].

Accurate estimates of the clinical stage and prognosis are essential for patient counseling and informed decision making. This is of great importance in UUT-UC treatment, since this cancer is invasive at diagnosis in over 65% of cases [13]. In respect of indisputable major significance of LVI, the aim of our research was to investigate the predictive impact of Ki-67 and E-cadherin expression on LVI in UUT-UC.

Methods

We examined formalin-fixed, paraffin-embedded specimens from 106 patients who had undergone open type nephroureterectomy with removal of bladder cuff for UUT-UC between 1995 and year 2010. The mean age of patients was 64.2 ± 10.8 years; the youngest patient was 32 years old, the oldest 87 years. There were 70 (66.0%) male and 36 (34.0%) female patients. Of the investigated UUT-UC, 78 (73.6%) had pelvic localization and 28 (26.4%) were tumors of ureter. During nephroureterectomy, enlarged lymph nodes were resected; no standard lymphadenectomy was undertaken. All cases of UUT-UC were diagnosed at the Institute of Pathology, Faculty of Medicine, Niš, Serbia.

The histological sections were processed by standard techniques, and stained with hematoxylin and eosin (HE). HE-stained slides were used to assess histological grade, pathologic stage, growth pattern of the tumor (papillary/solid), pattern of invasion (nodular/infiltrative), lymphovascular invasion and the presence of necrosis and metaplastic changes within the tumor. The 2002 Tumor Nodus Metasthasis (TNM) classification system [14] was used for pathologic staging, and the 2004 World Health Organization classification was used for histological grading of UUT-UC [15].

LVI was defined as the unequivocal presence of cancer cells in endothelium-lined lymphatic and vascular channels without underlying muscular walls [3]. By positive invasion was considered the presence of at least one well characterized malignant cell surrounded by endothelial cells. In the case of intravascular tumoral thrombus, it was usually floating completely free in the vascular lumen, with fibrin or plasma precipitate or erythrocytes around it. It was composed of tightly cohesive cells with a smooth border and a shrunk cytoplasm, and the cells in the periphery had a shell-like aspect [2]. Routine light microscopic examination was considered sufficient for LVI detection and no immunohistochemical staining was used to identify LVI particularly.

Immunohistochemical analysis

Tumors were analyzed using the mouse monoclonal antibody against E-cadherin (Takara Biomedical, Kyoto, Japan) at dilution of 1:1500, anti-Ki-67 antibody (Dako, Glostrup, Denmark) at 1:100 dilution, and a standard avidin–biotin immunoperoxidase complex detection system according to the manufacturer's protocol (Dako LSAB2R system-HRP). In brief, 4 μm tumor tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed in 0.1 M citrate buffer (pH 6.0) in a microwave oven. Endogenous peroxidase activity was quenched with 0.3% hydrogen

peroxide in methanol. After applying primary antibody, the slides were incubated for 60 minutes at room temperature. This step was followed by extensive washes with phosphate-buffered saline. Subsequently, sections were incubated with the secondary biotinylated antibody and with the streptavidin/avidin–biotin–peroxidase complex solution. Staining was developed using a liquid 3,3'-diaminobenzidine (DAB) substrate kit. Sections were counterstained with Mayer's hematoxylin. Negative controls were carried out by omitting the primary antibodies. The technique quality was assessed and areas with greater positivity were selected, avoiding peripheral area measurement, necrosis or artifact.

E-cadherin expression was scored according to the established criteria [16, 17] that classify tumors as normal if staining was similar to that of normal urothelium (> 90% of the cells are dyed). Aberrant tumor expression was defined as negative (complete absence of immunoreactivity), focally positive (< 10% of the cells were stained), and heterogeneous (10–90% of the cells were stained). In each case, it was determined whether the membrane or the cytoplasm was stained.

Ki-67 labeling index was calculated as the number of positive nuclei ×100 *per* the total number of nuclei in ten random high power fields (×400) in each tumor. This index was established by counting at least 2,000 cells in fields distant from necrotic areas. The results were classified into following groups: low Ki-67 expression (< 20% of cell nuclei stained positive for Ki-67) and Ki-67 overexpression (> 20%) [18].

Statistical analysis

All data analyses were processed using the Statistical Package for Social Sciences, version 15.0 statistical software (SPSS, Chicago, IL). A p value of 0.05 or less was considered indicative of a statistically significant difference. Continuous variables like age were represented as mean ± SD, and differences in the age of the patients among different groups were compared using Student's t-test. Categorical variables were analyzed by χ^2 and Fisher's exact test with Yates correction. Binary logistic analysis was performed in SPSS.

Results

Normal surrounding transitional cell epithelium displayed exclusively membranous expression of E-cadherin, with staining of the cell-cell borders. Fifty eight tumors (54.7%) maintained normal staining pattern, while 48 (45.3%) showed altered E-cadherin immunoexpression (Figure 1A). Within the aberrant group, the expression of E-cadherin was positive heterogeneous in 40 (37.7%), positive focally in 3 (2.8%) and negative in 5 (4.7%) of tumors. All tumors positive for E-cadherin displayed membranous positivity, while in 14 (13.2%) of the tumors with altered expression, significant cytoplasmic staining was also observed. High Ki-67 labeling index was found in 40 (37.7%) of the tumors (Figure 1B).

Lymphovascular invasion was detected in 37 (34.9%) UUT-UC (Figures 1c and d). The association of LVI with pathological features, Ki-67 labeling index, and E-cadherin expression of the examined tumors are shown in Table 1.

LVI demonstrated strong correlation with tumor characteristics associated with high grade ($\chi^2 = 19.485$; $p < 0.001$), advanced stage ($\chi^2 = 24.580$; $p < 0.001$) and architecture of the tumor, with higher LVI occurrence in sessile tumors with solid growth ($\chi^2 = 19.485$; $p < 0.001$), than in papillary neoplasms. Moreover, LVI was associated with nodular type of invasion ($\chi^2 = 30.883$; $p < 0.001$) and the presence of necrosis

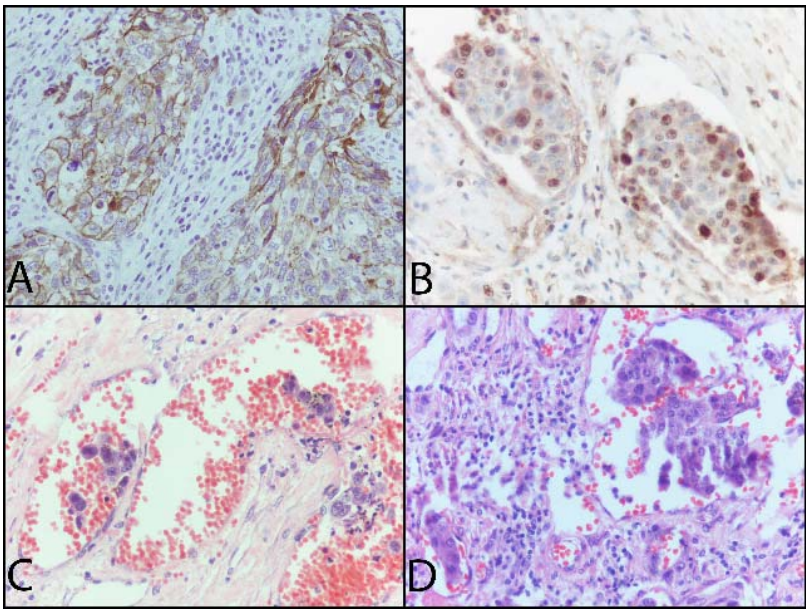

Fig. 1 – Representative photomicrographs of upper urinary tract urothelial carcinoma (UUT-UC), original magnification ×200: (A) Aberrant heterogeneous staining pattern of E-cadherin in UUT-UC; (B) Ki-67 immunostained nuclei of cancer cells in lymphovascular invasion; (C) and (D) Vascular invasion in UUT-UC infiltrating renal parenchyma, haematoxylin-eosin stain.

Table 1

The association of lymphovascular invasion with pathological characteristics, Ki-67 labeling index and E-cadherin expression and of UUT-UC

UUT-UC characteristics	LVI, n (%)		χ^2-test
	no	yes	*p*
Localization			5.835
pyelon	56 (52.8)	22 (20.8)	< 0.05
urether	13 (12.3)	15 (14.2)	
Multifocality			
no	47 (44.3)	26 (24.5)	0.052
yes	22 (20.8)	11 (10.4)	0.819
Grade			
low	40 (37.7)	5 (4.7)	19.485
high	29 (27.4)	32 (30.2)	< 0.001
Stage			
low	32 (30.2)	0 (0.0)	24.580
high	37 (34.9)	37 (34.9)	< 0.001
Growth pattern			
papillary	40 (37.7)	5 (4.7)	19.485
solid	29 (27.4)	32 (30.2)	< 0.001
Invasion pattern			
nodular	14 (13.2)	28 (26.4)	30.883
infiltrative	55 (51.9)	9 (8.5)	< 0.001
Necrosis			
no	47 (44.3)	15 (14.2)	7.543
yes	22 (20.8)	22 (20.8)	< 0.01
Ki-67 index			
< 20	53 (50.0)	13 (12.3)	17.805
> 20	16 (15.1)	24 (22.6)	< 0.001
E-cadherin staining			
normal	48 (45.3)	10 (9.4)	17.589
aberrant	21 (19.8)	27 (25.5)	< 0.001
E-cadherin type of expression			
negative	2 (1.9)	3 (2.8)	
focal	3 (2.8)	0 (0.0)	22.045
heterogeneous	16 (15.1)	24 (22.6)	< 0.001
homogeneous	48 (45.3)	10 (9.4)	
E-cadherin M/C			
negative	2 (1.9)	3 (2.8)	3.687
membrane	59 (55.7)	26 (24.5)	0.158
membrane + cytoplasm	8 (7.5)	8 (7.5)	

UUT-UC – upper urinary tract urothelial carcinoma; LVI – lymphovascular invasion.

($\chi^2 = 7.543$; $p < 0.01$), and was more frequently observed in neoplasms with ureteral than pelvic localization ($\chi^2 = 5.835$; $p < 0.05$). In addition, the presence of LVI was significantly associated with high Ki-67 labeling index (χ^2=17.805; $p < 0.001$) and the loss of homogeneous membranous E-cadherin staining ($\chi^2 = 17.589$; $p < 0.001$), as well as with type of E-cadherin expression ($\chi^2 = 22.045$; $p < 0.001$).

Tumor characteristics and the level of immunohistochemical expression of the investigated markers, for which the association with LVI was observed, were tested in logistic regression analysis models. Stage, grade, growth and invasion pattern ($\chi^2 = 35.113$; $p < 0.001$), as well as the expression of Ki-67 and E-cadherin ($\chi^2 = 17.765$; $p < 0.001$) significantly predicted the presence of LVI. However, in the first model only solid growth ($p < 0.05$) and nodular type of invasion ($p < 0.05$) were good predictors of LVI (Table 2). In the second model, only Ki-67 overexpression was found to improve the prediction of LVI ($p < 0.05$), while E-cadherin staining alteration did not demonstrate such quality (Table 3).

Discussion

UUT-UC is relatively rare disease, yet with a significant impact to mortality due to urothelial neoplasms. Poor prognoses have been reported for patients with tumors invading beyond the muscularis (pT3) and adjacent organs/perinephric fat (pT4), with 5-year survival rates of 54% and 19%, respectively [19]. Such outcomes indicate the importance of selecting patients at higher risk of disease-specific death, as well as adequate treatment strategies [20].

Invasion of tumor cells into blood vessels is an essential and important step in initiating metastatic cascade. LVI in primary tumor indicates that neoplastic cells have already invaded surrounding tissues [7]. Although further genetic altera-

Table 2

Binary logistic regression analysis of upper urinary tract urothelial carcinomas: tumor characteristics as model predictors

Tumor characteristics	B	S.E.	Sig.	Odds ratio	95.0% C.I.for odds ratio	
					lower	upper
Grade	-0.274	1.048	0.794	0.760	0.097	5.932
Stage	20.476	8.451E3	0.998	7.809E8	0.000	.
Growth pattern (solid)	1.692	0.864	0.050	5.432	1.000	29.514
Invasion pattern (nodular)	1.622	0.779	0.037	5.061	1.100	23.295
Constant	-42.502	1.690E4	0.998	0.000		

Table 3

Binary logistic regression analysis of upper urinary tract urothelial carcinomas: Ki-67 and E-cadherin immunohistochemical staining as model predictors

Parameters	B	S.E.	Sig.	Odds ratio	95.0% C.I.for odds ratio	
					lower	upper
Ki-67 index	1.459	0.607	0.016	4.303	1.309	14.151
E-cadherin expression (aberrant)	1.379	0.932	0.139	3.970	0.639	24.671
E-cadherin type of expression (heterogeneous)	0.092	0.428	0.830	1.096	0.474	2.536
Constant	-3.531	1.052	0.001	0.029		

tions are necessary for these disseminated cells to acquire the phenotype which will result in overt clinical metastases, LVI is unambiguous sign of disease progression and increased cancer mortality [2]. In recent study that comprised large international series of patients treated with radical nephroureterectomy for UUT-UC, LVI has been observed in 24% (349/1453) of the investigated patients [2]. In an investigation that *a priori* excluded lymph node positive patients from the study population LVI was found in only 13% (31/238) of patients [3]. In the present research LVI was detected in 34.9% of UUT-UC. The discrepancy may be due to study limitation caused by a relatively small number of investigated patients. This is a major reason for contemporary research of low-incidence diseases like UUT-UC to require multicentric collaboration.

There is a positive correlation between LVI and well established features of biologically aggressive UUT-UC [2]. Proportion of LVI increased with advancing tumor stage, high tumor grade, the presence of necrosis, sessile tumor architecture and the presence of squamous differentiation [2, 3]. The results of this study are in accordance with previous findings: LVI was associated with advanced stage, high tumor grade, solid growth and nodular invasion pattern. Moreover, the location of the primary tumor (renal pelvis/ureter) [21] and multifocality [22] were also proven to be independent variables affecting cancer-specific survival in UUT-UC. In the present study LVI was more frequently observed in ureteral tumors, but significant correlation between LVI and multifocality was not found.

LVI has been reported to be closely associated with metastases and a poor prognosis in urological malignancies [3, 23]. Recent study identified LVI as an independent predictor of clinical outcomes in non-metastatic patients who underwent radical nephroureterectomy for UUT-UC [2, 24]. In patients with localized UUT-TCC, LVI status may be a predictive marker for recurrence-free and cancer-specific survivals [3]. Considering the importance of LVI assessment in UUT-UC diagnosis, we aimed to investigate the correlations between LVI and immunohistochemical expression of two well-known and frequently routinely applied immunohistochemical biomarkers: Ki-67 and E-cadherin.

Ki-67 is expressed during all phases of the cell cycle except G_0, rendering cellular expression of Ki-67 as a measure of tumor proliferation [25]. In UUT-UC, a significant association was observed between the overexpression of Ki-67 and the pathologic stage and tumor grade [4, 26], which was confirmed in our study. In a study by Fromont et al. [5] Ki-67 was the only one of the numerous investigated markers significantly associated with tumor stage in UUT-UC. In addition, besides the pathologic stage, Ki-67 overexpression was found to be an independent predictor of cancer specific survival in UUT-UC [4]. Our findings demonstrated significant association of Ki-67 labeling index and the presence of LVI in primary tumor. In logistic regression analysis Ki-67 expression, besides tumor growth and invasion pattern, was found to be the variable that improves the prediction of LVI. Our results strongly indicate mitotic index as a significant independent predictor of LVI. This emphasizes the importance of Ki-67 staining of UUT-UC sections in routine pathological practice, regardless if LVI has been observed in HE slides. We also found that Ki-67 index was significantly and independently associated with E-cadherin expression and tumor high grade, which concurs with previous results [4, 5, 26].

Mutation and inactivation of E-cadherin enables metastasis through induction of an epithelial-to-mesenchymal transition, invasiveness, and anoikis resistance [8]. In human tumors, loss or reduction of E-cadherin expression can be caused by somatic mutations, chromosomal deletions, proteolytic cleavage, and silencing of the CDH1 promoter [8, 27]. Loss of E-cadherin

expression leads to a dissociation of cells from cohesive tissues and correlates with dedifferentiation and generation of invasive phenotype [9]. In the present study, preserved immunoreactivity of E-cadherin was recognized in 54.7% of the samples, which is similar to the results of previous investigations [4, 28]. Moreover, we observed that the majority of tumors with aberrant expression had a heterogeneous staining pattern with positive and negative areas within the tumor, in accordance with other studies [16, 29].

A reduced expression of E-cadherin has been linked not only with high grade and advanced stage, but also with disease progression and poor survival in UUT-UC [6, 12, 16]. Regardless of upper tract treatment modality, recurrence in the bladder consistently occurs in 20–50% of patients, necessitating the use of routine cystoscopic surveillance [30, 31]. Decreased expression level of E-cadherin was found to be the only independent predictor for intravesical recurrence [30]. However, despite the prognostic significance attributed to E-cadherin alteration, a recent study has not confirmed the association of E-cadherin with the parameters of biological aggressiveness and LVI [4]. Our findings implied that altered expression of E-cadherin is more frequent in UUT-UC with LVI. However, in logistic regression analysis aberrant E-cadherin staining was not recognized as independent predictor of LVI.

Conclusion

This study investigated the impact of Ki-67 and E-cadherin expression on lymphovascular invasion in primary UUT-UC. The choice to correlate these two markers to LVI was based on their well-established role in cancer growth, invasiveness and dissemination and, in addition, their availability in routine practice of immunohistochemical laboratories. Only Ki-67 expression was found to be a significant independent predictor of LVI in UUT-UC, while E-cadherin staining added no valuable information to LVI probability assessment. The evaluation of Ki-67 could identify a subset of patients with urothelial carcinoma of the upper urinary tract that might require closer follow-up after surgery.

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