Low PHLDA3 Expression in Oesophageal Squamous Cell Carcinomas Is Associated with Poor Prognosis

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Abstract. *Background/Aim: Patients with oesophageal squamous cell carcinoma (ESCC) have a poor prognosis. Akt has been associated with malignant potential in several cancers, including ESCC. Pleckstrin homology-like domain, family A, member 3 (PHLDA3) has been identified as a direct target gene of p53 and as a potent inhibitor of Akt activation. The present study investigated the role of PHLDA3 expression and its ability to predict prognosis in patients with ESCC who did not receive induction therapy. Materials and Methods: The intensity of PHLDA3 expression was immunohistochemically analyzed in tumor and adjacent normal tissue samples from 84 patients with ESCC, 22 who underwent endoscopic submucosal dissection and 62 who underwent thoracic oesophagectomy. Results: High expression of PHLDA3 was observed in 60 (71.4%) patients and low expression in 24 (28.6%). Cancer-specific (p=0.029) and disease-free (p<0.001) survival rates were significantly lower in the PHLDA3 low-than in the PHLDA3 high-expression group, and low PHLDA3 expression was an independent predictor of postoperative recurrence (relative risk (RR)=0.38; 95% confidence interval (CI)=0.166-0.78; p=0.0074). Conclusion: Low PHLDA3 expression in ESCC may be predictive of tumor recurrence suggesting that Akt activation may be a therapeutic target in ESCCs.*

Oesophageal squamous cell carcinoma (ESCC), the major histological type of oesophageal cancer in East Asian countries, is the eighth most common form of cancer in the world and one of the most lethal (1). Despite improvements in surgical techniques for reduced perioperative mortality and the introduction of multi-modal therapies, oesophageal cancer remains difficult to cure (2, 3). Patients with oesophageal cancer have a poor prognosis with a 5-year survival rate less than 20% (4). Tumor markers that could predict tumor progression, recurrence or overall prognosis would, therefore, be useful. Since conventional therapeutic methods have been limited in their effects on treatment outcomes, innovative strategies for treating ESCCs are being explored, especially those that are molecularly targeted.

Akt and its downstream agents have been recently reported to be important in ESCC tumorigenesis. The phosphatidylinositol-3 kinase (PI3K)-Akt signaling pathway plays a critical role in human cancer cell growth, survival and proliferation (5-9). Akt translocates to the cell membrane, where its pleckstrin homology (PH) domain interacts with PI (3,4,5)P3, converted from PI (4,5)P2 by PI3K, resulting in Akt phosphorylation on Thr308 and Ser473. This activated Akt transduces downstream survival signals, promoting carcinogenesis (6, 10). Activation of the PI3K/Akt pathway has also been reported in oesophageal cancers, promoting cancer cell survival, tumorigenicity and metastasis (11-13). Inhibition of Akt activation may, therefore, be a target of treatment of ESCCs. Although the mammalian target of rapamycin complex (mTORC) PP2A and PHLPP have been reported to inhibit Akt, pleckstrin homology-like domain, family A, member 3 (PHLDA3), which is directly targeted by p53, was shown to potently inhibit Akt activation (10, 14-18).

p53 negatively regulates Akt through several p53 target genes, including *PTEN* and *PHLDA3* (19). PHLDA3 induced by p53 directly inhibits Akt binding to PIP3 at the cell membrane resulting in cell apoptosis. PHLDA3 expression was found to inhibit Akt translocation to the plasma membrane and subsequent Akt phosphorylation and activation. Repression of PHLDA3 was found to enhance Akt activation and inhibit p53-mediated apoptosis. PHLDA3 was also found to inhibit anchorage-independent cell growth, with *PHLDA3* expression being frequently lost in human endocrine tumors.

Despite its identification as a tumor suppressor gene, the clinicopathological significance of PHLDA3 expression in

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Key Words: Oesophageal cancer, PHLDA3, Akt, prognostic factor.

human ESCCs remains unclear. This study was designed to assess the clinical significance of PHLDA3 in ESCCs, by immunohistochemically assessing PHLDA3 expression in ESCCs and by analyzing the relationships between PHLDA3 expression with clinicopathological factors and patient survival.

Materials and Methods

Patients' characteristics. Primary ESCC tissue samples were obtained from 84 consecutive patients who underwent curative resection for oesophageal cancer at the Dokkyo Medical University Hospital between May 2009 and September 2012. None of the patients received induction therapy and none had evidence of distant metastasis. Oesophageal cancer was classified according to the 7th edition of the TNM classification of the International Union against Cancer. Patient demographic and clinical characteristics are summarised in Table I. Clinical follow-up data, including cancer-specific survival (CSS) and disease-free survival (DFS), were available for all patients. All patients provided written informed consent and the study protocol was approved by our institutional review board.

The mean patient age was 68.2±9.7 years (range, 40 to 86 years). The 84 patients included 72 males (85.7%) and 12 females (14.3%), with 22 (26.2%) undergoing endoscopic submucosal dissection (ESD) and 62 (73.8%) undergoing thoracic oesophagectomy. Primary tumors were located in the cervical oesophagus (Ce) of 2 patients (2.4%), the upper thoracic oesophagus (Ut) of 10 (11.9%), the mid-thoracic oesophagus (Mt) of 44, (52.4%) and the lower thoracic oesophagus (Lt) of 28 (33.3%). Out of these 84 patients, 61 were negative (72.6%) and 23 positive (27.4%) for lymph node metastasis. Staging showed that 15 patients (17.9%) were Stage 0, 38 (45.2%) were Stage I, 13 (15.5%) were Stage II and 18 (21.4%) were Stage III. CSS was defined as the time interval from oesophageal resection to the last contact or death and DFS was defined as the time interval from oesophageal resection to first recurrence. Patients were followed-up after resection every 2 to 3 months until November 29, 2013. Median follow-up time was 877.6 days (range=208-1,647 days).

Immunohistochemistry. Primary ESCC was diagnosed by two pathologists at our Institution after examination of tissue specimens. Tissues were sectioned to a thickness of 4 μm, fixed in 10% formalin, embedded in paraffin, deparaffinised with xylene, hydrated through a series of graded alcohol solutions and immersed in 3% hydrogen peroxide in absolute methanol for 30 min. The sections were washed with distilled water and phosphate-buffered saline (PBS; pH 7.4). Antigens were retrieved by immersion in preheated 0.01 M citrate buffer (pH 6.0) and heating in a microwave at 98°C for 20 min. The sections were incubated with normal goat serum for 30 min to minimize background staining, followed by incubation with a rabbit polyclonal anti-PHLDA3 antibody (1:20; Atlas Antibodies AB, Stockholm, Sweden) at room temperature for 1 h in a high humidity chamber. After washing in PBS, the sections were incubated with biotinylated anti-rabbit IgG for 30 min at room temperature, followed by incubation with an avidin-biotin peroxidase complex solution (VECTASTEIN® ABC KIT; Vector laboratories, Burlingame, CA, USA). Peroxidase activity was visualized by incubation in 0.02% 3,3'-diaminobenzidine tetrahydrochloride solution containing 0.005% H_2O_2. All slides were counterstained with haematoxylin, dehydrated and mounted in aqueous medium. PHLDA3 staining was evaluated by two pathologists blinded to the clinical and pathological background of

Table I. *Relationship between PHLDA3 expression and clinicopathological features*.

Factors	High expression n=60	Low expression n=24	p-Value
Age	69.3±9.68	65.5±9.56	0.11
Gender			
Male	53	19	0.28
Female	7	5	
Location			
Cervical/ Upper	10	2	0.26
Middle	33	11	
Lower	17	11	
Depth			
Tis	15	0	<0.001*
T1	32	4	
T2	6	3	
T3	6	16	
T4	1	1	
Lymph node metastasis			
Absent	52	9	<0.001*
Present	8	15	
Lymphatic invasion			
Absent	46	7	<0.001*
Present	14	17	
Venous invasion			
Absent	37	5	<0.001*
Present	23	19	
Stage			
0, I	46	3	<0.001*
II, III	14	21	

*$p<0.05$.

the patients. Normal epithelium tissue, present on each slide, was used as internal control. Low PHLDA3 expression was defined as identical or weaker staining than the internal control and high PHLDA3 expression was defined as stronger staining than the internal control.

Statistical analysis. Correlations between PHLDA3 expression and clinicopathological parameters were assessed using Chi-square tests and *t*-tests. Survival parameters were determined using the Kaplan-Meier method and compared using the log-rank test. Univariate and multivariate survival analyses were performed using the Cox proportional hazards regression model. A probability (*p*) <0.05 was considered statistically significant. All statistical analyses were performed using the JMP 5.0 software (SAS Institute Inc., Cary, North, Carolina).

Results

Expression of PHLDA3 in ESCC tissues. High PHLDA3 expression was observed in 60 (71.4%) out of the 84 ESCC samples and low expression in the other 24 (28.6%); representative results are shown in Figure 1. In normal squamous epithelium, PHLDA3 was detected in the cytoplasm of the basal layer (Figure 1b).

Table II. *Results of multivariate analysis of clinicopathological factors affecting disease-free survival rate following surgery.*

Clinicopatliologic variable	Univariate analysis			Multivariate analysis		
	RJR	95%CI	*p*-Value	RR	95%CI	*p*-Value
PHLDA3 expression (high/low)	0.32	0.15-0.6	<0.001	0.38	0.166-0.78	0.007
Age (<68/≥68)	0.8	0.45-1 43	0.44	-	-	-
Gender (male/female)	0.71	0.16-1 61	0.47	-	-	-
Depth (Tis, T1/T2, T3)	1.97	1.1-3.83	0.021	2.73	0.37-17.5	0.31
Lymph node metastasis (negative/positive)	2.57	1.44-4.98	0.0015	2	0.99-4.47	0.053
Lymphatic invasion (negative/positive)	1.61	0.91-2.96	0.1	-	-	-
Venous invasion (negative/positive)	1.89	1.03-4.0	0.04	1.56	0.67-3.93	0.3

RR: Relative risk; CI: Confidence interval. *$p<0.05$.

Clinical significance of PHLDA3 expression in ESCC patients. The correlations between PHLDA3 expression and clinicopathological characteristics of ESCC patients (age, gender, location, tumor depth, lymph node metastasis, lymphatic invasion, venous invasion and TNM stage) are shown in Table I. There were significant correlations between PHLDA3 expression and tumor depth ($p<0.001$), lymph node metastasis ($p<0.001$), lymphatic invasion ($p<0.001$), venous invasion ($p<0.001$) and TNM stage ($p<0.001$).

Kaplan–Meier analysis showed that CSS ($p=0.029$) (Figure 2) and DFS ($p<0.001$) (Figure 3) were significantly lower in the PHLDA3 low than in the PHLDA3 high group. The 4-year CSS rates of the high and low expression groups were 96.3% and 81.3%, respectively, and their 4-year DFS rates were 94.2% and 55.6%, respectively.

Univariate analysis showed that low expression of PHLDA3 was a significantly prognostic factor of poor survival, but multivariate analysis showed that low PHLDA3 expression was not an independently prognostic factor of poor survival (data not shown). In contrast, low PHLDA3 expression was an only independent predictor of postoperative recurrence (relative risk (RR)=0.38: 95% confidence interval (CI)=0.166–0.78: $p=0.0074$) (Table II).

Discussion

This study showed that the level of expression of PHLDA3 was higher in early than in advanced ESCC and that low expression of PHLDA3 was associated with ESCC. Interestingly, low PHLDA3 expression was an independent predictor of tumor recurrence but not of patient prognosis.

Immunohistochemical analysis of PHLDA3 expression in prostate and breast cancer showed strong PHLDA3 immunostaining in normal glands but weak or absent immunostaining of prostate and breast cancer cells (20, 21). The PHLDA3 gene was frequently lost and its expression down-regulated in large cell neuroendocrine carcinomas (15).

These findings, together with our results in ESCC, suggest that PHLDA3 may act as tumor suppressor in many types of cancer.

We found that low PHLDA3 expression in ESCCs was associated with tumor progression and recurrence and with poor patient prognosis. Although PHLDA3 expression in lobular breast cancer was reported associated with nuclear accumulation of p53 (21), this study, to our knowledge, is the first to assess the relationships between *PHLDA3* expression in ESCCs and clinicopathological factors, patient prognosis and tumor recurrence. The down-regulation of PHLDA3 expression in ESCCs is likely due to its involvement in the Akt pathway. Akt, which is activated by receptor tyrosine kinases (RTKs), has been found to regulate cell survival by the phosphorylation of numerous downstream substrates that directly or indirectly control the apoptotic machinery (5, 6). Akt plays a key role in ESCC tumorigenesis and development (22). Indeed, our findings suggest that activation of Akt followed by repression of PHLDA3 is associated with ESCC progression and patient prognosis.

PHLDA3 expression has been reported to depend on transcriptional regulation by *p53* and loss of heterozygosity (LOH) of PHLDA3 (11). Point mutations in p53 have been observed in at least 50% of oesophageal carcinomas (23) but this, and its effect on p53 expression, would not be sufficient to explain the down-regulation of PHLDA3 expression in ESCCs. In contrast, Akt has been reported to suppress mouse double minute 2 (MDM2)-mediated p53 expression in ESCCs (13). Akt activation in clinical specimens decreases the induction and expression of p53-dependent PHLDA3. Gains of chromosome 1q32, which includes *PHLDA3*, have also been reported in ESCCs (24-26). These results are consistent with our finding, that most ESCC tissues express higher levels of PHLDA3 than normal oesophageal tissues. The relevance of LOH of *PHLDA3* and its low expression to tumor progression, however, are unclear. Detailed analyses of micro-RNAs and PHLDA3 methylation are necessary to determine the mechanisms by which PHLDA3 promotes ESCC progression and recurrence.
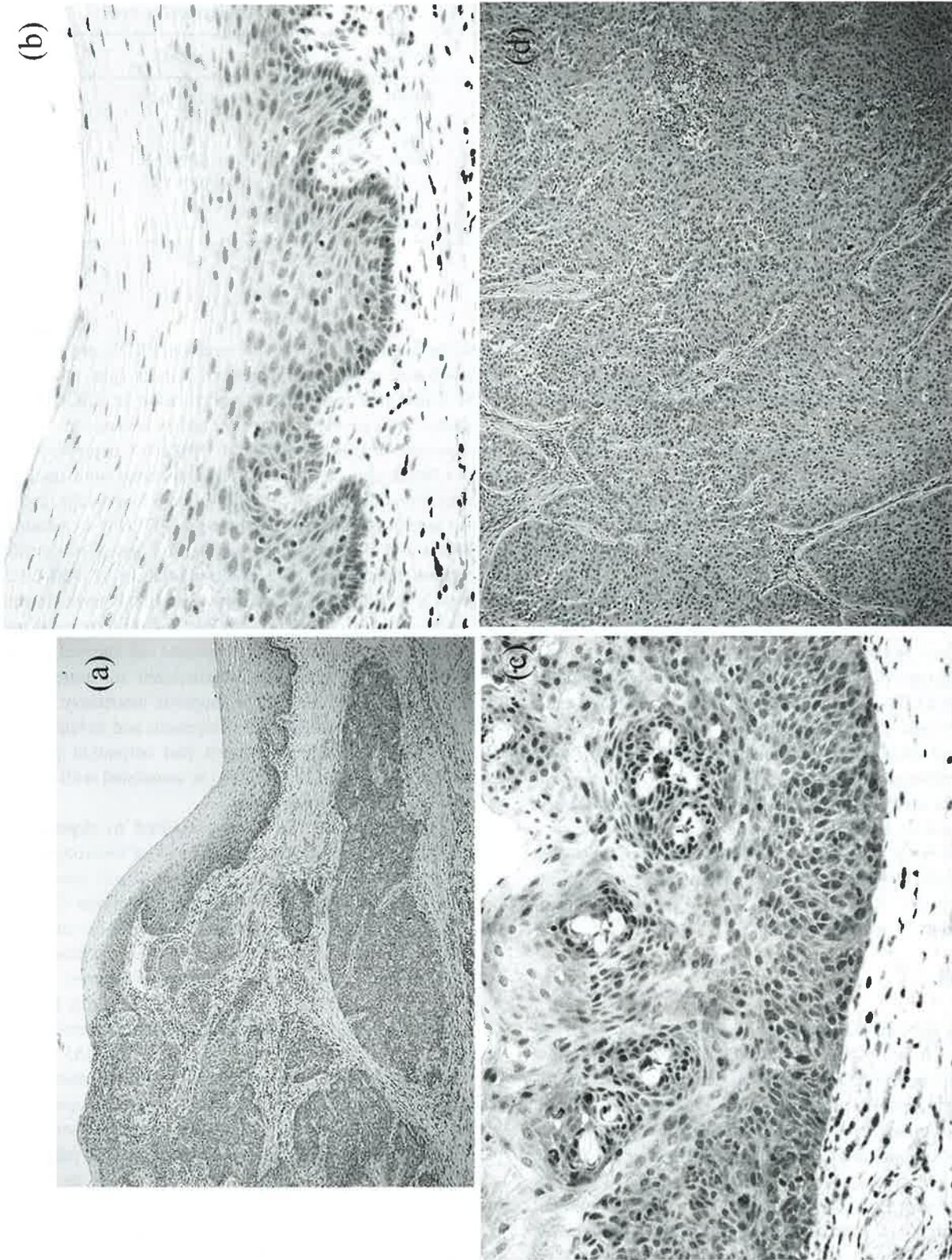
Figure 1. a-d. Representative photomicrographs of tissue sections immunostained for PHLDA3. a, Normal oesophageal squamous epithelium and squamous cell carcinoma showing expression of PHLDA3 (×100). b, Normal squamous epithelium showing expression of PHLDA3 (×400). c, A primary oesophageal tumor (T1a) showing high PHLDA3 expression (×400). d, A primary oesophageal tumor (T3) showing no PHLDA3 expression in the cytoplasm of cancer cell nests (d:×100).
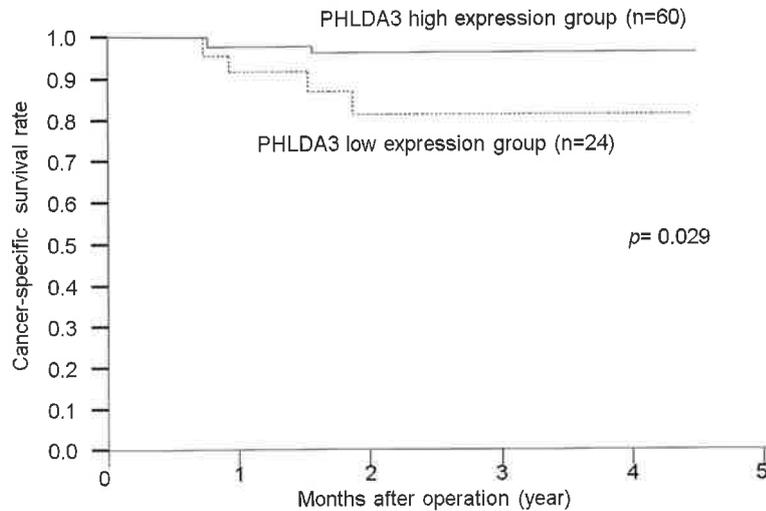
Figure 2. *Relationship between PHLDA3 expression and postoperative cancer-specific survival. The 4-year cancer-specific survival rate was significantly lower in ESCC patients with low compared to those with high PHLDA3 expression (p=0.029).*
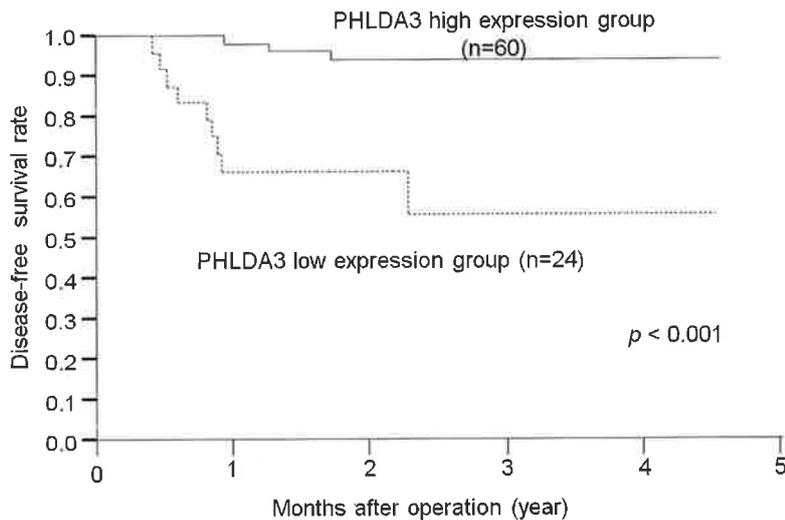

Figure 3. *Relationship between PHLDA3 expression and postoperative disease-free survival. The 4-year disease-free survival rate was significantly lower in ESCC patients with low compared to those with high PHLDA3 expression (p<0.001).*

PHLDA3 has been identified as a tumor suppressor in breast cancer and large cell neuroendocrine carcinoma. Similarly, clinical stage progression of ESCCs was accompanied by decreased PHLDA3 expression. Over-expression of PHLDA3 *in vitro* increased cell death (sub-G_1) and induced p53-dependent apoptosis (15). Down-regulation of PHLDA3 expression in cells transfected with siRNAs targeting PHLDA3 also decreased p53-dependent apoptosis (15). Aberrant activation of Akt has been observed in many tumor types and the tumor suppressive activity of PHLDA3 was shown to be due to its inhibition of Akt (6, 15). Akt inhibition has been shown to suppress the growth of Akt-activated cancer cells and also found to promote carcinogenesis and malignant grade of oesophageal cancer (5-11, 13). Because PHLDA3 is a potent inhibitor of Akt, the specific inhibition of Akt by PHLDA3 may be useful in treating patients with Akt-activated ESCCs.

Conclusion

In conclusion, the levels of expression of PHLDA3 were lower in advanced than in early ESCCs and normal oesophageal epithelium, with low PHLDA3 expression correlating with

postoperative tumor progression and recurrence, as well as poor patient prognosis. PHLDA3 expression may be a useful marker in patients with high risk ESCCs and an effective therapeutic target for Akt-activated ESCCs.

Conflicts of Interest

The Authors declare that they have no conflict of interest.

Acknowledgements

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