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ORIGINAL RESEARCH

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TfR associated with cervical cancer with or without neoadjuvant chemotherapy

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Abstract

The objectives of this study was to examine the role of transferrin receptor (TfR) in the treatment of cervical cancer. Demographic and cancer-specific data were collected prospectively. Cancerous and adjacent mucosa tissues with neoadjuvant chemotherapy (NACT) or surgery alone were collected. TfR mRNA and protein expression was measured by Quantitative reverse transcription PCR (RT-qPCR) and immunoblots. We measured the cell viability by the MTT (3- [4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay with or without TfR-siRNA transfection. All 42 patients aged 49.31 $\pm$ 6.30 years with locally advanced cervical cancer were included: NACT (n = 22), surgery alone (n = 20). Quantitative results showed that the levels of TfR mRNA & protein in cancerous tissues were higher than those in adjacent mucosal tissues. We also demonstrated that the levels of TfR mRNA and protein were lower in tumor tissues collected from patients with NACT than in those collected from patients without NACT. We also observed that silencing of the TfR gene suppressed the survival of HeLa cells (48 h, $p < 0.05$). Our findings suggest a potential value for TfR as a diagnosis or evaluation marker for cervical cancer. The present study suggests that si-TfR may have an antitumou effect against cervical cancer cells, probably by limiting cell proliferation and reducing the TfR mRNA and protein levels.

Keywords

Neoadjuvant chemotherapy; Transferrin receptor; Cervical cancer; Clinical characteristics

1. Introduction

Surgery (S) or concomitant chemo-radiotherapy (CT-RT) are standard treatments for locally advanced cervical cancer [1, 2]. Neoadjuvant chemotherapy (NACT) has been applied in clinical practice in some countries [1, 2]. Neoadjuvant chemotherapy followed by surgery (NACT + S) has the potential to erase micrometastases, reducing both surgical complexity and systemic failure during radical hysterectomy [3]. In this way, NACT + S represents an affordable alternative approach [3]. However, the NACT brings about serious toxicity or side effects. Therefore, a more selective therapeutic approach for the treatment of cancer by delivering therapeutic molecules into malignant cells is needed [4].

Transferrin receptor (TfR) is a serum glycoprotein that participates in the transport of ferric ions *via* its interaction with transferrin [5]. TfR is a cofactor for DNA synthesis and can also be utilized for the delivery of therapeutic molecules into cancer cells [6–9]. It has been reported that the expression level of TfR is greatly increased on the surface of tumor cells [10]. Similarly, our team previously found elevated TfR expression in cervical cancer cells [6]. Clinical characteristics such as tumor size, vasoinvasion, parametrial invasion, and lymph node metastasis are prognostic factors for cancer. These factors correlate with patient outcomes, such as the 5-year survival rate [11]. However, the association between TfR expression and clinical characteristics in squamous carcinoma of human cervical cancer remains unknown. The expression of TfR in cervical cancer with or without NACT is not clear. The relationship between TfR expression and known prognostic factors is unknown. In this study, we quantified the gene expression of TfR in cervical cancer in the presence or absence of NACT and assessed the association between TfR expression and tumor size, vasoinvasion, parametrial invasion and lymph node metastasis.

Although our team has found elevated TfR expression in cervical cancer cells [7], the potential correlation between TfR function and cervical cancer cell death (HeLa cells) remains unknown. Additionally, the relationship between proliferation and TfR is still unclear. In this study, the association between TfR and tumor proliferation was tested with small interfering RNA (siRNA) transfection. We examined cell viability by using the MTT assay while blocking the natural function of TfR in the cervical cancer cell line.

The findings in our study may assist in the future determination of whether TfR can serve as a prognostic factor for cervical cancer and/or a therapeutic target.

2. Materials and methods

2.1 Patient samples

From January 2021 to June 2021, 42 locally advanced cervical cancer patients were included for NACT prior to an operation in the Department of Gynecology of West China Second Hospital. Patients with poor general conditions, for example, severe side effects or who were unfit for general anesthesia or chemotherapy were excluded (n = 10). Finally, 42 women were included.

All patients were fully informed about the potential toxic and adverse effects of NACT. Patients with tumors up to 5 cm in size were given a combination of cisplatin and paclitaxel once every 3 weeks, followed by cold knife conization and lymphadenectomy. The cervical tumor samples from 42 patients included 34 squamous cell carcinomas and 8 adenocarcinomas. Cancerous and adjacent mucosa tissues with NACT (n = 22, 49.8 $\pm$ 3.2 years old) or surgery alone (n = 20, 48.8 $\pm$ 4.3 years old) were collected. All of them provided informed consent before the experiments.

2.2 RT-qPCR

To determine the mRNA levels of human TfR, primers were designed according to the National Center for Biotechnology Information (NCBI) GenBank of humans and synthesized by Shanghai Sangon Co. Total RNA was isolated from cervical tissues by TRIzol (Invitrogen, USA) and reverse transcribed into cDNA with the Revert Aid™ First Strand cDNA Synthesis Kit (lot number: K1622, Thermo Scientific, Rockford, Illinois, USA). TfR expression was detected with RT-qPCR. The details had been described in our previous research [7].

2.3 Small interfering RNA (siRNA) transfection

TfR siRNA (RiboBio, China) was transfected into HeLa cells (50% confluence) with Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA) as previously reported [7]. The medium was replaced with Dulbecco's Modified Eagle Medium (DMEM) after 6 h of incubation as described in our previous research [7].

2.4 Cell viability assay

Cell viability was determined through the MTT assay (Amresco, 0793-250) as previously reported by our team [7]. Briefly, HeLa cells (5×10^4 cells/mL) were incubated with control or TfR siRNA in complete medium. MTT solution (5 mg/mL in Phosphate buffered saline [PBS]) was then added, and the resultant formazan crystals were dissolved in Dimethyl Sulfoxi (DMSO) as described in our previous research [7]. Cell growth inhibitory ratio and the Ct number were calculated as described in previous research [7]. The half-maximal (50%) inhibitory concentration (IC50) was calculated with the curve. In the cell assay, three repeats were used in each group.

2.5 Western blot

Proteins from cervical tissues or cell lines were separated by 4–20% gradient Sodium Dodecyl Sulphate -polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The PVDF membranes were blocked in a solution containing Tris Buffered Saline with Tween (TBST) (10 mm Tris-Hydrochloride, pH 7.4, 150 mm NaCl, and 0.05% Tween 20) and 5% nonfat dried milk at room temperature for 1 h and incubated with TfR (13113, 1:1000 dilution; Cell Signaling Technology, USA) and β-Tubulin (700608, 1:1000 dilution; Zen Biotechnology, China) at 4°C overnight. After washing with TBST buffer, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit antibody (511203, 1:10000 dilution; Zen Bio Technology, China) at room temperature for 1 h. After washing with TBST buffer, signals were detected by using a chemiluminescence reagent (lot number: A5305-1G, Millipore, , Billerica, Massachusetts, USA), imaged with a gel imaging system (Tanon, China), and quantified using Tanon imaging. Data are presented as the ratio of phosphorylated proteins to total proteins and are expressed as the fold-change compared to the control group.

2.6 Data analysis

All statistical analyses were performed using SPSS 20.0 (IBM, Armonk, NY, USA). The statistical significance of TfR expression in relation to clinicopathological features, including parametrial invasion, tumour size, vasoinvasion, histopathological subtype or lymph node metastasis, was assessed using the independent-samples *t*-test and one-way Analysis of Variance (ANOVA). A two-tailed p value < 0.05 was considered statistically significant.

3. Results

We first examined the mRNA levels of TfR in tissue samples using RT-qPCR. The results in Fig. 1 show the PCR products (99 bp) of TfR in patient tissues from cervical carcinoma (lane 1), cervical carcinoma with NACT (lane 2), and paracarcinoma (lane 3). The blank (negative) showed no cDNA reaction (data not shown). The data indicated that the samples were free of contamination and that the TfR cDNA was correctly collected.

The RT-qPCR results showed that the TfR mRNA levels were slightly decreased in the group treated with NACT compared with those treated with surgery alone (Fig. 1). A significant difference was observed between cancer nests and paracancer tissues either with or without NACT (p = 0.012, Fig. 1).

Representative immunoblots showed that the levels of TfR protein were slightly decreased in the group treated with NACT compared with the group treated with surgery alone (Fig. 2). A slight difference was observed between cancer nests and normal tissues (Fig. 2).

The western blot results showed that the protein levels of TfR were decreased in the group with si-TfR (Fig. 3).

We further questioned whether the expression of TfR is correlated with the clinical manifestation. The clinicopatho-

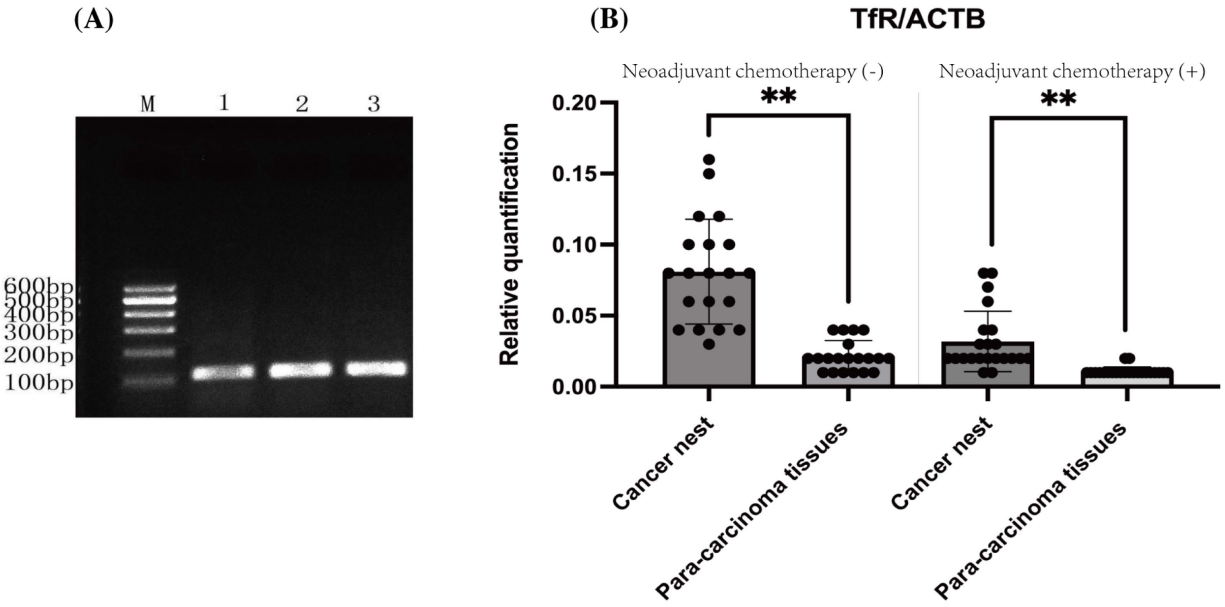

FIGURE 1. TfR expression. (A) Ethidium bromide staining of PCR products of TfR on a 2% agarose gel. Total RNA prepared from cervical carcinoma (lane 1), cervical carcinoma with NACT (lane 2), and para-carcinoma tissues (lane 3) was subjected to 45 cycles of PCR after reverse transcription. The amplification of TfR cDNA generated a fragment of 99 bp, and the amplification of *ACTB* cDNA produced a fragment of 111 bp. M indicates the marker. (B) TfR expression in tumour and pericarcinous tissues with or without NACT. Ethidium bromide staining of PCR products of TfR on a 2% agarose gel. Total RNA prepared from cervical carcinoma (lane 1), cervical carcinoma with NACT (lane 2), and para-carcinoma tissues (lane 3) was subjected to 45 cycles of PCR after reverse transcription. ** $p < 0.01$. TfR: transferrin receptor; *ACTB*: Actin Beta.

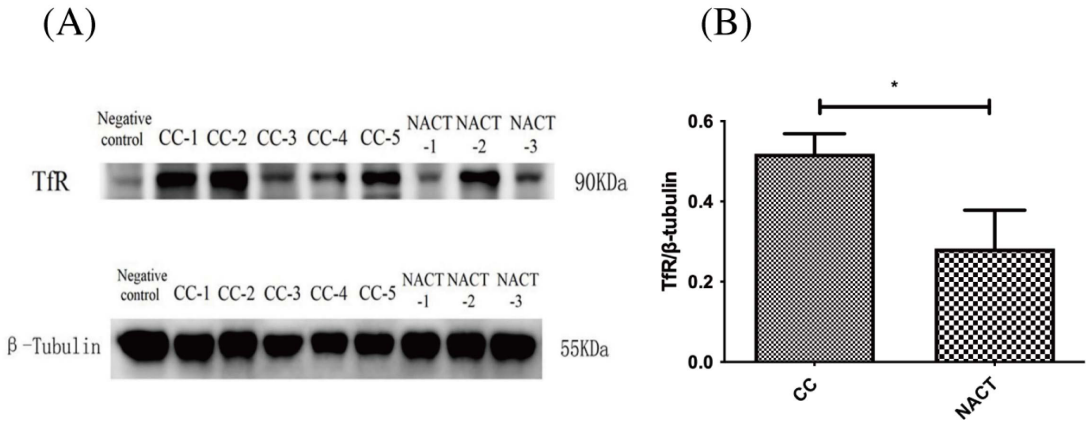

FIGURE 2. TfR protein in tumour tissues with or without NACT. Representative immunoblots showed that the protein levels of TfR were slightly decreased in the group treated with NACT compared with the group treated with surgery alone. (A) TfR protein in tumour tissues with or without NACT. (B) Quantitative analyses for TfR protein were performed by Western blot. A slight difference was observed between cancer nests and normal tissues (Fig. 2a–b). * $p < 0.05$. TfR: transferrin receptor; NACT: neoadjuvant chemotherapy.

logical characteristics of the 42 cervical cancer samples are summarized in Table 1. The results did not reveal significant differences among groups concerning clinical classification. As shown in Table 1 we did not find significant correlations between TfR mRNA expression and tumor size, parametrial invasion, vasoinvasion, histopathological subtype, or lymph node metastasis ($p > 0.05$). Future investigation with a larger sample size may be warranted with regard to the relationship.

We further examined the effects of TfR gene silencing with an MTT assay. HeLa cells were transfected with TfR-siRNA. As shown in Fig. 3, we observed that silencing TfR further decreased cell survival after 48 h ($p < 0.05$, Fig. 3), suggesting that knockdown of TfR promotes cancer cell death.

4. Discussion

In the present study, we reported that TfR was upregulated in carcinoma tissues compared with normal paracancerous tissues ($p < 0.05$). The mRNA and protein levels of TfR were slightly decreased in tumour tissues after NACT compared with surgery alone.

NACT is a common treatment approach for cervical tumours

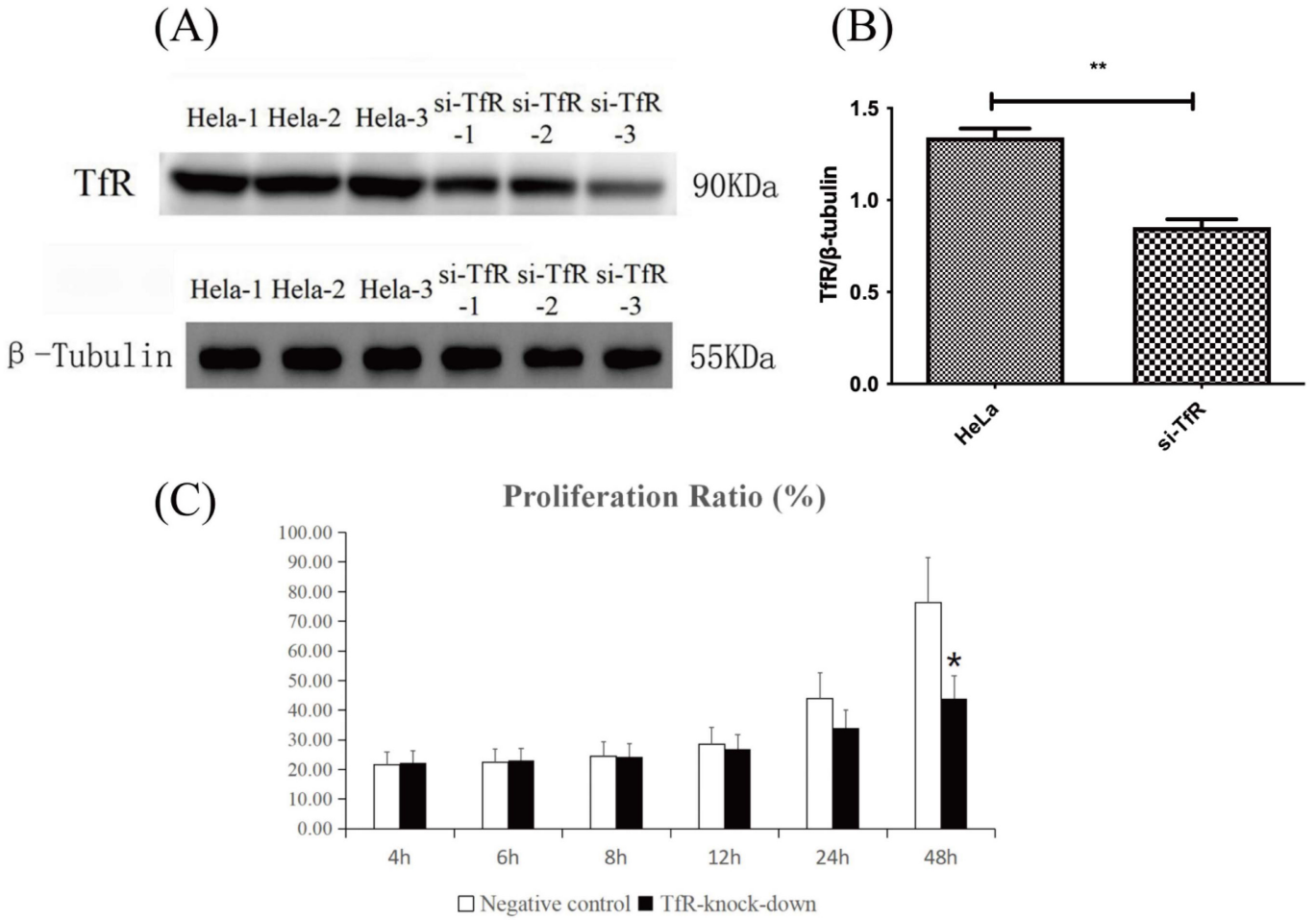

FIGURE 3. TfR expression and DHA-induced cell death. (A) TfR protein levels in HeLa cells with or without si-TfR. (B) Quantitative analyses of TfR protein in HeLa cells were performed by Western blot. (C) Knockdown of TfR further enhances DHA-induced cell death. Fig. 3. The protein levels of TfR were decreased in the group with si-TfR (Fig. 3a). HeLa cells were transfected with TfR-siRNA (10 μM) as indicated. The protein levels of TfR were decreased in the TfR-siRNA group compared with HeLa cells. HeLa cells were transfected with TfR-siRNA (10 μM) as indicated (Fig. 3b). The MTT assay was conducted to determine the surviving cells, which are presented as the percent inhibition ratio. The results presented are the mean ± SD from 3 independent experiments. Compared with the control, * $p < 0.05$, ** $p < 0.01$. TfR: transferrin receptor.

and is widely used in destroying tumours before surgical removal. NACT could turn cervical tumors from untreatable to treatable by shrinking the volume, thus effectively reducing the difficulty and morbidity of more extensive procedures [12]. The downregulation of TfR represents a potential surrogate marker for the efficacy of NACT on undetected dissemination [13].

The relationship between TfR mRNA expression and tumour size, vasoinvasion, parametrial invasion, histopathological subtype or lymph node metastasis was also assessed; however, no significant correlation was observed. However, previous study showed that high TfR expression was associated with incrementally advanced stage, tumor status, and lymph nodes in cervical cancers [14]. While multivariate analysis revealed that TfR remained an independent prognostic variable for poor overall survival [14]. However, the use of TfR in staging, grading or prognostic assessment is quite doubtful [15]. Future investigations with larger sample sizes may be warranted regarding the relationship.

We also found that the decrease in TfR gene expression levels led to reduced cell proliferation after RNA interference. Similarly, the amount of TfR protein decreased, leading to reduced cell proliferation after RNA interference.

TfR, as a type II transmembrane glycoprotein, serves as a carrier protein for transferrin, which is essential for the import of iron into the cell [16]. Iron is one of the trace elements essential for cell growth, proliferation and function. Compared with normal cells, tumour cells have higher demands for iron uptake to meet rapid proliferation [17]. Disorders of iron metabolism can induce tumorigenesis and cancer progression [16, 17]. TfR, which is the most important iron transporter, participates in tumor onset and progression [16, 17]. As the endocytosis of iron was disturbed by si-TfR, inadequate iron led to inhibited cervical cancer cell proliferation.

Aberrant TfR is thought to have an important role in the immunology of cervical cancer [8, 14]. Iron endocytosis mediated by TfR is essential for lymphocyte development and proliferation [18]. However, insufficient iron uptake results in defective lymphocyte activation and a lack of antibody production [18, 19]. Enriched TfR erythroid cells in neonates

TABLE 1. Correlation between TfR expression level and clinical characteristics.

Variables	N (%)	Mean difference ± Standard error	*p*-value
Tumor size			
<40 mm	34 (80.95)	0.03 ± 0.02	0.12
≥40 mm	8 (19.05)	0.05 ± 0.03	
Infiltration depth			
<15 mm	32 (76.19)	0.05 ± 0.03	0.50
≥15 mm	10 (23.81)	0.07 ± 0.06	
Vasoinvasion			
Negative	32 (76.19)	0.05 ± 0.03	0.50
Positive	10 (23.81)	0.07 ± 0.06	
Lymph node metastasis			
Negative	36 (85.71)	0.03 ± 0.02	0.12
Positive	6 (14.29)	0.05 ± 0.03	
FIGO stage			
<IIa2	32 (76.19)	0.05 ± 0.03	0.49
≥IIa2	10 (23.81)	0.06 ± 0.05	
Histopathological subtype			
Squamous cell carcinoma	34 (80.95)	0.04 ± 0.02	0.36
Adenocarcinoma	8 (19.05)	0.05 ± 0.03	

Correlation between TfR expression level and clinical characteristics in cervical cancer. Increased expression of TfR was not associated with tumour size, parametrial invasion, vasoinvasion or lymph node metastasis, neoadjuvant chemotherapy, or FIGO staging. FIGO: International Federation of Gynaecology and Obstetrics.

suppress systemic immune cell activation and compromise neonatal host defense [19, 20]. Further studies are warranted to explore the exact mechanisms via immune cell activation, especially in cervical cancer.

5. Conclusions

Overall, our work reveals TfR as a new relevant gene and highlights the potential perspective value for uncovering relationships between cervical carcinoma and their potential normal cellular counterpart.

AUTHOR CONTRIBUTIONS

MRX, TT and QC—Conception and design; MRX, QJX and TT—Administrative support; MRX and TT—Provision of study materials or patients; QJX, QC, JHY and SYJ—Collection and assembly of data; TT, JHY, SYJ, QJX, MRX and QC—Data analysis and interpretation. All authors manuscript writing and final approval of manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by ethics committee of West China Second Hospital of Sichuan University (NO.2021052) and all participants provided informed consent before the experiments.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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