MicroRNA-186-5p inhibits the metastasis of cervical cancer by targeting FZD3

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Summary

Purpose: *The purpose of this study was to detect the differentially expressed microRNA/miR-186-5p in cervical cancer samples and to uncover its influence on the development of cervical cancer.*

Methods: *Differentially expressed microRNA-186-5p was detected in cervical cancer tissues and normal cervical tissues. Its influences on clinical features and prognosis of cervical cancer patients were analyzed. Regulatory effect of microRNA-186-5p on migratory potential in HeLa and C33-A cells was examined. In addition, the target gene binding to microRNA-186-5p was searched and its involvement in the malignant development of cervical cancer was determined.*

Results: *MicroRNA-186-5p was downregulated in cervical cancer patients, especially those with lymphatic metastasis*

or distant metastasis. High metastasis rate and poor prognosis were seen in cervical cancer patients expressing a low level of microRNA-186-5p. Overexpression of microRNA-186-5p inhibited migratory ability of cervical cancer cells. FZD3 was the downstream gene binding to microRNA-186-5p, which was upregulated in cervical cancer samples. Besides, it was capable of reversing the role of microRNA-186-5p in inhibiting migratory ability of cervical cancer cells.

Conclusions: *MicroRNA-186-5p is lowly expressed in cervical cancer samples, and it inhibits the metastasis in cervical cancer cells by targeting FZD3.*

Key words: *microRNA-186-5p, FZD3, cervical cancer, metastasis*

Introduction

Cancer incidence has gradually increased with the development of the society and economy [1]. Breast cancer and cervical cancer are the most prevalent ones in females, which seriously threaten females' health and even lives [2-4]. The incidence of cervical cancer is much higher in Asia than in other regions [5-7]. Compared with the last century, the average age of cervical cancer cases at diagnosis in China has been advanced by at least 13 years. At present, the average age of diagnosing cervical cancer in China is about 45 years, which is 10 years earlier than non-Chinese women [7]. Squamous cell carcinoma and adenocarcinoma are the cervical cancer with the highest incidence [8]. It is necessary to clarify the pathogenesis and etiology of cervical cancer, thus improving the prognosis [9-11].

MiRNAs are vital regulators in cancer development [12,13]. They are able to mediate key genes associated with cancer cell behaviors [14,15]. Abnormally expressed miRNAs have been identified in different types of tumors [16-18]. These cancer-associated miRNAs exert their biological functions by binding downstream target genes [19,20]. Previous studies have shown that microRNA-186-5p is lowly expressed in colorectal cancer and NSCLC [21,22]. Here, we explored the potential role of microRNA-186-5p and its downstream gene in influencing cervical cancer development.

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Methods

Cervical cancer patients and tissue samples

Cervical cancer tissues (n=72) and adjacent ones (n=72) were collected during surgery. The average age of included patients was (42±9.2) years old. All samples were pathologically confirmed and staged based on the FIGO standard, 2014. This study got approval by Ethics Committee of West China Second Hospital of Sichuan University and it was conducted after obtaining the informed consent of each subject.

Cell lines and reagents

Cervical cancer cell lines (HeLa and C33-A) and a human immortalized epidermal cell line (HaCaT) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C. Cell passage was conducted every 2-3 days.

Transfection

After cell culture to 60-70% confluence in 6-well plates, they were transfected with plasmids constructed by GenePharma (Shanghai, China), using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Forty-eight h later, cells were collected for the following use.

Transwell migration assay

A total of 200 μL of suspension ($5.0×10^5$/mL) and 500 μL of medium containing 10% FBS were added in the upper and bottom of a Transwell chamber (Millipore, Billerica, MA, USA), respectively. Forty-eight h later, fixed cells in the bottom were dyed with crystal violet for 20 min. Migratory cell number was finally counted in 5 fields that were randomly chosen in each sample.

Wound healing assay

Cells were inoculated in 6-well plates and cultured to 90% confluence. After creation of an artificial wound in a cell monolayer, medium with 1% FBS was replaced. Twenty-four h later, wound closure was captured for calculating the percentage of wound healing.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Extracted RNAs by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using Primescript RT Reagent (TaKaRa, Otsu, Japan). The obtained cDNAs underwent qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Japan). The experiment for each sample was performed in triplicate, and the relative level was calculated by $2^{-\Delta\Delta Ct}$ and normalized to that of glyceraldheyde 3-phosphate dehydrogenase (GAPDH) or U6. microRNA-186-5p: forward: 5'-ACACTC-CAGCTGGGCAGCAGCACACT-3', reverse: 5'-CTCAACTG-GTGTCGTGGA-3'; U6: forward: 5'-CTCGCTTCGGCAG-CACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'; FZD3: forward: 5'-GGGGTCTGAGGATAGCATTTCT-3', reverse: 5'-ACCACATTCCCAAACCACAGA-3'; GAPDH: forward: 5'-GCACCGTCAAGGCTGAGAAC-3', reverse: 5'-ATGGTGGTGAAGACGCCAGT-3'.

Western blot

Cells were lysed for isolating proteins and electrophoresed. Protein samples were loaded on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h. Primary and secondary antibodies were applied for indicated time. Band exposure and analyses were finally conducted.

Dual-luciferase reporter assay

Cells were pre-seeded in 24-well plates, and they were co-transfected with NC mimic/microRNA-186-5p mimic and FZD3-WT/FZD3-MUT, respectively. After 48 h of cell culture, they were lysed for measuring luciferase activity (Promega, Madison, WI, USA).

Statistics

SPSS 22.0 software (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between groups were analyzed by the *t*-test. Chi-square test was conducted for analyzing the relationship between microRNA-186-5p level and clinical data of cervical cancer patients. Pearson correlation test was applied for assessing the correlation between expression levels of microRNA-186-5p and FZD3 in cervical cancer tissues. Kaplan-Meier curves and log-rank test were used for survival analysis and $p<0.05$ was considered as statistically significant.

Results

Downregulation of microRNA-186-5p in cervical cancer samples

Compared with normal ones, microRNA-186-5p was lowly expressed in cervical cancer tissues (Figure 1A). Included cervical cancer patients were classified into two groups based on their median level of microRNA-186-5p. It was shown that microRNA-186-5p level was correlated with the rates of lymph node metastasis and distant metastasis, rather than the other clinical features. Identically, a lower level of microRNA-186-5p was seen in cervical cancer patients with lymph node metastasis or distant metastasis than those without metastases. MicroRNA-186-5p levels in cervical cancer cell lines were lower than those in human immortalized epidermal cell line (HaCaT) (Figure 1B). Survival analysis illustrated that lowly expressed microRNA-186-5p was unfavorable to prognosis in cervical cancer. It is suggested that microRNA-186-5p may be a promising hallmark of cervical cancer.

MicroRNA-186-5p inhibited the migratory ability of cervical cancer cells

In vitro abundance of microRNA-186-5p was similarly lower in cervical cancer cell lines. We constructed microRNA-186-5p overexpression and knockdown models in HeLa and C33-A cells, re- spectively (Figure 2A). Migratory cell number and wound closure percentage decreased in HeLa cells overexpressing microRNA-186-5p than those of controls (Figure 2B and 2C, left). Conversely, knock- down of microRNA-186-5p enhanced the migratory ability of C33-A cells (Figure 2B and 2C, right).
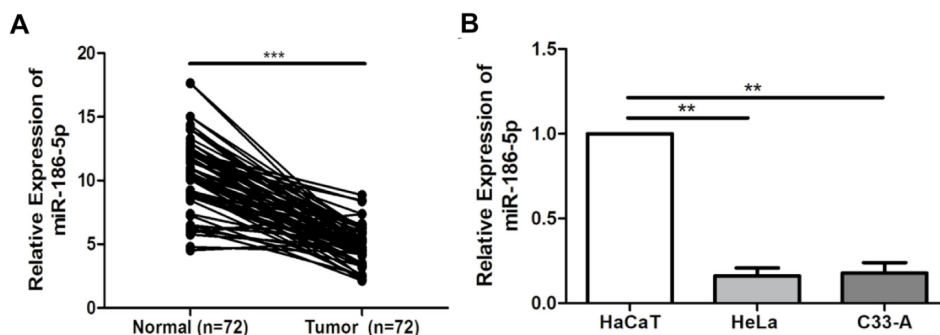

Figure 1. Downregulation of microRNA-186-5p in cervical cancer samples. **A:** MicroRNA-186-5p levels in normal cer- vical tissues and cervical cancer tissues. **B:** MicroRNA-186-5p levels in cervical cancer cell lines. Data were expressed as mean±SD. **p<0.01, ***p<0.001.
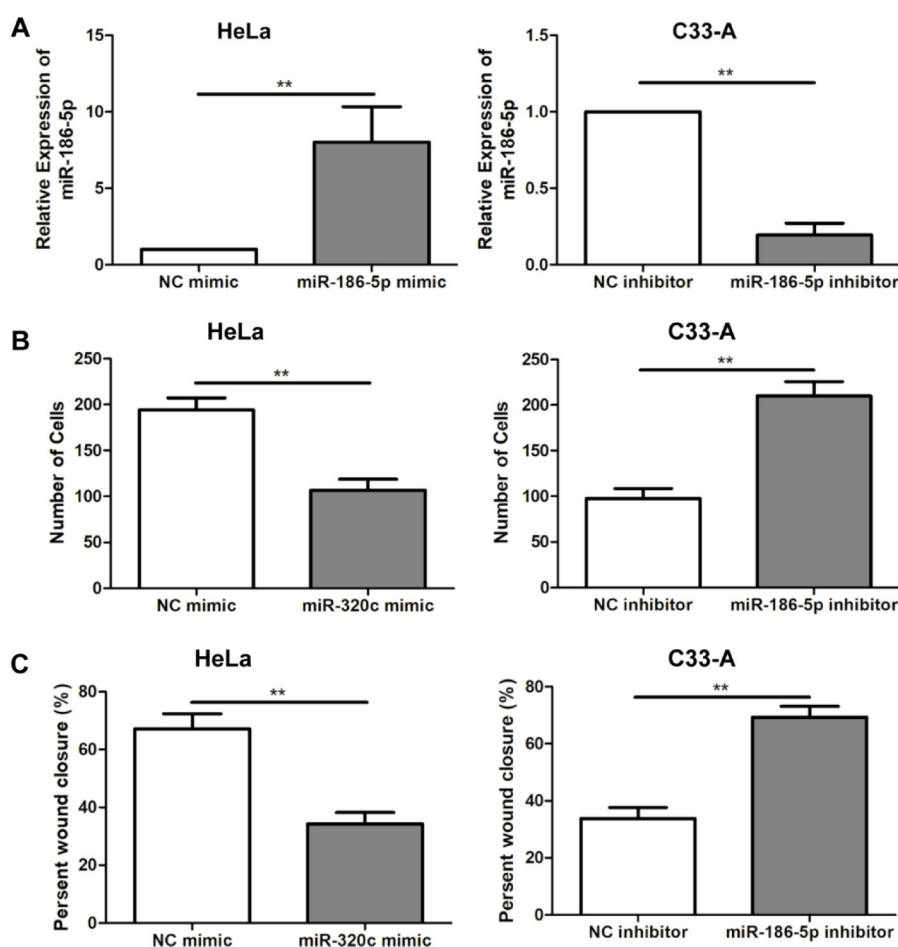

Figure 2. MicroRNA-186-5p inhibited migratory ability of cervical cancer cells. **A:** Transfection efficacy of microRNA- 186-5p mimic and inhibitor in HeLa and C33-A cells, respectively. **B:** Migration in HeLa and C33-A cells with microRNA- 186-5p overexpression or knockdown, respectively. **C:** Wound healing in HeLa and C33-A cells with microRNA-186-5p overexpression or knockdown, respectively. Data were expressed as mean±SD. **p<0.01.

MicroRNA-186-5p bound FZD3

Protein levels of FZD3, N-cadherin, Vimentin, β-catenin and MMP-9 were markedly downregulated in HeLa cells overexpressing microRNA-186-5p, while E-cadherin was upregulated. Transfection of microRNA-186-5p inhibitor yielded the opposite expression trends of the abovementioned genes in C33-A cells (Figure 3A). Contrary to microRNA-186-5p, FZD3 was highly expressed in cervical cancer tissues and negatively related to microRNA-186-5p level (Figure 3B). As predicted binding sequences depicted, we constructed wild-type and mutant-type FZD3 vectors. Overexpressed microRNA-186-5p could decrease luciferase activity in FZD3-WT vector, verifying that FZD3 was the downstream gene of microRNA-186-5p (Figure 3C).

FZD3 abolished the regulatory role of microRNA-186-5p in cervical cancer metastasis

Subsequently, the potential functions of FZD3 in cervical cancer development were explored. Transfection efficacy of pcDNA3.1-FZD3 and si-
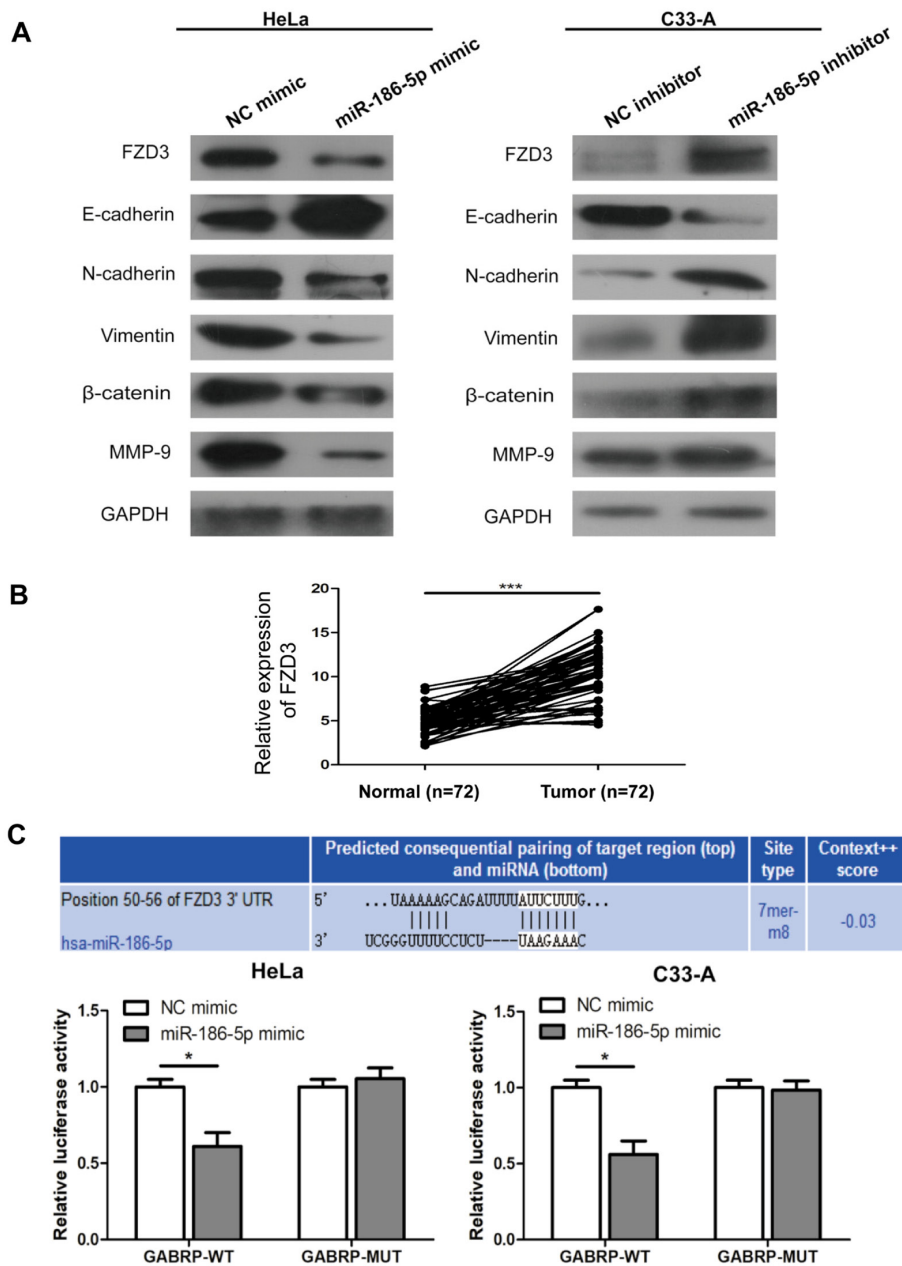

Figure 3. MicroRNA-186-5p bound to FZD3. **A:** Protein levels of FZD3, E-cadherin, N-cadherin, Vimentin, β-catenin and MMP-9 in HeLa and C33-A cells with microRNA-186-5p overexpression or knockdown, respectively. **B:** FZD3 levels in normal cervical tissues and cervical cancer tissues. **C:** Binding sequences in the 3'UTR of microRNA-186-5p and FZD3. Luciferase activity in HeLa and C33-A cells co-transfected with NC mimic/microRNA-186-5p mimic and FZD3-WT/FZD3-MUT, respectively. Data were expressed as mean±SD. *p<0.05, ***p<0.001.
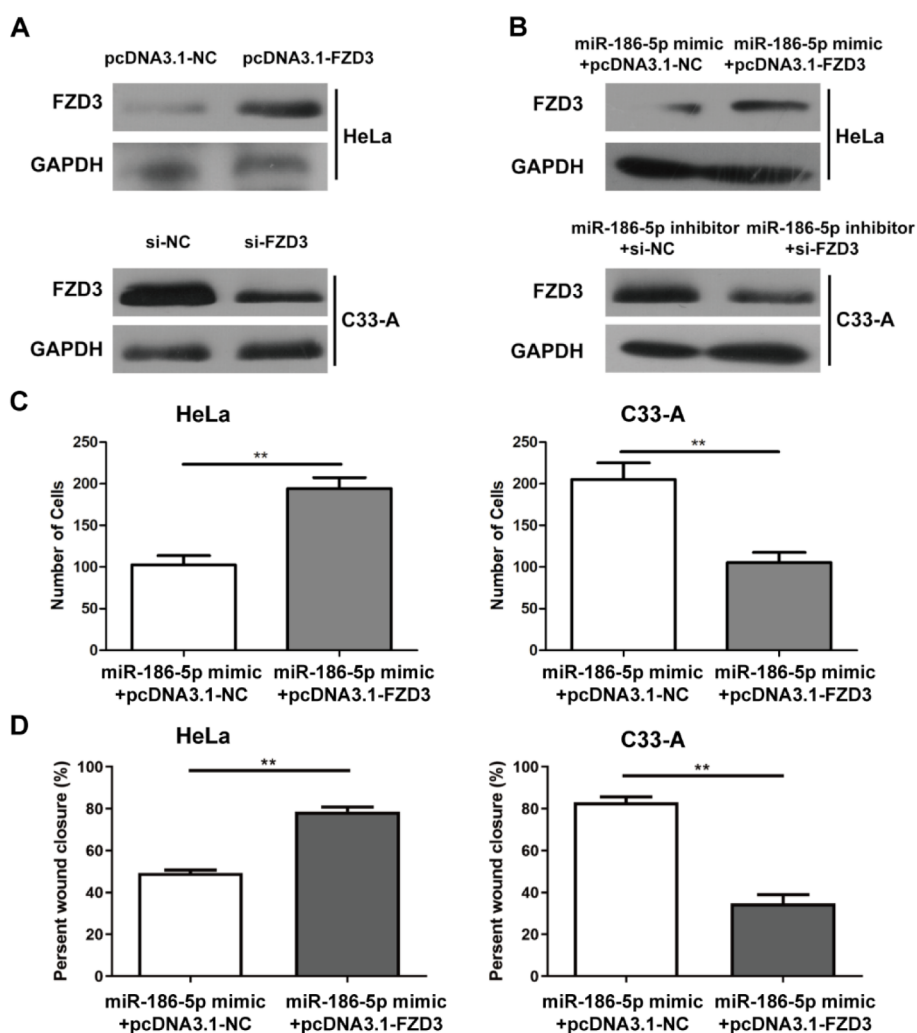
Figure 4. FZD3 abolished the regulatory role of microRNA-186-5p in cervical cancer metastasis. **A:** Transfection efficacy of pcDNA3.1-FZD3 and si-FZD3 in HeLa and C33-A cells, respectively. **B:** Protein level of FZD3 in HeLa and C33-A cells influenced by both microRNA-186-5p and FZD3. **C:** Migration in HeLa and C33-A cells influenced by both microRNA-186-5p and FZD3. **D:** Wound healing in HeLa and C33-A cells influenced by both microRNA-186-5p and FZD3. Data were expressed as mean±SD. **p<0.01.

FZD3 in HeLa and C33-A cells was tested, respectively (Figure 4A, 4B). Interestingly, larger migratory cell number and wound closure percentage were observed in HeLa cells co-overexpressing microRNA-186-5p and FZD3 than those solely overexpressing microRNA-186-5p (Figure 4C and 4D, left). On the contrary, lower migratory ability was found in C33-A cells with co-silenced microRNA-186-5p and FZD3 compared with those transfected with microRNA-186-5p inhibitor (Figure 4C and 4D, right).

Discussion

Genes transport genetic information from the parent to offspring [23]. In the long process of biological evolution, organisms are subject to environmental changes, such as temperature and geographic location. Some genes will adapt to the environmental changes and finally survive. However, some are mutant because of external changes. Accumulated mutations to a certain extent will finally result in qualitative changes and thus new species are formed [23,24]. Highly conserved genes during the continuous evolution are essential for the basic life activities of organisms [24,25]. Protein-encoding genes are of significance, and non-coding genes are also fundamental during biological processes [25].

About one third of human genome can be regulated by miRNAs. A complicated network involving miRNAs and their target genes contributes to influence pathological events [12-15]. Abnormally expressed miRNAs have been identified in tumor profiling [16-18]. Previous studies have shown the vital regulation of microRNA-186-5p in tumors [21,22].

Here, our findings showed that microRNA-186-5p was downregulated in cervical cancer tissues. Its level was negatively related to metastasis rate and predicted a poor prognosis in cervical cancer patients. It is suggested that microRNA-186-5p may be a tumor suppressor in cervical cancer. Moreover, microRNA-186-5p remarkably inhibited the migratory ability of cervical cancer cells. Transfection of microRNA-186-5p mimic downregulated FZD3, N-cadherin, Vimentin, β-catenin and MMP-9, and upregulated E-cadherin, indicating a potential interaction between microRNA-186-5p and the FZD3 signaling.

FZD3 was confirmed to be the downstream gene of microRNA-186-5p. The FZD3 gene is a member of the Frizzled family, and it encodes a frizzled (FZD) protein, which is a seven trans-membrane protein with a structure similar to the G protein-coupled receptor [26]. The extracellular N-terminus of FZD3 protein has a cysteine-rich domain (CRD) [26,27]. It was uncovered in this study that FZD3 was highly expressed in cervical cancer samples, which was negatively correlated with mi-croRNA-186-5p level. Notably, FZD3 was able to abolish the regulatory effect of microRNA-186-5p on the migratory ability of HeLa and C33-A cells. As a result, we have proven that microRNA-186-5p/FZD3 axis was responsible for inhibiting the deterioration of cervical cancer.

Conclusions

MicroRNA-186-5p is lowly expressed in cervical cancer samples, and it inhibits the metastatic ability of cervical cancer cells by targeting FZD3. Besides, the microRNA-186-5p/FZD3 axis is a promising therapeutic target for cervical cancer.

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Conflict of interests

The authors declare no conflict of interests.

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