

## Original Research Article

# Curcumin inhibits epithelial-mesenchymal transition in colorectal cancer cells by regulating miR-206/SNAI2 pathway

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### Abstract

**Purpose:** To examine the effects of curcumin on epithelial-mesenchymal transition (EMT) via regulation of miR-206 and SNAI2 in colorectal cancer (CRC) cells. Relationship between SNAI2 and miR-206 and the effects of curcumin on related mechanisms were also identified.

**Methods:** Transwell assays were used to analyze cellular migration and invasion. Genes associated with changes in protein and mRNA expression were evaluated by western blotting and quantitative reverse transcription PCR analyses, respectively. The relationship between SNAI2 and miR-206 was determined using a dual luciferase assay.

**Results:** Curcumin inhibited cell metastasis, upregulated miR-206 expression, and decreased SNAI2 levels. Furthermore, miR-206 directly targeted SNAI2 and inhibited EMT via downregulation of SNAI2 expression. Curcumin inhibited EMT in CRC cells by upregulating miR-206.

**Conclusion:** This study, for the first time, discovered the role of curcumin on epithelial-mesenchymal transition process in colorectal cancer cells by modulating miR-206/SNAI2 axis. These findings suggest that curcumin may be useful as a novel therapeutic agent to inhibit the metastasis of CRC.

**Keywords:** Epithelial-mesenchymal transition, Colorectal cancer, MiR-206, SNAI2, Cell migration, Curcumin

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## INTRODUCTION

Improper lifestyle and diet are the main risk factors for colorectal cancer (CRC) [1-3]. Despite advances in the study of CRC, it is still a common gastrointestinal malignancy with high mortality due to metastasis. Currently, the main challenge is the development of a clinically effective method to inhibit CRC metastasis. Metastasis is a complex process that is caused

by multiple gene interactions. However, little is known about the exact mechanism by which CRC metastasis occurs.

Curcumin has been widely studied in a variety of tumors as a potential chemotherapy agent [4]. It can inhibit cancer cell invasion, proliferation, inflammation, and angiogenesis, promote apoptosis, and increase the sensitivity of tumors to chemotherapeutic drugs [5-7]. Curcumin also

exert anticancer effects by regulating downstream genes or pathways via alterations in miRNA levels [8,9]. Bisphenol A-induced MCF-7 cell proliferation was found to be inhibited by the regulation of the miR-19/AKT/PTEN/p53 pathway [10]. The upregulation of miR-192-5p suppresses proliferation and promotes apoptosis in non-small cell lung cancer through preventing the PI3K/Akt pathway [11]. In CRC, curcumin inhibits cell invasion and migration by the regulation of the miR-21, miR-34a, and miR-27a expression. Moreover, via the regulation of miR-491 expression, curcumin was shown to inhibit the proliferation of HCT-116 cells and promote apoptosis [12]. However, in view of the complex pathogenesis of CRC, curcumin's mechanism of action requires further study.

Curcumin can upregulate miR-206 expression and decrease insulin signaling in fructose-induced podocytes [13], but few studies have investigated the relation of miR-206 expression as well as curcumin in cancer. miR-206 plays a role in tumor suppression in multiple tumors, especially in CRC, and has been shown to downregulate NOTCH3 and inhibit tumor proliferation and metastasis [14]. miR-206 can also inhibit CRC progression by targeting FMNL2 [15] and can increase 5-fluorouracil (5-FU) sensitivity via the inhibition of Bcl-2 expression [16]. However, in CRC, whether curcumin can regulate the miR-206 expression is unknown. This study investigated the role of curcumin on the epithelial-mesenchymal transition process in CRC cells, and discovered its function in regulating miR-206/SNAI2 axis.

## EXPERIMENTAL

### Cell culture and reagents

Sigma-Aldrich (St. Louis, MO, USA) provided the curcumin (99% purity). American Type Culture Collection (CCL-247, USA) provided HCT116

CRC cell. Cells were incubated in DMEM (Gibco, USA) containing 10% FBS (Gibco) and 100 U/ml penicillin-streptomycin (Gibco), and maintained in an incubator: humidified, 37 °C, 5% CO<sub>2</sub>.

### HCT116 cell transfection

GenePharma (Shanghai, China) synthesized miR-206 mimics, miR-206 inhibitor, SNAI2 plasmid, respective negative controls. Table 1 shows the primers sequences. According to the manufacturer's instructions, the miR-206 mimics (50 nM), miR-206 inhibitor (50 nM), SNAI2 plasmid (50 nM), and respective negative controls were transfected into differentiated podocytes by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 2 days of growth, the original medium was discarded, and RPMI-1640 culture medium (Gibco, USA) was selected to the transfected cells.

### Transwell assay

HCT116 cells ( $1 - 2 \times 10^5$ ) were seeded into the top chambers (pore size of 8- $\mu$ m) of Transwell plates (Costar, UK). After 12 h, cells from at least six fields of view were photographed under a microscope and counted for the migration assay. The same number of HCT116 cells were also seeded into top chambers precoated with Matrigel (BD Biosciences, USA) and incubated for one day for the invasion assay.

### qRT-PCR

The total cells RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA) and the SuperScript RT kit from Invitrogen (Invitrogen, Carlsbad, CA) was used to reverse-transcribed. Using the PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific, USA) and the ABI PRISM7900 Sequence Detection System (Applied Biosystems, USA),

**Table 1:** Sequence of miRNA and mRNA primers

Gene	Forward (5'-3')	Reverse (5'- 3')
miR-206	CAGA TCCGATTGGAATGTAAGG	TATGCT TGTTCTCGTCTCTGTGTC
SNAI2	ATGAGGAATCTGGCTGCTGT	CAGGAGAAAATGCCTTTGGA
U6	GTAGTCGGCCGAAGGTCTCAC	ACCGTGGATGCAATGCCTAA
miR-206 mimics	UGGAAUGUAAGGAAGUGUGUG	ACACACUUCUUACAUUCCA
Negative control	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
miR-206 inhibitor	CCACACACUUCUUACAUUCCA	
SNAI2 plasmid	GGACTAGTATGCCGCGCTCCTTCCTGGTC	CGGAATTCTCAGTGTGCTACACAG
GAPDH	AAGGAAATGAATGGGCAGCC	CAGCCAGATTC TAGGAAAAGCATCACCCGGA

qRT-PCR was subsequently performed using the primers shown in Table 1. The  $2^{-\Delta\Delta Ct}$  method was used for miRNA and mRNA expression analyses [17]; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 snRNAs served as the positive controls.

### Luciferase reporter assay

The mutant (MUT) SNAI2 3'-untranslated region (UTR) and wild-type (WT) SNAI2 3'-UTR, which both contained a putative binding site of miR-206, were synthesized and inserted into the reporter vector of pmirGLO dual luciferase (YouBio, Changsha, China). Reporter vectors containing the MUT and WT SNAI2 3'-UTRs, and the NC-mimic/miR-206 mimic, were co-transfected into HEK293T cells and cultured until the cells reached 45 % confluence. After 48 h, the assay of dual luciferase system (Promega, Madison, USA) was used to evaluate the levels of luciferase, which were normalized to those of the *Renilla* luciferase.

### Western blot

HCT116 cells were homogenized on ice for half an hour in RIPA buffer (Sigma-Aldrich, USA) with protease inhibitors. The homogenate was then centrifuged, 12,000 rpm, 15 min; and collected the supernatant and stored at  $-20^{\circ}\text{C}$ . Electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gels using Tris-glycine running buffer was performed to separate total proteins, which were then immobilized onto PVDM (Sigma-Aldrich, USA). Nonspecific binding was blocked by gentle shaking in Tris-buffered saline with 3 % bovine serum albumin and 0.1 % Tween-20 for one hour at room temperature. Then the membranes were cultured with the following primary antibodies: anti-Zeb1 (1:1000, Applygen), anti-SNAI2 (1:1000, Santa Cruz, USA), anti-E-cadherin (1:1000, Abcam, USA), anti-Vimentin (1:1000, Applygen, Beijing, China), anti-Snail (1:1000, Abcam), and anti-GAPDH (1:1000, Applygen) overnight at  $4^{\circ}\text{C}$ . Proteins were visualized using a LAS-4000 mini system (Fujifilm, Japan) and quantified using Quantity one software. Protein expression levels were standardized to those of GAPDH.

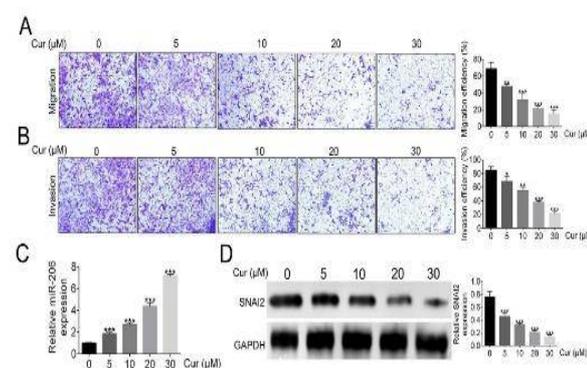
### Statistical analysis

GraphPad 8.0 software was used to analyze the data. Results are presented as mean  $\pm$  standard deviation (SD) and all experiments were repeated three times.  $P < 0.05$ , was considered statistically significant, ANOVA was performed to determine the significance of differences among experimental groups.

## RESULTS

### Curcumin inhibits cell metastasis, upregulates miR-206 expression, and decreases SNAI2 levels

The effects of graded concentrations (0 – 30  $\mu\text{mol/L}$ ) of curcumin on the HCT116 cells migration and invasion were investigated. The results showed that both invasion and migration were suppressed by curcumin (Figure 1 A and B). Increased concentrations of curcumin led to significant changes in the inhibitory efficacy (all  $p < 0.05$ ). A biological analysis was used to determine whether curcumin had any effects on miR-206 and SNAI2. HCT116 cells were treated of different concentrations of curcumin for one day, the miR-206 mRNA expression and SNAI2 protein levels were evaluated by qRT-PCR and western blotting. The miR-206 mRNA levels were markedly increased (Figure 1C) and the protein levels of SNAI2 were decreased in HCT116 cells treated with curcumin (Figure 1D). These results suggest that curcumin suppresses cell metastasis, upregulates miR-206 expression, and decreases SNAI2 levels.

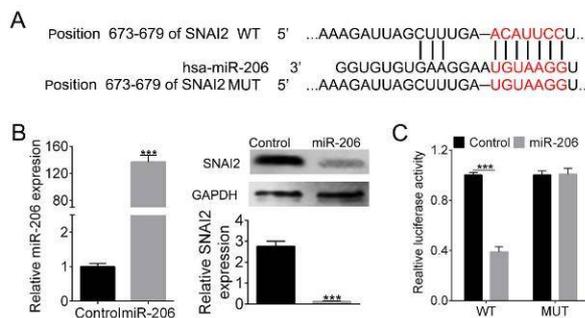


**Figure 1:** The effects of Curcumin on the migration, invasion and the expression of miR-206 and SNAI2 in HCT116 cells. (A) Migration and (B) invasion of HCT116 cells after curcumin treatment (0, 5, 10, 20, and 30  $\mu\text{M}$ ) for 24 h observed by the Transwell assay. (C) The mRNA expression levels of miR-206 in HCT116 cells after curcumin (0, 5, 10, 20, and 30  $\mu\text{mol/L}$ ) treated for 24 h were measured using qRT-PCR. (D) The protein expression levels of SNAI2 in HCT116 cells after curcumin (0, 5, 10, 20, and 30  $\mu\text{mol/L}$ ) treated for 24 h were evaluated by western blotting; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. HCT116 cells treated with curcumin (0  $\mu\text{mol/L}$ ), respectively

### MiR-206 binds SNAI2 and inhibit SNAI2 expression

SNAI2, as a target of miR-206, was indicated to contain an miR-206-binding site in the TargetScan database. To verify this prediction, both MUT and WT SNAI2 3'UTRs were cloned

into the reporter system (Figure 2 A). To validate whether SNAI2 expression could be directly regulated by miR-206, SNAI2 protein levels in HCT116 cells that were overexpressing miR-206 by western blot analysis were examined. Figure 2 B shows that SNAI2 levels were reduced in these cells. In addition, Figure 2 C shows that WT SNAI2 3'-UTR luciferase activity was inhibited when miR-206 mimics were co-transfected with the vector ( $p < 0.001$ ), whereas MUT SNAI2 3'-UTR luciferase activity was not significantly affected by co-transfection of miR-206 mimics. These data suggest that miR-206 can directly target SNAI2 and suppress SNAI2 expression levels in HCT116 cells.

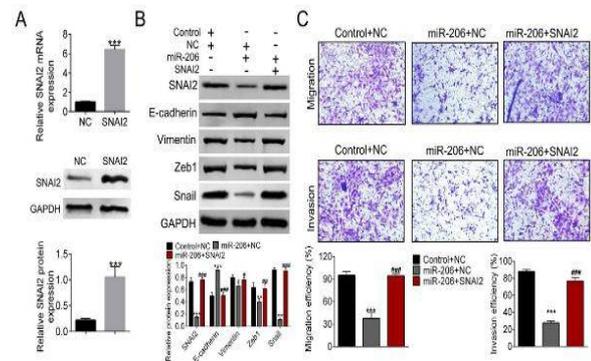


**Figure 2:** MiR-206 binds SNAI2 and inhibits SNAI2 expression. (A) A binding site of miR-206 in the 3'-UTR of SNAI2 was identified by TargetScan. (B) The levels of miR-206 mRNA in HCT116 cells overexpressing miR-206 and SNAI2 expression levels in HCT116 cells transfected with miR-206 mimics were evaluated by qRT-PCR and western blotting, respectively. (C) The MUT and WT SNAI2 3'-UTR luciferase activities constructs were measured in HCT116 cells transfected with miR-206 mimics; \*\*\* $p < 0.001$  vs. control

**MicroRNA-206 inhibits EMT in HCT116 cells via downregulating SNAI2**

Next, this study overexpressed SNAI2 in HCT116 cells. Western blot and qRT-PCR results showed a successful overexpression ( $p < 0.001$ , Figure 3 A). To further determine the mechanism by which miR-206 affects CRC progression, levels of proteins associated with the EMT were measured. As shown in Figure 3 B, miR-206 overexpression markedly increased E-cadherin levels, whereas in HCT116 cells, SNAI2, vimentin, Zeb1, and Snail levels were reduced as compared to the control + NC group. While a significant reduction in E-cadherin protein level and increases in SNAI2, Vimentin, Zeb1, and Snail protein levels were found in HCT116 cells transfected with SNAI2 and miR-206 mimics. Moreover, results in Figure 3C showed that both the migration and invasion of HCT116 cells were blocked through miR-206 mimics ( $p < 0.001$ ), whereas the number of cells undergoing invasion

and migration were dramatically increased after transfection of miR-206 mimic + SNAI2 ( $P < 0.001$ ). These data suggest that miR-206 blocks CRC progression via the regulation of proteins associated with EMT.



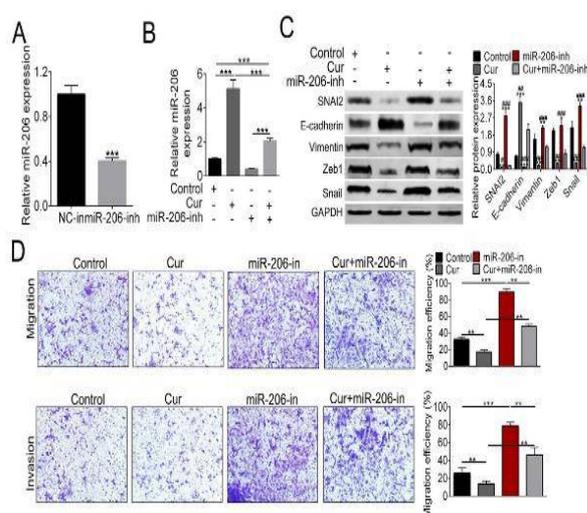
**Figure 3:** The EMT is inhibited via the downregulation of SNAI2 expression by miR-206. (A) Overexpression of SNAI2 in HCT116 cells was evaluated by qRT-PCR and western blotting. (B) SNAI2, E-cadherin, Zeb1, Vimentin, and Snail protein expression in HCT116 cells was evaluated by western blotting after transfection of miR-206 + NC, miR-206 mimics or miR-206 + SNAI2. (C) HCT116 cells invasion and migration were evaluated using the Transwell assay after transfection of miR-206 + NC, miR-206 mimics or miR-206 + SNAI2; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , compared with Control + NC group. # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$ , compared with miR-206 + SNAI2 group

**Curcumin inhibits EMT of CRC cells upregulated miR-206**

The results of qRT-PCR expressed that the miR-206 knockdown in HCT116 cells caused a reduction in the mRNA expression of miR-206 levels ( $p < 0.001$ , Figure 4 A). miR-206 overexpression was observed in HCT116 cells after treatment with curcumin compared with control ( $p < 0.001$ ). In addition, we found a marked reduction in miR-206 expression levels in HCT116 cells after miR-206 knockdown in combination with curcumin compared with treatment with curcumin alone ( $p < 0.001$ ). However, expression of miR-206 levels were increased in HCT116 cells after miR-206 knockdown and treatment with curcumin compared to miR-206 knockdown alone ( $p < 0.001$ ), as shown in Figure 4 B. Furthermore, after treatment with curcumin, E-cadherin protein levels were markedly increased, and SNAI2, Vimentin, Zeb1, and Snail protein expression levels were decreased in HCT116 cells. However, knockdown of miR-206 had the opposite effect on the levels of these proteins.

Compared to miR-206 knockdown alone, the levels of E-cadherin were reduced and the

SNAI2, Vimentin, Zeb1, and Snail levels were increased in HCT116 cells by knockdown of miR-206 and treatment with curcumin (Figure 4 C). The invasion and migration of HCT116 cells after treatment with curcumin or miR-206 knockdown were further analyzed in this study (Figure 4D). After treatment with curcumin, the number of cells undergoing migration and invasion was dramatically decreased (all  $p < 0.01$ ), whereas treatment with curcumin or knockdown of miR-206 markedly improved the number of HCT116 undergoing migration and invasion. Intriguingly, after knockdown of miR-206 and treatment with curcumin, cellular migration and invasion were inhibited in HCT116 cells. These data demonstrate that curcumin inhibits the EMT in CRC cells via upregulating miR-206.



**Figure 4:** The effect of Curcumin on CRC cell EMT. Expression of miR-206 mRNA in HCT116 cells was evaluated by qRT-PCR after knockdown of miR-206 (A) and after treatment with curcumin (20  $\mu\text{mol/L}$ ), knockdown of miR-206, or both (B). (C) Western blot analyses of SNAI2, E-cadherin, Zeb1, Vimentin, and Snail expression in HCT116 cells after treatment with curcumin (20  $\mu\text{mol/L}$ ), knockdown of miR-206, or both. (D) HCT116 cells migration and invasion was observed using the Transwell assay after treatment with curcumin (20  $\mu\text{mol/L}$ ), knockdown of miR-206, or both; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$ , statistically significant

## DISCUSSION

In the present experiment, treatment of CRC HCT116 cells with curcumin inhibited cell metastasis, upregulated miR-206 expression, and decreased SNAI2 levels. miR-206 was shown to bind SNAI2 and inhibit SNAI2 expression. Furthermore, miR-206 inhibited the EMT by downregulating SNAI2 expression. After treatment with curcumin, the EMT of HCT116 cells was inhibited and the expression of miR-206 was upregulated. These results suggest that

curcumin inhibits the EMT by regulating the miR-206/SNAI2 pathway in CRC cells.

Colorectal cancer is considered the 3rd-most usual type of tumor worldwide the development and progression of CRC are known to involve several genetic and environmental risk factors including chromosomal abnormalities, epigenetic changes, and unhealthy lifestyles. Various biomarkers, such as RNAs, DNAs, epigenetic changes, glycoproteins and proteins, and microRNAs, have been investigated for the prognosis, diagnosis, and treated with CRC patients. Previous reports have suggested that miR-206 can regulate different target genes that function in various cell processes, for example, cell differentiation, apoptosis and proliferation [18-20]. As a unique transcription factor for androgens, SNAI2 coordinates androgen receptors and promotes resistance to prostate cancer [21]. Curcumin has been reported to inhibit the activation of carcinogens and elicit anti-metastatic and anti-invasive effects in breast, lung, and prostate cancers [22,23]. In the present study, HCT116 cells were treatment of graded concentrations of curcumin, resulting in the strong inhibition of cellular migration and invasion. Additionally, the study firstly report that curcumin upregulates miR-206 expression and downregulates SNAI2 expression.

SNAI2 belongs to the Snail superfamily and is a highly conserved zinc finger transcription factor participated in both normal development and carcinogenesis [24-26]. Therefore, it is of great importance to demonstrate the SNAI2-mediated regulation of cancer cell migration and invasion. Various miRNAs were found to be downregulated in many types of tumors and may mediate tumorigenesis by targeting tumor suppressor genes.[27] Prior work reported that decreased miR-206 expression levels were related to the clinical progression of CRC [28]. However, the roles and expression levels of miR-206/SNAI2 in CRC have not been clearly reported until now. In our study, we used bioinformatics analyses and transfection assays to confirm that miR-206 exhibited a tumor suppressor effect via the downregulation of SNAI2. In our opinion, we firstly explore the post-transcriptional regulation of SNAI2 by miR-206 in CRC.

The EMT is involved in many stages of cancer progression. Tumor cells are endowed with more aggressive phenotypes during the EMT, such as stem cell-like and mesenchymal features [29]. This transition results in cellular migration and invasion. The EMT reduces the proteins expression that promote cell-cell contact, for

example,  $\alpha$ -catenin and E-cadherin, and increases the mesenchymal markers levels, including Vimentin, Zeb1, and Snail [30]. In the current experiment, we identified the role and mechanism of action of miR-206/SNAI2 in the suppression of the EMT in CRC. Snail family transcriptional repressor 2 expression was down-regulated and the EMT process was inhibited by miR-206 in CRC cell lines. As for the EMT markers, Vimentin, Zeb1, and Snail expression levels were reduced after transfection with miR-206 mimics, whereas E-cadherin expression was markedly improved. The miR-206 overexpression was shown to inhibit invasion and migration of cell by down-regulation the SNAI2 expression.

Curcumin has been shown to suppress miR-21 transcription via AP-1, inhibit cancer migration and invasion *in vivo*, and stabilize the cancer suppressor PDCD4 in CRC [31]. Moreover, curcumin has been shown to inhibit metastasis via the regulation of miR-181b expression by targeting the down-regulation of CXCL1 and CXCL2 levels in breast cancer [32]. Curcumin has also been shown to inhibit metastasis via the up-regulation of miR-7 expression, as well as the down-regulation of SET8, in pancreatic cancer [8]. This study appears to be the first to confirm that the anti-metastatic roles of curcumin are related to the miR-206/SNAI2 pathway and the EMT in CRC. Curcumin was shown to strongly inhibit CRC via upregulating miR-206 expression, the suppression of both EMT and SNAI2 expression, and the inhibition of cancer cell invasion and migration.

## CONCLUSION

The findings of this study show, for the first time, that curcumin serves as a suppressor of EMT in CRC cells by modulating miR-206/SNAI2 pathway. Hopefully, these results provide a new candidate target for the future development of therapeutic interventions against CRC metastasis.

## DECLARATIONS

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### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was performed by the authors of this article. All liabilities related with the content of this article will be borne by the authors. Pan Zhao and Chunjie Zhang designed all the experiments and revised the manuscript. Dafei Xie and Maowei Pei formed the experiments, and Pan Zhao wrote the paper.

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