Transcription factor E2F3a regulates \textit{CASP8AP2} transcription and enhances sensitivity to chemotherapeutic drugs in acute lymphoblastic leukemia

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Abstract

**Background:** Low expression of E2F3a and caspase 8 associated protein 2 (CASP8AP2) are associated with poor prognosis of childhood acute lymphoblastic leukemia (ALL).

**Methods:** Dual-luciferase reporter assay and wild type as well as four mutated types of reporter plasmids were used to demonstrate the activation of E2F3a on \textit{CASP8AP2} transcription. The direct binding of E2F3a with the promoter of \textit{CASP8AP2} was shown by Chromatin Immunoprecipitation (ChIP). Cell proliferation activity and cell cycle were determined by MTS and flow cytometry in leukemic cells after treating with common chemotherapeutic drugs vincristine and daunorubicin.

**Results:** In this study, we found that up-regulation of E2F3a in leukemic cells led to increased fraction of cells in S and G2/M phase, accelerated proliferation, and enhanced sensitivity to vincristine and daunorubicin. ChIP and luciferase assay indicated that E2F3a could directly bind to two fragments in the wild type of \textit{CASP8AP2} promoter (−206 to −69 and −677 to −507), and activate its transcription activity which was reduced in mutated promoters. The effect of E2F3a on chemotherapeutic sensitivity of leukemic cells could be reversed by down-regulating \textit{CASP8AP2}.

**Conclusions:** E2F3a could promote transcription and expression of \textit{CASP8AP2}. The effect of E2F3a on chemotherapeutic sensitivity of ALL cells was implemented by regulating \textit{CASP8AP2} expression to a great extent.

**Keywords:** Childhood acute lymphoblastic leukemia, E2F3a, CASP8AP2, Transcription regulation, Chemotherapeutic sensitivity
residual disease (MRD) and relapse [8–11]. However, it was unclear about the mechanisms of down-regulation of CASP8AP2 in childhood ALL.

E2F family was thought of as one of the main transcription regulators of replication dependent histone, and played an important role in regulation of cell cycle progression [12]. Furthermore, the members of E2F family have been reported to be involved in genesis and progression of many types of tumors [13, 14]. Gene expression profiling in childhood ALL showed E2F3 expressed in most patients, whereas other members’ expression was low or undetectable [15, 16]. There were two E2F3 isoforms, E2F3a and E2F3b [17, 18]. The latter could be found in G0 phase, whereas E2F3a played a pivotal role in transition of G1/S and cell proliferation [19]. Our previous study has showed that similar to CASP8AP2, low expression of E2F3a was common and related to poor treatment outcome in childhood ALL [20].

In the present study, we showed that E2F3a could activate CASP8AP2 transcription directly; its over-expression enhanced the sensitivity of leukemic cells to chemotherapeutic drugs, and could be counteracted by knock down of CASP8AP2. Thus, in childhood ALL, the prognostic significance of E2F3a was largely implemented through regulating CASP8AP2 expression.

Methods
Plasmids
Recombinant plasmid pcDNA3-E2F3a was a kind gift from Professor Kiyoshi Ohtani at Kansai University, Japan. shCASP8AP2, a tetracycline-inducible short hairpin RNA (Tet-on shRNA) expression plasmid, was kindly given by Professor Vincenzo de Laurenzi of Sapienza University of Rome, which was generated from human CASP8AP2 nucleotides 416 to 434 (5′-GATTGTCTGAGTTTCCACA-3′). Tet-on recombinant lentivirus for over-expression of E2F3a and negative control (lentivirus encoding red fluorescent protein) were products of Shanghai Genechem Co., Ltd.

There were three E2F binding sites (TTTSSCGC) in the 1000 bp sequence upstream of the transcription start site of CASP8AP2 (GenBank AL353692.14), at −614, −169, −131 respectively. The DNA fragment containing these three binding sites was amplified using 5′-CATATGCCATGGGCCCCTATGACAACTTACCC-3′ as forward primer and 5′-GGAACCACTGCTTCAACCGTGTTTTTTTTTT-3′ as reverse primer. Underlined letters represented the two introduced restriction digestion sites (NcoI in forward primer and BglII in reverse primer respectively). After digested by NcoI and BglII, PCR product was subcloned into vector pGL3-basic to make luciferase reporter CASP8AP2-wild.

Based on CASP8AP2-wild, four mutated reporters were generated using Fast Mutagenesis System (TransGen Biotech Co., Ltd, China): CASP8AP2-mutant1, -mutant2, -mutant3, -mutant4, which were mutated at binding site −169, −131, −617, and all the three sites respectively (Fig. 1). All binding sites in the four reporters were mutated to 5′-ctctctct-3′ confirmed by sequencing. The primers used in mutagenesis were listed in Table 1.

**Table 1 The Primers used in mutagenesis**

| Mutation sites | Primers |
|----------------|---------|
| −169           | F: 5′−AGGAAGATTACTGGAGCTCTCTCAACCGTGTTTTTTTTTTT-3′  
|                | R: 5′−AGAGAGAGCTCCAGTAACTTCTCTAGGCTTAAAGG-3′  |
| −131           | F: 5′−TTTTTTCCTCAACAGGCTCTCTTTATGTGGGGCTCTG-3′  
|                | R: 5′−AGAGAGAGCTTTGGAAAGGAAAAAAGACTGGTTG3′  |
| −614           | F: 5′−AGCTCTGTCGCTTCTATATCTCTTCATCTATCAATGCTGGGCTG-3′  
|                | R: 5′−AGAGAGAGATAGAGGCCACCCAGATCTCGAGGCCCGGCTA-3′  |

**Fig. 1** Diagram of the wild type and 4 types of mutant promoters of CASP8AP2. The vertical lines represented the binding sites of E2F3a. The crosses represented the mutated binding sites. The arrow represented the transcription start site. Luc luciferase.

Cell lines
Human ALL cell lines 697, REH, MHH-CALL-2, Nalm-6 cells were cultured in RPMI 1640 (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Human leukemia SUP-B15 cells were cultured in Iscove’s modified Dulbecco’s media (IMDM) supplemented with 20% FBS. Human colorectal cancer HCT116 cells were cultured in DMEM supplemented with 10% FBS. All cells were maintained at 37 °C in a humidified atmosphere containing 5% CO2.
Transfection
To transfect plasmids, Lipofectamine 2000 (Invitrogen, USA) and ECM830 Electroporation System (BTX, USA) were used for HCT116 and human ALL cell lines respectively.

Dual-luciferase reporter assay
Transfected cells were collected after 48 h. Activities of firefly luciferase (FL) and renilla luciferase (RL) were determined using Dual-Luciferase® Reporter Assay System (Promega, USA). Relative luciferase activity was represented by FL/RL.

Determination of cell proliferation
Cell proliferation was determined by MTS method. Briefly, cells were plated into 96-well plate, 100 μl/well. For HCT116, cell confluence should be about 30%; for leukemic cells, the number of cells should be about 10^4 per well. After treatment with Doxycycline (0.5 μg/ml), chemotherapeutic drugs (10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10 μg/ml of Vincristine or Daunorubicin), or plasmid transfection, the cells were cultured for 24 h at 37 °C in a humidified atmosphere containing 5% CO₂. Twenty microlitre of CellTiter 96 AQueous One Solution Reagent (Promega, USA) was added to each well and mixed gently. After incubation for 2–4 h, absorbance at 490 nm was determined using microplate reader. After calibrated with non cellular background, cell viability was calculated using a non treatment control regarded as 100% of cell viability.

E2F3a stable infection
697 and REH cells were infected by inducible Tet-on E2F3a lentivirus (multiplicity of infection = 100). Puromycin (0.4 μg/ml) was added after 48 h. Culture medium was changed every two days until the sixth or eighth day, at that time the stable infected cells were screened out. These cells could be induced to over-express E2F3a by Doxycycline (Dox, 0.5 μg/ml).

Determination of cell cycle phases
Flow cytometry was used to determine the changes in cell cycle phase fractions. Briefly, Dox was firstly tested for its effect on cell cycle phase fractions in 697 cell. After serum deprivation and induction of E2F3a over-expression by Dox, the 697 cell stably infected with inducible Tet-on E2F3a lentivirus was cultured for 24 h in complete medium containing 10% fetal bovine serum. The harvested cells were washed using phosphate-buffered saline, fixed by 75% ethanol, and digested by RNase A. After nucleus staining by 5 μl of propidium iodide (PI), the cell cycle phase fractions was determined by flow cytometry.

Chromatin Immunoprecipitation (ChIP)
The ChIP assay was carried out with ChIP assay kit (Beyotime Institute of Biotechnology, China), anti-E2F3 or rabbit IgG antibody. The separated DNA was amplified with the PCR primer pairs, 5'-GCCCCTCTCTTAAA GCCC-3' and 5'-GAGTGCCCTACGGCCAATCAT-3' for fragment −206 to −69, 5'-GGGTGGAATACCT GACTTG-3' and 5'-GCAAGTCAGGTATTCCACC-3' for fragment −677 to −507 in CASP8AP2 promoter respectively.

Western blotting
Total proteins were extracted from cells lysed with RIPA lysis buffer and separated using SDS-PAGE. Nuclear proteins were separated with NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, USA) and NuPAGE Bis–Tris Mini Gels System (GIBCO, USA). Western blotting was carried out with antibodies described below.

Antibodies
The antibodies used in this study included anti-E2F3 (Abcam, UK), rabbit polyclonal anti-CASP8AP2 (Abcam, UK), rabbit polyclonal anti-β-actin (MBL, Japan), goat polyclonal anti-Lamin B (Santa Cruz, USA), Dylight 800 AffiniPure goat anti-rabbit IgG and anti-mouse IgG(H+L, EarthOx, USA).

Statistics
Independent-samples T test was used to compare cell numbers in 697 cell with or without over-expression of E2F3a at different time points. Nonlinear regression curve fit was used to draw the curve and independent-samples T test was used to compare the regression coefficients of the two curves after VCR or DNR treatments in 697 and REH cells with or without over-expression of E2F3a, and in 697 cell over-expressing E2F3a with or without knock-down of CASP8AP2. Relative fluorescence intensities among the groups transfected with different plasmids were compared using one-way Analysis of Variance (ANOVA) or Fisher’s Least Significant Difference (LSD) test. It was regarded as statistically significant when P<0.05. The statistical analyses were carried out using SPSS16.0 software package. The fitting curves of inhibitory effects of chemotherapeutic drugs on cell proliferation were plotted by GraphPad Prism 5, and half maximal inhibitory concentration (IC50) was also calculated by the software.
Results

**E2F3a over-expression led to an increased fraction of leukemic cells in S and G2/M phases and accelerated proliferation**

E2F3a was significantly up-regulated by 24 h induction of Dox (0.5 μg/ml) in 697 stably infected with Tet-on lentivirus (Fig. 2a). Flow cytometric determination showed no effect of Dox on cell cycle phase fraction (P > 0.05, Fig. 2b). It was noteworthy that there was a significant decrease in the fraction of cells in G0/G1 phases upon induction of E2F3a expression by Dox (44.64% ± 2.18% vs. 32.51% ± 1.34%, P = 0.001). By contrast, obvious increases in S and G2/M phases cells were observed (51.48% ± 2.21% vs. 55.27% ± 0.34%, P = 0.043; 3.88% ± 2.43% vs. 12.22% ± 1.02%, P = 0.005, Fig. 2c). MTS assay also showed that after Dox induction and 1–3 days culture, the proliferation of infected cells was significantly accelerated since day 1 (P value was 0.007, 0.003, 0.004 for day 1–3 respectively, Fig. 2d).

**Over-expression of E2F3a enhanced sensitivity of leukemic cells to chemotherapeutic drugs vincristine and daunorubicin**

Vincristine (VCR) and daunorubicin (DNR) were two main chemotherapeutic drugs in induction therapy of ALL. Different concentrations of VCR and DNR were used to treat leukemic cells for 24 h. In 697 and REH cells, over-expression of E2F3a (Fig. 2a) could obviously decrease cell viability; half maximal inhibitory concentrations (IC50) were also reduced (Table 2, Fig. 3a–d). These suggested the role of E2F3a in enhancing sensitivity of leukemic cells to chemotherapeutic drugs.

**E2F3a could activate CASP8AP2 transcription**

The leukemic cell line 697 was treated with Dox for over-expression of E2F3a, then transfected using electroporation method with plasmid CASP8AP2-wild. For HCT116, pcDNA3-E2F3a and CASP8AP2-wild were co-transfected using Lipofectin method. When E2F3a was induced to over-express (Figs. 2a, 4a), the relative luciferase activity of the wild type of CASP8AP2 promoter was increased significantly (P < 0.05, Fig. 4b, c).

| Cell lines | Drugs   | E2F3a over-expression | IC50 (μg/ml) | P     |
|------------|---------|-----------------------|--------------|-------|
| 697        | VCR     | Dox −                 | 0.1313       | < 0.001|
|            |         | Dox +                 | 0.0548       |       |
|            | DNR     | Dox −                 | 0.0211       | < 0.001|
|            |         | Dox +                 | 0.0075       |       |
| REH        | VCR     | Dox −                 | 0.0658       | < 0.05 |
|            |         | Dox +                 | 0.0211       |       |
|            | DNR     | Dox −                 | 0.0266       | < 0.05 |
|            |         | Dox +                 | 0.0108       |       |

**Table 2 IC50 after treatment with VCR and DNR in leukemic cells over-expressing E2F3a**

![Fig. 2](image.png) **Fig. 2** Effects of over-expression of E2F3a on the distribution of cell cycle phases and proliferation of 697. a E2F3a was significantly up-regulated after 24 h induction of Dox (0.5 μg/ml) in 697 (left) and REH (right) cells. b In 697 cells, Dox had no effect on the cell cycle phase fraction. c In 697 cells, over-expression of E2F3a resulted in decrease or increase in the fraction of cells in G0/G1, or S and G2/M phases respectively. d Over-expression of E2F3a led to accelerated proliferation. The standard errors of the means are shown (n = 3 experiments for each time point); independent samples T test, *P < 0.05**
pcDNA3-E2F3a was co-transfected into HCT116 or 697 with CASP8AP2-mutant 1–4 respectively, the luciferase activities of all the mutated types of promoters were obviously enhanced, however, all were lower than the wild type \((P<0.05 \text{ for all, Fig. 4b, c})\). These results indicated that E2F3a was able to enhance the transcriptional activity of CASP8AP2; the three E2F binding sites at \(-614, -169, \text{ and } -131\) were all involved in the activation role of E2F3a. Accordingly, over-expression of E2F3a resulted in significant up-regulation of CASP8AP2 in 697 cell (Fig. 4d).

**E2F3a could bind CASP8AP2 promoter directly**

ChIP assay was used to determine whether endogenous E2F3a was capable of binding the two fragments \((-206 \text{ to } -69 \text{ and } -677 \text{ to } -507\) of CASP8AP2 promoter directly in six leukemic cell lines, including MHH-CALL-2, REH, RS4;11, SUP-B15, Nalm-6 and 697. In all the cell lines, there were clear specific bands in the samples of input and anti-E2F3a, whereas no or weak bands were noticed in the control samples (Fig. 5). Sequencing and alignments indicated that the amplification products matched the sequences of CASP8AP2 promoter completely. Together, these results showed that E2F3a was able to directly bind the two promoter regions of CASP8AP2.

**Down-regulation of CASP8AP2 reversed the advancing effect of E2F3a on chemotherapeutic sensitivity of 697 cell**

As described above, E2F3a could enhance the sensitivity of 697 and REH cells to chemotherapeutic drugs. It was noticed that high expressions of both E2F3a and CASP8AP2 were associated with better treatment response and prognosis; moreover, E2F3a could bind CASP8AP2 promoter and activate its transcription. Thus, the role of CASP8AP2 played in the effects of E2F3a on chemotherapeutic sensitivity was investigated further.

shCASP8AP2 led to down-regulation of CASP8AP2 in 697 stably infected with Tet-on E2F3a lentivirus (Fig. 6a). When these cells were treated by different concentrations
Fig. 4 E2F3a over-expression enhanced the transcriptional activity of CASP8AP2. 

- **a** E2F3a was up-regulated 24 h after transfection of pcDNA3-E2F3a in HCT116 cell.
- **b, c** In HCT116 and 697, relative luciferase activity of CASP8AP2-wild was higher than that of the 4 types of mutant promotor reporters when co-transfected with pcDNA3-E2F3a. The standard errors of the means are shown (n = 3 experiments for each mutant); independent-samples T test, *P < 0.05.
- **d** E2F3a over-expression resulted in up-regulation of CASP8AP2 in 697 cell.

Fig. 5 E2F3a could bind the two fragments of −206 to −69 (a) and −677 to −507 (b) in CASP8AP2 promotor.
of VCR, the cell viability and IC50 were significantly increased (Fig. 6b, shControl vs. shCASP8AP2, 0.0076 vs. 0.0145, P = 0.0105). Increase tendency was also found after treatment with DNR (Fig. 6c, shControl vs. shCASP8AP2, 0.0173 vs. 0.0188, P = 0.056). Taken together, the advancing effect of E2F3a on chemotherapeutic sensitivity of ALL cells was implemented by regulating CASP8AP2 expression to a considerable degree, and could be reversed by down-regulating CASP8AP2.

**Discussion**

The expression level of CASP8AP2 was an important independent prognostic factor in childhood ALL. The patients with low expression of CASP8AP2 at diagnosis were at greater risk of relapse [11]. However, it was unclear about the mechanisms of low expression of CASP8AP2 in childhood ALL. Our previous study has shown that in childhood ALL, hypermethylation of two CpG sites located at −1189 and −1176 in CASP8AP2 promoter was correlated to mRNA low expression, MRD positivity before consolidation therapy (day 78th) and poor prognosis [21]. It was worthy to note that the methylation of these two CpG sites was not the key factor to affect gene expression. Since the methylation of core promoter of CASP8AP2 decreased gene expression and induced resistance to chemotherapeutic drugs [22, 23], it was deduced that the specific transcription factors capable of binding core promoter and inducing transcription played the pivotal role in regulating CASP8AP2 expression. Thus, the key to clarify the mechanism of low expression of CASP8AP2 in childhood ALL was to identify the transcription factors capable of binding core promoter of CASP8AP2.

There were three binding sites of E2F located at −614, −169, and −131 in CASP8AP2 promotor. The studies of gene expression profiling of St. Jude Children’s Research Hospital and us have indicated that only E2F3 expressed, whereas the expression of other members including E2F1, E2F2, and E2F 4–6 could not be detected in most samples [15, 16]. Therefore, E2F3a was probably an important factor to regulate CASP8AP2 expression in childhood ALL. In present study, E2F3a over-expression could activate CASP8AP2 transcription; mutations of the three E2F binding sites at −614, −169, and −131 made transcription activity decreased significantly. Meanwhile, the binding of E2F3a with the two fragments of CASP8AP2 promotor, −206 to −69 and −677 to −507, was identified by ChIP (Fig. 5). Thus, this study uncovered the fact that E2F3a was able to bind CASP8AP2 promoter and activate its transcription. It should be noted that
as dual luciferase assay showed, relative luciferase activity was still higher in the reporter with three mutations at -614, -169, and -131 than in the control. This was probably due to the existence of other binding sites of some transcription factors, such as c-Myb, c-Myc, and AP-1, which had the potential activating function on the promoter. Thus, further studies were needed to clarify the mechanism of low expression of CASP8AP2 in childhood ALL.

E2F3a was the key factor of controlling DNA replication and transition of G1 to S phase [19]. In the other hand, CASP8AP2 was essential to progression of S phase, transcription and 3’end processing of replication dependent histone mRNA [5, 7]. In this scenario, the positive regulation of E2F3a on CASP8AP2 transcription could coordinate genomic DNA replication and histone biosynthesis, with the result of chromatin integrity and stability. The similar results in colorectal cancer cells and leukemic cells suggested that this regulation probably existed in many types of tissues and cells, and was essential to cell proliferation.

Abnormal high expression of E2F3a has been found in many types of solid tumors [13, 14, 19], often associated with poor prognosis [24, 25]. However, our previous study has shown that E2F3a was generally low-expressed and associated with leukemic relapse or induction failure in childhood ALL [20]. Accordingly, in present study, we showed that E2F3a over-expression made leukemic cells proliferate more rapidly, and more sensitive to VCR and DNR. It has been long recognized that slow-proliferating leukemic cells were resistant to common chemotherapeutic drugs including VCR, DNR, cytarabine, methotrexate, and thioguanines [26]. As E2F3a plays an important role in G1/S transition and cell proliferation [19], its down-regulation probably lead to slow proliferation and chemotherapeutic resistance of leukemic cells in childhood ALL. Many groups have reported similar results. Flotho et al. have reported the association of the low expression of some genes essential for cell cycle progress with the high level of MRD and relapse in childhood ALL, such as CCNB2, CDC2, CKS1B, and interestingly, CASP8AP2 [8, 9].

It was noteworthy that although E2F3a overexpression reduced IC50 of leukemic cells, there was no significant difference in the viability of leukemic cells at high concentrations of chemotherapeutic drugs. It meant that the effect of E2F3a expression level on chemoresistance was related to drug concentration. High concentration of drugs or combined chemotherapy would play a bigger role in eliminating leukemic cells.

Although our results were contrary to that of studies in solid tumors; the reason behind this contradiction was unclear. Based on the results of present study, down-regulation of CASP8AP2 which was also associated with leukemic relapse in childhood ALL [11] may be one of the main reasons of chemotherapeutic resistance of low-expression of E2F3a.

To test this hypothesis, CASP8AP2 was knocked down in 697 with over-expression of E2F3a and sensitivity to VCR and DNR was determined. As expected, IC50 of VCR and DNR treatment significantly increased or tended to increase respectively. Therefore the advancing effects of E2F3a on sensitivity to chemotherapeutic drugs were implemented via up-regulating CASP8AP2 to a great extent. On the other hand, as VCR and DNR were cell cycle specific or non-specific drugs respectively, these results also suggested that the effects of CASP8AP2 on sensitivity to drugs was mainly implemented via influencing cell cycle progression, whereas E2F3a may make effects on chemotherapeutic sensitivity via other ways to be investigated.

Many mechanisms and factors were involved in resistance to chemotherapeutic drugs of tumor cells, such as DNA damage and repair, cell apoptosis, metabolism and absorption of drugs, and abnormal cell cycle [27]. Except the effects on cell cycle progression revealed in the present study, there may be other ways by which E2F3a and CASP8AP2 affected chemotherapeutic sensitivity in childhood ALL. In these processes, interactions of E2F3a and CASP8AP2 with other proteins needed to be clarified. In addition, development of novel small molecules and techniques for target therapy against low expression of E2F3a and CASP8AP2 was an important direction for further study.

Conclusions

Our data suggest that E2F3a increased leukemic cells in S and G2/M phases, accelerated proliferation and enhanced sensitivity to chemotherapeutic drugs. Furthermore, E2F3a could bind CASP8AP2 promoter directly and activate CASP8AP2 transcription; CASP8AP2 down-regulation reversed the advancing effect of E2F3a on chemosensitivity.

Abbreviations

CASP8AP2: caspase 8 associated protein 2; ALL: acute lymphoblastic leukemia; ChIP: chromatin immunoprecipitation; EFS: event free survival; MRD: minimal residual disease; Tet-on shRNA: tetracycline-inducible short hairpin RNA; FBS: fetal bovine serum; IMDM: Iscove’s modified Dulbecco’s media; FL: firefly luciferase; RL: renilla luciferase; Dox: doxycycline; PI: propidium iodide; ANOVA: analysis of Variance; LSD: Fisher’s Least Significant Difference; VCR: vincristine; DNR: daunorubicin; IC50: half maximal inhibitory concentrations.

Authors’ contributions

FFL and K LW performed the experiments, analyzed the data and drafted the paper. LD and XL performed some experiments and analyzed the data. MYW and TYW provided administrative support and supplied the clinical data. ZGL and LC provided the designs of the study, revised the article critically and provided final approval. All authors read and approved the final manuscript.
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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The datasets used and/or analysed during this study are available from the corresponding authors on reasonable request.

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Not applicable.

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Not applicable.

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