microRNA 421 induces apoptosis of c-33a cervical cancer cells via down-regulation of Bcl-xL

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ABSTRACT. Cervical cancer is a life-threatening condition. MicroRNAs (miRNAs) can promote or inhibit cell death and proliferation. The present study investigated the effect of miRNA 421 on the growth and apoptosis of cervical cancer cells. miRNA 421 and control miRNA were synthesized and transfected into c-33a cervical cancer cells. A thiazolyl blue tetrazolium bromide assay, caspase-3 activity, and flow cytometry were used to study the effects of miRNA 421 on c-33a cell growth, and apoptosis. Small interfering RNA targeting Bcl-xL was synthesized and transfected into c-33a cells along with miRNA 421. Bcl-xL expression and cell apoptosis were then measured by western blot and flow cytometry, respectively. Transfection of miRNA 421 into c-33a cells reduced their growth, promoted their apoptosis (measured by increased phosphatidylserine eversion), activated caspase-3, and decreased Bcl-

xL expression. Silencing and overexpression of Bcl-xL enhanced and inhibited miRNA 421-induced apoptosis of c-33a cells, respectively. miRNA 421 induces c-33a cell apoptosis via down-regulation of Bcl-xL, suggesting that this latter might be used as a potential clinical target.

INTRODUCTION

Cervical cancer is a malignancy of the female reproductive system with an incidence rate of 0.03% (Kong et al., 2015). A variety of causes have been identified, of which, viral infections, sex, and frequency of childbirth are the main etiological factors (Jia et al., 2016). Cervical cancer is a life-threatening disease (Cong et al., 2015; Li et al., 2015). Although appropriate individualized treatment plans and integrated treatment regimens are effective, these approaches have shortcomings, including bleeding and other side effects (Bonetta et al., 2015; Bumrungthai et al., 2015; Song et al., 2015). Improving the specificity and success rate of cervical cancer treatment is an important and challenging goal for the medical and scientific communities. Targeted molecular therapy has become a focus of research concerning the treatment of cervical cancer (Deng et al., 2015; Lin et al., 2015; Liu et al., 2015). Targeting inhibitors of apoptosis proteins as a treatment for this condition has yielded poor results (Honegger et al., 2015); therefore, more effective molecular targets are needed (Bhattacharjya et al., 2015; Honegger et al., 2015). Moreover, targeting microRNAs (miRNAs) has not been attempted in cervical cancer treatment (Bhattacharjya et al., 2015).

miRNAs perform a variety of functions. miRNA-218 can inhibit the growth of cervical cancer cells, and miRNA-34a is associated with tumor metastasis (Geng et al., 2015; Kogo et al., 2015). Thus, miRNAs may be involved in the occurrence and development of cervical cancer (Lao et al., 2014; Geng et al., 2015; Kogo et al., 2015). In previous studies, specific primers were designed to measure miRNA expression in cervical cancer tissues and adjacent normal tissues by real-time polymerase chain reaction. Expression of miRNA 421 was found to be significantly higher in tumor tissues than in healthy tissue specimens, suggesting that it may play a role in cervical oncogenesis and its progression (Chang et al., 2014; Yuan et al., 2014).

The present study used c-33a cervical cancer cells to investigate the role of miRNA 421 in this malignancy. The aim of anti-cancer therapy is to kill tumor cells without affecting normal cells. Apoptosis is regulated by both anti- and pro-apoptotic proteins (Pedroza-Torres et al., 2014; Wen et al., 2015); therefore, the ideal anti-cancer drug would increase the effects of the latter and decrease those of the former. Bcl-xL is a widely studied anti-apoptotic protein (Zhang et al., 2013; Yu et al., 2014). Although many anti-cancer drugs target Bcl-xL, the effects of its down-regulation have not been as beneficial as expected (Huang et al., 2013). This study also tested Bcl-xL as a potential target, by using c-33a cells as a model to investigate the role of miRNA 421 in cervical cancer, providing a theoretical basis for targeted molecular therapy for this disease.

MATERIAL AND METHODS

Reagents and cells

c-33a cervical cancer cells were purchased from the American Type Culture Collection

(Manassas, VA, USA). Fetal bovine serum and cell culture medium were sourced from Hualan Biological Co. (Beijing, China). A liposome transfection kit (Lipofectamine 2000) was provided by Invitrogen (Carlsbad, CA, USA). Thiazolyl blue tetrazolium bromide (MTT) assay reagents were manufactured by Beijing Dingguo Co. (Beijing, China). Apoptosis detection reagents, a caspase-3 assay kit, a protein extraction kit, and a bicinchoninic acid (BCA) protein assay kit were purchased from Biyuntian Biotechnology Institute (Haimen, China). Mouse anti-human Bcl-xL antibody was provided by Santa Cruz Biotechnology (Dallas, TX, USA). miRNA 421, control miRNA, and Bcl-xL plasmids were made by Jima Biotechnology Co., Ltd. (Shanghai, China). Sequences for miRNA 421 and control miRNA were 5'-TCA GGT CAT CAC TAT CGG CAA T-3' and 5'-AAA GAA AGG GTG TAA AAC GCA-3', and sequences for Bcl-xL small interfering RNA (siRNA) and siRNA control were 5'-CTA TGA GGC TAC CAT CGT GC-3' and 5'-TTT CAC TAC GAT TAA GCG T-3'.

Cell culture

c-33a cervical cancer cells were cultured in high-glucose Dulbecco's modified Eagle's medium according to a previously published method (Liu et al., 2015).

Transfection

miRNA 421 and control miRNA were transfected into c-33a cells following the manufacturer protocol. One microgram of miRNA or control miRNA was mixed with Lipofectamine 2000 and transfected into cells when they had reached 80% confluency.

MTT assay of cell viability

The MTT test was performed according to standard methods (Lin et al., 2015). MTT solution was placed in each well and incubated with cells for 24 h. Dimethyl sulfoxide (100 µL per well) was then added to stop the reaction. Optical density at 560 nm was recorded using a microplate reader to plot a cell growth curve (Honegger et al., 2015).

Measurement of apoptosis by flow cytometry

Flow cytometry was performed according to established methods to detect apoptosis of c-33a cells. Briefly, c-33a cells were transfected with either miRNA 421 or control miRNA and cultured for 48 h. Cells were then collected and resuspended. Reaction buffer (100 µL) and fluorescein isothiocyanate-conjugated annexin V (2 µL) were added to a 500-µL cell suspension, which was then mixed and incubated for 25 min in the dark for flow cytometry analysis (Kogo et al., 2015).

Western blot

c-33a cells transfected with either miRNA 421 or control miRNA were collected and their proteins extracted and quantified using a BCA kit following the manufacturer protocol. Equal amounts of protein were electrophoresed and transferred to membranes, which were subsequently blocked. After being washed with Tris-buffered saline with Tween 20 (TBST),

membranes were incubated with primary antibodies overnight at 4°C. Following further washing with TBST, membranes were incubated with secondary antibodies (diluted 1:2500) for 3 h at 37°C, and proteins subsequently visualized by enhanced chemiluminescence. Bcl-xL expression levels were analyzed with the Gel-Pro Analyser 4.0 software (Bio-Rad Laboratories, Hercules, CA, USA; Lao et al., 2014).

Caspase-3 activity assay

Caspase-3 activity was detected following the kit manufacturer protocol. Briefly, c-33a cells were transfected with either miRNA 421 or control miRNA and cultured for 48 h. Cells were then collected, lysed, mixed with chromogenic substrate, and incubated at room temperature. The absorbance of each sample was recorded with a microplate reader (Yuan et al., 2014). Absorbance of c-33a cells transfected with control miRNA was subtracted from that of those transfected with miRNA 421 to determine relative caspase-3 activity.

Effect of Bcl-xL up- or down-regulation on miRNA 421-transfected c-33a cells

c-33a cells were transfected with either Bcl-xL expression plasmids or siRNA targeting Bcl-xL. These cells were then further transfected with miRNA 421 or control miRNA using Lipofectamine 2000. One microgram of Bcl-xL siRNA or Bcl-xL plasmid, and separately, 4 μg control miRNA or miRNA 421, was suspended in Lipofectamine 2000. These DNA/liposome mixtures were then added drop-wise to the cell culture medium.

Statistical analysis

SPSS 11.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. All experiments were repeated three times, and data are reported as means ± standard errors. Differences between groups were analyzed using the *t*-test, with $P < 0.05$ being considered statistically significant.

RESULTS

miRNA 421 transfection reduced viability of c-33a cells and inhibited their growth

The MTT assay showed that miRNA 421 transfection significantly reduced the viability of c-33a cells, compared with those transfected with control miRNA ($P = 0.0066$). No significant difference was observed between c-33a cells transfected with control miRNA and non-transfected c-33a cells. Therefore, the former were used as controls in this study (Figure 1).

miRNA 421 transfection increased the apoptosis of c-33a cells

Flow cytometry analysis revealed (Figure 2) that transfection with miRNA 421 significantly increased the presence of phosphatidylserine on c-33a cells, compared to those transfected with control miRNA ($P = 0.009$).
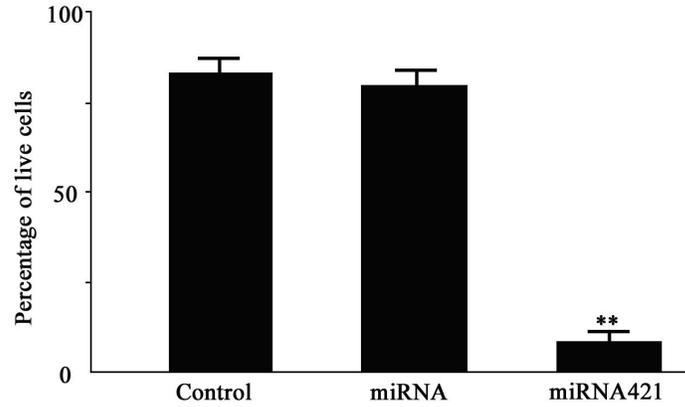
Figure 1. miRNA 421 transfection inhibited the growth of c-33a cells. A thiazolyl blue tetrazolium bromide assay was used to analyze the viability of non-transfected c-33a cells and those transfected with control miRNA or miRNA 421. **P < 0.01 compared to the control miRNA group.
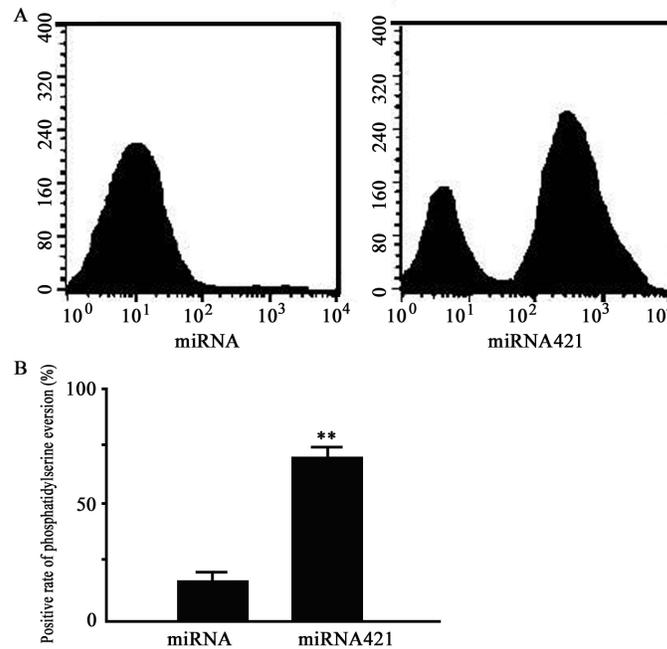

Figure 2. miRNA 421 transfection induced apoptosis of c-33a cells. Flow cytometry was used to analyze apoptosis of non-transfected c-33a cells and those transfected with control miRNA or miRNA 421. **A.** Flow cytometry analysis. **B.** Presence of phosphatidylserine on c-33a cells. **P < 0.01 compared to the control miRNA group.

miRNA 421 transfection activated caspase-3 in c-33a cells

The caspase-3 activity assay demonstrated (Figure 3) that miRNA 421 transfection significantly activated this enzyme in c-33a cells, compared to the control miRNA-transfected group (P = 0.031).
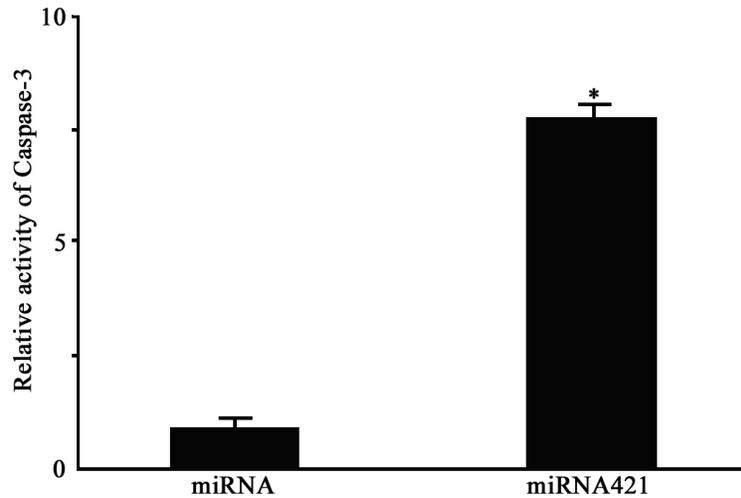
Figure 3. miRNA 421 transfection activated caspase-3 in c-33a cells. A caspase-3 assay was used to measure the activity of this enzyme in non-transfected c-33a cells and those transfected with control miRNA or miRNA 421. *P < 0.05 compared to the control miRNA group.

miRNA 421 transfection decreased Bcl-xL protein expression in c-33a cells

Western blotting showed that transfection with miRNA 421 significantly reduced expression of Bcl-xL protein in c-33a cells, compared with those transfected with control miRNA (Figure 4).
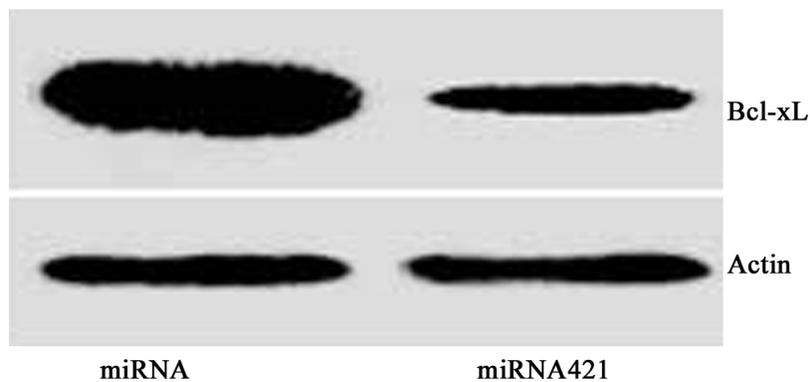

Figure 4. miRNA 421 transfection reduced Bcl-xL expression in c-33a cells.

Silencing of Bcl-xL enhanced miRNA 421-induced apoptosis of c-33a cells

Bcl-xL was silenced to investigate its role in miRNA 421-induced apoptosis of c-33a cells. Western blotting revealed that Bcl-xL levels were significantly reduced by siRNA (Figure 5 and Table 1). Expression of Bcl-xL in the c-33a-miRNA 421-siBcl-xL group was

significantly lower than that in the c-33a-miRNA 421-control siRNA group (P = 0.025). The results showed that decreased Bcl-xL expression resulting from siRNA significantly increased miRNA 421-induced apoptosis of c-33a cells, in comparison to that of cells transfected with and miRNA 421 (P = 0.014; Figure 5).
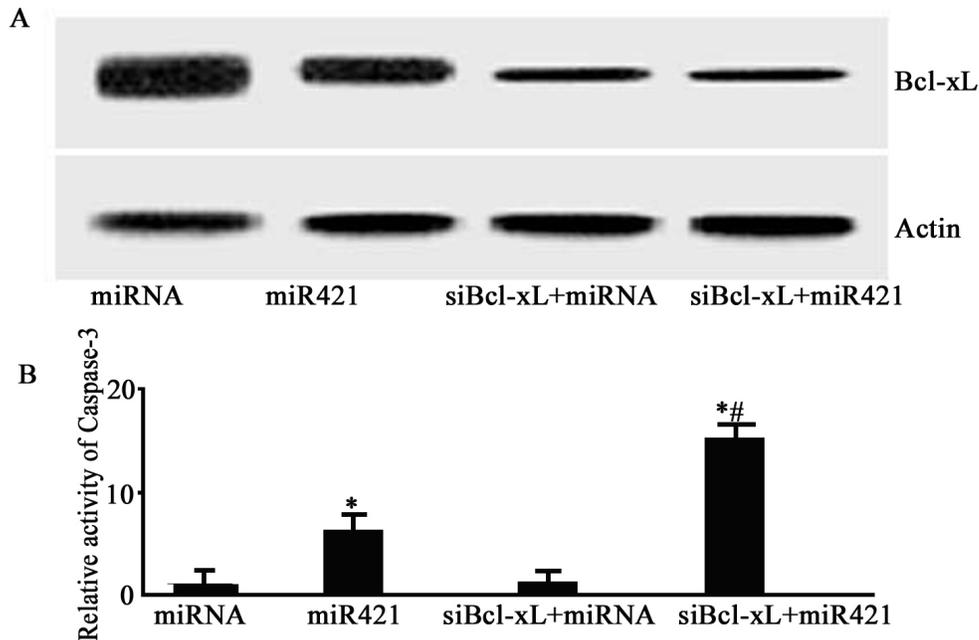

Figure 5. Silencing of Bcl-xL enhanced miRNA 421-induced apoptosis of c-33a cells. c-33a cells transfected with control miRNA, miRNA 421, miRNA 421 + Bcl-xL small interfering RNA (siBcl-xL), or control miRNA + siBcl-xL. **A.** Expression of Bcl-xL. **B.** Relative activity of caspase-3. *P < 0.05 compared to the control miRNA group; #P < 0.05 compared to the control miRNA + siBcl-xL group.

Table 1. Relative expression of Bcl-xL protein in c-33a cells transfected with small interfering RNA targeting Bcl-xL (siBcl-xL).

	miRNA	miRNA 421	siBcl-xL + miRNA	siBcl-xL + miRNA 421
Bcl-xL/actin	2.54 ± 0.22	1.31 ± 0.41*	0.57 ± 0.15*	0.42 ± 0.20*

miRNA = microRNA. *P < 0.05 compared to the miRNA group.

Overexpression of Bcl-xL inhibited miRNA 421-induced apoptosis of c-33a cells

Bcl-xL was overexpressed in c-33a cells in order to examine its effect on their miRNA 421-induced apoptosis. The results of Figure 6 and Table 2 suggested that Bcl-xL was significantly decreased in cells transfected with miRNA 421 plasmid (P < 0.05). As shown in Figure 6, Bcl-xL was significantly overexpressed in cells transfected with both miRNA 421 and the Bcl-xL plasmid, compared to those transfected with miRNA 421 alone (P = 0.018). The caspase-3 activity assay revealed that Bcl-xL overexpression significantly inhibited miRNA 421-induced apoptosis of c-33a cells (P = 0.027; Figure 6).
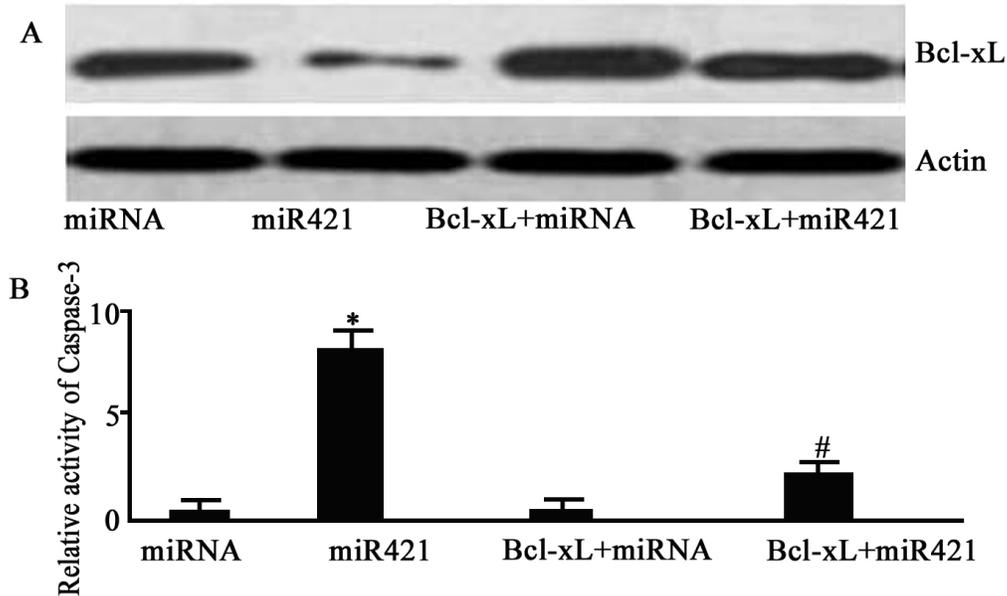
Figure 6. Overexpression of Bcl-xL inhibited miRNA 421-induced apoptosis of c-33a cells. A caspase-3 assay was used to analyze apoptosis of c-33a cells transfected with control miRNA, miRNA 421, or miRNA 421 + a Bcl-xL expression plasmid. **A.** Western blot of Bcl-xL expression. **B.** Caspase-3 activity. *$P < 0.05$ compared to the control miRNA group; #$P < 0.05$ compared to the miRNA 421 group.

Table 2. Relative expression of Bcl-xL protein in c-33a cells transfected with a Bcl-xL expression plasmid.

	miRNA	miRNA 421	Bcl-xL + miRNA	Bcl-xL + miRNA 421
Bcl-xL/actin	1.24 ± 0.13	0.35 ± 0.07*	1.48 ± 0.25	1.31 ± 0.18

miRNA = microRNA. *$P < 0.05$ compared to the miRNA group.

DISCUSSION

In this study, c-33a cervical cancer cells were used as a model to study the role of miRNA 421 in this malignancy at the molecular level. Our data demonstrated that transfection with miRNA 421 reduced the viability and promoted the apoptosis of c-33a cells. This is consistent with previous studies showing that miRNAs are involved in cell growth and survival (Li et al., 2015). Few studies have examined the effects of miRNAs in cervical cancer (Li et al., 2015). miRNA-218 has been shown to inhibit the growth of cervical malignancies, and miRNA-34a influences metastasis (Geng et al., 2015; Kogo et al., 2015). Thus, miRNAs may also be involved in the occurrence and development of cervical cancer (Lao et al., 2014; Geng et al., 2015; Kogo et al., 2015).

Bcl-xL is an inhibitor of apoptosis proteins (Yang et al., 2014). Whether miRNA 421 regulates the growth of c-33a cells through regulation of Bcl-xL remains unclear (Chen et al., 2014; Liu et al., 2015). The present study showed that transfection with miRNA 421 decreased Bcl-xL levels. Moreover, suppression and overexpression of Bcl-xL promoted and inhibited miRNA 421-induced apoptosis of c-33a cells, respectively.

In the current study, three different methods were employed to demonstrate that Bcl-xL plays a highly important role in the apoptosis of c-33a cells affected by miRNA 421. First, western blotting identified a significant reduction in Bcl-xL protein levels in c-33a cells. Second, suppression of Bcl-xL enhanced miRNA 421-induced apoptosis of these cells. Last, Bcl-xL overexpression was shown to inhibit such apoptosis. These results indicate that Bcl-xL performs a crucial function in the mechanism by which miRNA 421 promotes c-33a cell apoptosis, suggesting that it might constitute a novel target for the molecular therapy of cervical cancer (Luo et al., 2013). Other studies have reported that Bcl-xL inhibits apoptosis of cancer cells (Huang et al., 2013; Liu et al., 2015), but our investigation showed for the first time that miRNA 421 and Bcl-xL coordinate to regulate this process.

Having not included cervical tumor tissues and adjacent normal tissues in this study, measurement of Bcl-xL protein levels in such specimens, which would have demonstrated the relationship between Bcl-xL and cervical cancer in a clinical context, was not carried out. This study also lacked data from an animal model of cervical cancer, which would have allowed us to test the efficacy of targeting miRNA 421 as a treatment for this condition. These shortcomings will be addressed in future research.

CONCLUSIONS

Transfection with miRNA 421 reduced the viability of c-33a cervical cancer cells, and induced their apoptosis by suppression of Bcl-xL expression. Therefore, Bcl-xL may represent a therapeutic target in cervical cancer, and our study provides a useful theoretical basis for the treatment of this disease.

Conflicts of interest

The authors declare no conflict of interest.

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