MicroRNA-221-3p promotes proliferation and invasion in non-small cell lung cancer via targeting Axin2 to regulate Wnt/β-catenin signaling pathway

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Abstract

Objective

The mortality rate of lung cancer ranks first in malignant tumors. Among them, non-small cell lung cancer (NSCLC) accounts for about 85% of all lung cancer patients. In this study, we explore part of the mechanism of development and progression of NSCLC.

Methods/ Results

Firstly, there was an increase in microRNA-221-3p (miR-221-3p) expression and a decrease in Axin2 expression in NSCLC tissues using real-time reverse transcription polymerase chain reaction. Further studies showed that miR-221-3p inhibited the expression of Axin2, which negatively regulated the Wnt signaling pathway. With the method of inhibiting and overexpressing the expression of miR-221-3p and/or Axin2 respectively in NSCLC cell lines A549 and H1975, we found that inhibiting the expression of miR-221-3p leaded to a decrease in cell proliferation, migration and invasion, just like the results of overexpressing Axin2. Relatively speaking, overexpression of miR-221-3p in NSCLC cell lines showed the increase of proliferation as well as the decrease of apoptosis. Thus, we knew that miR-221-3p promoted the migration and invasion of NSCLC cells in vitro. What’s more, according to western blot and EdU assay, we demonstrated that overexpression of miR-221-3p inhibited the expression of Axin2 and subsequently activate classical Wnt/β-catenin signaling pathway. At last, a series of methods were used to identify that miR-221-3p inhibited Axin2 expression, increased cell proliferation, invasion and migration, and decreased cell apoptosis.

Conclusion

Our results suggest that miR-221-3p inhibits the expression of Axin2 and indirectly activates the typical Wnt/β-catenin signaling pathway, thus promoting tumor proliferation and invasion in NSCLC.

Introduction

Lung cancer is reported to be the most common cause of cancer deaths in human and its five-year survival rate of is below 15 percentage. Although, a mountain of works on the disease prevention, diagnosis and therapy improvement, the prognosis of lung cancer patients remains pessimistic. Lung cancer can be roughly divided into non-small cell lung carcinoma and small cell lung carcinoma (SCLC) by pathological classification. Over the past few decades, NSCLC accounts for more than 85% of lung cancer, clinically, the most portion of patients with NSCLC are diagnosed at locally advanced or metastatic stages. Therefore, new effective drugs and markers for early stage diagnosis are the keys to improving the cancer survival rates. Molecular networks and genetic mechanisms underlying the invasion and metastasis of NSCLC cells are the keys to identifying targets for therapeutic strategies.
MiRNA contains about 22 nucleotides, which belongs to small noncoding RNA molecules. In decades, multiple researches have been performed and revealed that miRNAs involve in various diseases by regulating genes expression in post-transcription process. In addition, miRNAs are applied as the biomarkers, prognostic markers and therapeutic targets of many diseases, especially for cancers. Several miRNAs have been identified to regulate the cisplatin chemosensitivity in NSCLC cells, such as miR-451, miR-379, and miR-185-5p. Besides, miR-382 and miR-381 inhibit cancer cell growth and metastasis in NSCLC.

It’s reported that miR-221-3p promotes the dysfunction of endothelial cells by suppressing PGC-1α expression in progressed atherosclerosis. Moreover, in another cardiovascular disease, miR-221-3p plays a role as a marker of early acute myocardial infarction. Emerging studies indicate that miR-221-3p functions as a key mediator of invasion and metastasis of various cancers, such as colon cancer, epithelial ovarian cancer, gastric carcinoma, prostate cancer, breast cancer and pancreatic cancer. Therefore, miR-221-3p was suggested to be a potential target for cancer prognosis and therapeutics.

The classical Wnt/β-catenin signaling pathway played a key role in the development of cancer and other diseases. Interaction between Wnt ligand and a heterodimeric transmembrane receptor complex results in the preservation and accumulation of β-catenin in the cytoplasm. This facilitates the translocation of β-catenin into the nucleus and forms a complex with T-cell specific transcription factor/lymphoid enhancer-binding factor to initiate the expression of downstream target genes. As a scaffold protein, Axin2 regulated GSK3β-dependent phosphorylation of β-catenin, which was a key component of APC complex mediated degradation of β-catenin. Axin2 was not only a target gene of Wnt signaling pathway, but also a Wnt inhibitors. It negatively regulates Wnt signaling pathway. At the same time, Axin2 functions as a tumor suppressor in many types of cancer.

Recently, we found that the expression level of miR-221-3p was dramatically increased in NSCLC tissues from 26 patients. In vitro, studies revealed that miR-221-3p promoted the proliferation and invasion of A549 and H1975 cells which were human NSCLC cell lines. We found that miR-221-3p activated the classical Wnt/β-catenin signaling pathway by inhibiting the expression of Axin2. Inhibition of miR-221-3p and overexpression of Axin2 effectively inhibited the proliferation and invasion of cancer cells in vitro. However, the positive effect of overexpression of Axin2 on inhibiting cancer cells would be weakened in the case of simultaneous overexpression of miR-221-3p.

**Methods**

**Patients and tissue sample**

A total of 26 tumor tissues and matched adjacent normal tissues were collected from 26 patients who had undergone surgery to treat NSCLC at the Affiliated Wujiang Hospital of Nantong University. Fresh tissue samples were confirmed via histopathological examination and then immediately stored in a -80
˚C refrigerator prior to subsequent experiments. Our project was approved by Research Ethics Committee of Affiliated Wujiang Hospital of Nantong University.

**Cell culture and transfection**

All cell lines involved in this study were purchased from ATCC and cultured in our central lab. Human NSCLC cell lines, including A549 and H1975, and human bronchial epithelioid cells 16HBE were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were then incubated in a humidified cell incubator maintained with 5% CO₂ at 37 ℃. Cells were plated in a 6-well dish and grown at 50%-60% density. All the transient transfections were conducted with Lipofectamine 2000 Reagent. The sequences were as followed: miR-221-3p mimics, 5′-AGCUACAUUGUCUGCGGCUUUC-3′, 5′-AACCCAGCAGACAAUGUAGCUU-3′; miR-221-3p inhibitor, 5′-GAAACCCAGCAGACAAUGUAGCU-3; and negative control, 5′-UUCUCCGAACGUGACGUTT-3′ and 5′-ACGUGACCGUUGCAGAACATT-3′. The sequences of siRNA and its negative control were as follows: Axin2, 5′-GCAGAGGGACAGGAATCAT-3′, and the negative control, 5′-GCAGGGACAGGAATCAT-3′.

**RT-qPCR**

Total RNA was extracted from patient tissue and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. cDNA was synthesized using PrimeScript® RT reagent Kit. RT-qPCR was performed using SYBR Premix ExTaq™ (Takara Bio, Inc.) on the platform of Applied Biosystems 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.). U6 and GAPDH were used as internal controls for miRNA and mRNA, respectively. Data analyses were performed using the comparative CT (∆∆CT) approach to calculating relative gene expression. Primers were synthesized by Sangon Biotech Co., Ltd. as follows: miR-221-3p, forward, 5′-CGGCTACATTGTCTGCCTG-3′ and reverse, 5′-CAGTGCGTGTCGTGGAGT-3′; and U6 forward, 5′-CGCTTCGGCAGCACATATAC-3′ and reverse, 5′-AACGCTTCACGAATTTGCGT-3′. The reverse universal miR qPCR primers were included in the PrimeScript™ miRNA RT-PCR kit (cat. no. RR716; Takara Biotechnology Co., Ltd.). The relative expression levels of miR-221-3p and were calculated using the 2−∆∆Cq method. All experiments were conducted in triplicate.

**Colony formation assay**

Colony formation assay was carried out to evaluate the proliferative potential of A549 and H1975 after transfections. Briefly, A549 and H1975 NSCLC cells with a density of 1 × 10³ cells/well were seeded in six-well plates and cultured at 37 ℃ with 5% CO₂. The medium was replaced with fresh culture medium every two or three days for two weeks. Subsequently, cells were fixed with 4% paraformaldehyde for 20 minutes and stained using 10% crystal violet for 30 minutes.

**Wound healing assay**
Wound healing assay was used to assess the cell migration ability of A549 and H1975 NSCLC cells \textit{in vitro}. The cells were cultured into six-well plates and incubated for 24 hours to full confluence. Before scratching, the medium was replaced with fresh culture medium without FBS. A scratch was then created using a sterile plastic tip, and the cells were incubated for 48 hours at 37 °C. The closure of the scratch was analyzed under the microscope and images were captured using an Olympus light microscope (Olympus Corporation, Tokyo, Japan).

\textbf{EdU assay}

EdU assay was performed to detect the proliferation of A549 and H1975 NSCLC cells. Cells were seeded in six-well plates and incubated for 48 hours after different treatments in a humidified cell incubator maintained with 5% CO$_2$ at 37 °C. After incubation with 10 μM EdU for 2 hours, cells were fixed in 4% paraformaldehyde. After that, Hoechst 33342 was used to stain the nuclei. Finally, cells were stained using a Cell-hour Light EdU Apollo 488 \textit{in vitro} Imaging Kit according to the manufacturer's recommendations.

\textbf{Cell apoptosis assay}

Cell apoptosis was performed using the Annexin V Apoptosis Detection Kit I. Briefly, the treated A549 and H1975 NSCLC cells were collected, washed twice with cold 1 × PBS and resuspended in 100μL binding buffer, followed by incubation with Annexin V-FITC and propidium iodide for 15 minutes at room temperature in the dark. Next, 200 μL of binding buffer was added. FACS Calibur was used to calculate the percentage of apoptotic cells.

\textbf{Transwell migration and invasion assays}

Migration and invasion assays were performed using transwell chambers. In migration assay, A549 and H1975 NSCLC cells at a density of 5 × 10$^4$ cells/well were added into the upper chamber. In invasion assay, Matrigel was inoculated into the upper chamber to form a gel at 37 °C, and then A549 and H1975 NSCLC cells were seeded into the upper compartments at a density of 1 × 10$^5$ cells/well. For transwell migration and invasion assays, the lower compartments were filled with 600 μL of medium with 20% FBS. After incubation for 48 hours, cells that had not migrated or invaded were removed from the upper surface while the cells that had migrated or invaded to the lower surface of the membrane were fixed with 4% paraformaldehyde and stained in 10% crystal violet. All experiments were conducted in triplicate.

\textbf{Western blot (WB) assay}

Proteins were separated by electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% milk at room temperature for one hour, then incubated with diluted antibodies against MMP-2, MMP-9, GAPDH, Bcl-2, Bax, Caspase-3, Caspase-7, Axin2, MMP-7, β-catenin, and β-actin. All antibody dilutions were 1:1000. After being washed, blots were incubated with secondary antibody and visualization by enhanced chemiluminescence. All experiments were conducted in triplicate.
Cell immunofluorescence

A549 NSCLC cells were incubated overnight at 4 °C with primary antibodies against β-catenin. The following day, cells were washed in PBS three times (5 minutes each wash) and incubated with secondary antibodies for 2 hours at room temperature. After incubation, cells were rinsed three times (5 minutes each wash) in PBS and mounted in 50% triglyceride. All experiments were conducted in triplicate.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Data were presented as the mean standard deviation from three independent experiments. Significant differences between means were analyzed by the two-tailed, unpaired, non-parametric Mann-Whitney test, and differences were considered significant at $P < 0.05$.

Results

MiR-221-3p expression is increased in tumor tissues from NSCLC patients

To identify the relationship between miR-221-3p levels and NSCLC, we measured the expression level of miR-221-3p in 26 NSCLC patients’ tumor tissues using RT-qPCR. Compared with the control group, the expression level of miR-221-3p was drastically upregulated in NSCLC tissues (Figure 1A). Then, we analyzed the expression level of miR-221-3p in NSCLC cell lines (A549 and H1975) and human bronchial epithelioid cells (16HBE). In contrast to 16HBE, miR-221-3p was significantly upregulated in A549 and H1975 (Figure 1B). In short, these results showed that miR-221-3p was increased in NSCLC tissues and cell lines, which suggested that miR-221-3p involved in biological processes of NSCLC.

MiR-221-3p affects the proliferation and apoptosis of NSCLC cells

To explore the role of miR-221-3p in NSCLC cells, miR-221-3p NC, miR-221-3p mimics, miR-221-3p inhibitors were transfected into NSCLC cells separately. RT-qPCR assays were employed to measure the expression level of miR-221-3p in NSCLC cells after infection, the data verified the transfection efficiency (Figure 2A). Both EdU staining assay and colony formation assay indicated that the proliferation of NSCLC cells was enhanced in miR-221-3p mimics group, which was contrast to miR-221-3p inhibitors group (Figure 2B, 2C). In addition, we calculated the apoptosis of NSCLC cells, it showed that inhibition miR-221-3p reduced the cell viability of NSCLC cells (Figure 2D). To verify this result, WB assays were performed to detect the expression level of apoptosis associated proteins. The expression of BAX, Caspase-3 and Caspase-7 were increased in miR-221-3p inhibitors group while Bcl-2 was downregulated (Figure 2E). Collectively, these findings demonstrated that overexpression of miR-221-3p promoted cell proliferation and enhance the NSCLC cell viability.

MiR-221-3p promotes the migration and invasion of NSCLC cells
To further investigate how miR-221-3p influences NSCLC cell migration and invasion abilities, wound healing and transwell assays were used to evaluate these abilities. Previous study showed that metastasis-related proteins, such as MMP-2 and MMP-9, are involved in tumor invasion[29]. We found that the expression levels of MMP-2 and MMP-9 were dramatically increased in miR-221-3p mimics group (Figure 3A). Compared with the miR-221-3p NC and miR-221-3p inhibitors, miR-221-3p mimics significantly increased the migration and invasion abilities of A549 and H1975 NSCLC cells (Figure 3B, 3C). It was proved that miR-221-3p was involved in tumor invasion, and then miR-221-3p-mimics were shown to significantly enhance the migration and invasion capabilities of A549 and H1975 non-small cell lung cancer cells. These data verified that miR-221-3p played a positive role in the migration and invasion of NSCLC cells.

**Axin2 as a directly target of miR-221-3p is downregulated in tumor tissues from NSCLC patients**

Though we had identified that miR-221-3p played an important role in the NSCLC development, the biological effect of miR-221-3p was unknown in NSCLC cells. In order to seek the downstream target of miR-221-3p, we used online tool StarBase and luciferase reporter assay to show miR-221-3p bound to Axin2 and negatively modulated the luciferase activity in wildtype Axin2-containing cells (Fig. 4A and B). What’s more, both mRNA and protein expression levels of Axin2 were markedly declined in human NSCLC tissues compared with normal tissues, contrary to expression trend of miR-221-3p (Figure4C, 4D). Western blot assay also revealed that the expression level of Axin2 was suppressed in NSCLC cells (Figure 4E), which demonstrated that Axin2 was inhibited by miR-221-3p in NSCLC cells.

**MiR-221-3p activates the Wnt/β-catenin signaling pathway by inhibiting Axin2**

Axin2 was an intracellular protein and reported as a potential tumor suppressor in breast cancer [26, 30]. In order to investigate whether Axin2 acted as a tumor suppressor in NSCLC, we overexpressed miR-221-3p and Axin2 in A549 NSCLC cell respectively. We found that overexpression of miR-221-3p reduced the mRNA and protein levels of Axin2 (Figure 5A). Interestingly, we observed the nuclear localization of β-catenin in miR-221-3p mimics group and pcDNA-Axin2 +miR-221-3p mimics group (Figure 5B 5C). This implied the activation of classical Wnt/β-catenin signaling pathway. Through detecting expression level of downstream target gene MMP-7 of Wnt/β-catenin signaling pathway, we observed that overexpression of miR-221-3p increased the expression level of MMP-7. In conclusion, our results suggested that overexpression of miR-221-3p inhibited the expression of Axin2 and subsequently activated classical Wnt/β-catenin signaling pathway.

**MiR-221-3p attenuates the effects of Axin2 on the proliferation and apoptosis in NSCLC cells**

Then we wanted to know what happened when Axin2 and miR-221-3p were overexpressed at the same time. First, we examined the expression level of Axin2 in NSCLC cells under different treatments (Figure 6A). EdU staining assay showed that the proliferation was decreased in Axin2-overexpressing cells, but was restored in pcDNA- Axin2 +miR-221-3p mimics group (Figure 6B). Our statistical results on the number of apoptotic cells showed that overexpression of Axin2 resulted in more apoptotic cells, but this
condition was inhibited in pcDNA- Axin2 +miR-221-3p mimics group. At the same time, we found a slight decrease in the expression level of BAX, Caspase-3 and Caspase-7, and a significant increase in the expression level of Bcl-2 in NSCLC cells compared to pcDNA- Axin2 group (Figure 6C). According to using wound healing and Transwell, we found that after overexpression of Axin2, the migration and invasion abilities of cells decreased significantly, but if both miR-221-3p and Axin2 were overexpressed at the same time, these abilities of NSCLC cells returned to near NC group (Figure 7A, B). To explore the possible causes of this phenomenon, we examined the expression levels of migration-related proteins. We observed a decrease in the expression level of MMP-2 and MMP-9 in pcDNA- Axin2 group, but a significant increase in the expression of MMP-2 and MMP-9 in pcDNA- Axin2 +miR-221-3p mimics group compared with pcDNA- Axin2 group in NSCLC cells (Figure 7C). In conclusion, our results suggested that miR-221-3p inhibited Axin2 expression, increased cell proliferation, invasion and migration, and decreased cell apoptosis. It suggested that miR-221-3p might be one of the main causes of NSCLC growth and metastasis.

Discussion

In this study, we found for the first time an increase in the expression level of miR-221-3p in NSCLC. We demonstrated that inhibiting the expression of miR-221-3p effectively suppressed cell proliferation, accompanied by an increase in apoptosis, in NSCLC cell lines A549 and H1975 in vitro. On the contrary, overexpression of miR-221-3p showed an increase in proliferation and a decrease in apoptosis in both cell lines. At the same time, experiments also proved that miR-221-3p promoted the ability of migration and invasion of NSCLC cells in vitro. Further bioinformatics analysis suggested that Axin2 that down-regulated in NSCLC tissues might be a target gene of miR-221-3p, and then confirmed by luciferase and western blot experiments. Subsequently, we found that overexpression of Axin2 in vitro resulted in decreased cell proliferation, increased apoptosis, and decreased migration and invasiveness. These results were consistent with those obtained when inhibiting the expression level of miR-221-3p. At the same time, overexpression of Axin2 significantly reversed the changes caused miR-221-3p overexpression. Surprisingly, we observed that overexpression of Axin2 resulted in the accumulation of β-catenin in the cytoplasm. Meanwhile, overexpression of miR-221-3p and Axin2 made β-catenin enter the nucleus (Fig. 5C). As was known to all, β-catenin was an integral structural component of cadherin-based adherent junctions and the critical nuclear effector of canonical Wnt signaling in the nucleus. While activation of the Wnt pathway is based on the change and translocation of β-catenin; once β-catenin enters the nucleus and binds to TCF/LEF, the pathway will be initiated immediately [35]. We also found that this process made downstream target gene MMP-7 start to express. This might be a new mechanism for the involvement of miR-221-3p in the regulation of NSCLC. In conclusion, our results suggested that targeting Axin2 and inhibiting its expression might be one of the main reasons for the proliferation and invasion of NSCLC. It provided us with a new and key perspective in understanding and treating NSCLC.

The mortality rate of lung cancer remains high in all types of cancer [31]. NSCLC accounts for about 85% of all lung cancer patients. In recent years, with the development of gene analysis and molecular diagnosis technology [36], some cancer-related genes can be detected in patients' tiny tumor biopsy
specimens. Researchers have found that epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), fibroblast growth factor receptor (FGFR), phosphatidylinositol 3-kinase catalytic subunit alpha (PIK3CA) and so on become more and more important [37]. There have been some fruitful studies in clinical practice for these targets [38–40]. However, we still had a long way to fully understand and cure NSCLC.

Increasing evidence proved that miRNAs not only inhibited tumors, but also promoted tumorigenesis and maintenance by regulating various physiological processes of tumor cells. Previous studies have revealed the functions of miRNAs in NSCLC, such as Let-7 family members, miR-126, miR-144, miR-145 and miR-34a, which have been proved to inhibit NSCLC, while miR-17-92, miR-21, miR-31, and miR-222 are thought to promote the tumorigenesis of NSCLC [41–42]. These all suggested that miRNAs might be a new perspective in the diagnosis and treatment of NSCLC. MiR-221-3p is a member of the miRNAs family that regulates anti-angiogenesis genes. Its coding gene cluster is located on the X chromosome and mainly expressed in the intimal layer of human atherosclerotic vascular endothelial cells [43].

Previous reports have shown that miR-221-3p plays a key role in abnormal cell proliferation and differentiation in cancer [19, 21, 25]. Wu et al. reports that miR-221-3p directly targets ART4 to regulate epithelial ovarian cancer [22]. In contrast, in our study, we found that miR-221-3p was highly expressed in NSCLC tissues. In vitro experiments demonstrated that overexpression of miR-221-3p resulted in increased cell proliferation, decreased apoptosis and increased invasiveness, suggesting that miR-221-3p as a tumor promotes miRNA played an important role in the regulation of NSCLC.

There is increasing evidence that typical Wnt signaling pathway may be destroyed in various cancer states [33, 44–46]. Our results suggested a positive correlation between miR-221-3p expression and nuclear aggregation of β-catenin, suggesting that it played an important role in regulating typical Wnt/β-catenin signaling pathway. The expression of MMP-7, a downstream target of Wnt pathway was also positively correlated with the expression of miR-221-3p. Axin2, as a scaffold protein, regulates GSK3β-dependent phosphorylation of β-catenin, which is a key component of APC complex degradation of β-catenin and negatively regulates the Wnt signaling pathway.29, 30 At the same time, Axin2 exists as a tumor suppressor in many types of cancer. 31 Therefore, our results provided strong evidence that miR-221-3p directly targeted Axin2 and inhibited its expression. The result was a typical activation of the Wnt/β-catenin signaling pathway, which in turn showed an increase in cell proliferation, a decrease in apoptosis, and an increase in invasiveness in vitro.

In summary, we found a new miRNA involved in the regulation of NSCLC, miR-221-3p. Further studies showed that inhibition of miR-221-3p increased the Axin2 expression, thus inhibiting the activation of the classical Wnt/β-catenin signaling pathway, effectively inhibiting the proliferation and invasion of cancer cells. However, Axin2 might not be the only target of miR-221-3p for regulating Wnt signal over-activation in NSCLC. Alternatively, miR-221-3p regulated a set of genes that directly or indirectly regulated Wnt signaling. Whether this involved more complex regulation remained to be further explored by us and other researchers.
Abbreviations

| Abbreviation | Full name                                |
|--------------|------------------------------------------|
| NSCLC        | non-small cell lung cancer               |
| miR-221-3p   | microRNA-221-3p                          |
| SCLC         | small cell lung carcinoma                |
| FBS          | fetal bovine serum                       |
| DMEM         | Dulbecco's modified Eagle's medium       |
| WB           | Western blot                             |
| EGFR         | epidermal growth factor receptor         |
| ALK          | anaplastic lymphoma kinase               |
| FGFR         | fibroblast growth factor receptor        |
| PIK3CA       | phosphatidylinositol 3-kinase catalytic subunit alpha |

Declarations

Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by Research Ethics Committee of Affiliated Wujiang Hospital of Nantong University. Written informed consent was obtained from individual or guardian participants.

Consent for publication

Not applicable. The authors agree to publication in the Journal.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no conflicts of interest.

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Authors' contributions

YZ conceived and designed the study. JZ and LD performed the literature search and data extraction. XH and YX drafted the manuscript. QW and YW conducted the experiment. All authors read and approved the final manuscript.

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