**Original Article**

**MiR-499 is a diagnostic biomarker of paroxysmal atrial fibrillation involved in the development of atrial fibrillation**

Runwei Ma¹, Jiyang Wang¹, Xingjian Wu², Zongning Chen³, Xiaoyu Zhang¹, Xu Yang¹, Yi Song¹

¹Department of Cardiovascular Surgery, The Second People’s Hospital of Yunnan Province, Kunming, Yunnan Province, China; ²Department of Cardio-thoracic Surgery, People’s Hospital of Wen Shan, Yunnan Province, China; ³Department of Cardiology, People’s Hospital of Li Jiang, Yunnan Province, China

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**Abstract:** Background: microRNAs (miRNAs) are associated with cardiovascular disease, regulate gene expression, and are detectable in blood samples. Objective: We sought to determine the diagnostic value of miRNAs in paroxysmal atrial fibrillation (AF) and the mechanisms underlying miRNA involvement in the pathophysiological of AF. Methods: miRNA profiles in plasma samples from AF patients and healthy individuals were obtained using a microarray assay and validated by qPCR. Subsequently, the diagnostic performance of selected miRNAs was analyzed using receiver operating characteristic (ROC) curves. In vitro, western blot, and luciferase reporter assays were conducted to determine downstream targets of miR-499 in HL-1 cells. The whole-cell patch-clamp technique was then performed to observe the function of miR-499 on Ca²⁺ channels. Results: miRNA microarray data revealed 10 aberrantly expressed miRNAs between groups and qPCR validated three upregulated miRNAs, miR-21, miR-208, and miR-499. ROC analysis showed miR-499 had a higher diagnostic performance than the other two, with an area under the curve value of 0.83 (95% CI, 0.74-0.90). Furthermore, miR-499 directly regulated the expression of the L-type Ca²⁺ channel Cav1.2 (encoded by the CACNA1C gene). The results from the whole-cell patch-clamp technique showed a reduction in L-type Ca²⁺ current in HL-1 cells. Conclusions: miR-499 could serve as a diagnostic biomarker for paroxysmal AF and targets the CACNA1C gene regulating Cav1.2 expression and function, which is involved in the development of AF.

**Keywords:** Diagnosis, Cav1.2, miRNA, paroxysmal atrial fibrillation

**Introduction**

Atrial fibrillation (AF) is a global healthcare problem affecting 2.5% of the current population worldwide and its incidence increases with age [1]. Currently, the prevalence of AF is 5.2 million in the US and is predicted to rise to 12.1 million by 2030 [2]. Despite progress in the management of AF patients, this arrhythmia remains one of the major causes of stroke, heart failure, sudden death, and cardiovascular morbidity in the world [3]. Approximately one in five ischemic strokes is attributable to cardioembolic events from AF. Unfortunately, strokes due to AF are associated with increased disability and mortality compared to strokes from other etiologies [4, 5]. Because AF, especially paroxysmal AF, may be undetected, there is a critical need to identify those at greatest risk for the arrhythmia.
show that miRNAs are measurable in blood and body fluids [13], which makes them ideal potential diagnostic and prognostic biomarkers for diseases such as AF [14]. However, thus far, published studies have provided a limited understanding of the factors involved in early AF development.

The aim of the present study was to determine the feasibility of detecting and quantifying the expression level of miRNAs in plasma samples from patients at different stages of AF, to find practical diagnostic biomarkers for paroxysmal AF in clinical settings, and to ascertain the molecular mechanism of miRNAs involved in the development of AF. We identified dysregulated miRNAs in plasma using an miRNA microarray that were validated by qPCR. Receiver operating characteristic (ROC) curves were used to determine the diagnostic values of the selected miRNAs: miR-21, miR-499, and miR-208. In addition, the effects of miR-499 on Ca2+ channel expression were tested to determine how the candidate miRNA integrates into AF pathophysiology. We determined the diagnostic value of miRNAs for paroxysmal AF and identified a crucial association of miR-499 upregulation with AF development.

### Materials and methods

**Patients and plasma samples**

Between November 2010 and September 2014, 312 consecutive patients diagnosed with atrial fibrillation were considered for inclusion in the Second People’s Hospital of Yunnan Province. The exclusion criteria were: a) history or symptoms of heart failure; b) cardiac valvular disease, left atrial or left atrial appendage thrombosis; c) major surgical procedure or trauma within 60 days; or d) malignancy, immune system diseases, serious liver or renal dysfunctions, cerebrovascular accident, or nervous system diseases. Thus, 259 patients presenting with paroxysmal AF (64 patients), persistent AF (95 patients), and permanent AF (100 patients) were included. Local and regional research ethics committees approved the study. The medical history of each AF patient was recorded. The clinical characteristics of study subjects are shown in Table 1. Plasma samples from 259 patients with AF and 76 healthy volunteers were centrifuged at 3000×g for 10 min within 1 h of acquisition and stored at -80°C. Written informed consent was obtained from each patient.

| Table 1. Characteristics of study subjects |
|------------------|------------------|------------------|------------------|------------------|------------------|
| Characteristic   | Healthy          | Paroxysmal AF    | Persistent AF    | Permanent AF     | P value          |
| Number           | 76               | 64               | 95              | 100              |                  |
| Male             | 41 (54%)         | 31 (48%)         | 52 (54%)        | 43 (43%)         | 0.873            |
| Mean age         | 64±6.2           | 67±9.0           | 66±8.7          | 70±7.4           | 0.105            |
| BMI kg/m²        | 22.5±1.1         | 21.2±2.3         | 21.8±1.9        | 22.0±2.8         | 0.584            |
| Diabetes         | 11 (14%)         | 20 (31%)         | 28 (30%)        | 34 (34%)         | 0.004*           |
| Hypertension     | 13 (17%)         | 39 (61%)         | 41 (43%)        | 62 (62%)         | 0.019*           |
| TC (mmol/L)      | 4.6±0.8          | 4.9±0.5          | 5.2±0.4         | 5.1±0.5          | 0.137            |
| TSH mIU/mL       | 1.8±0.2          | 1.9±0.3          | 1.7±0.3         | 1.8±0.4          | 0.213            |
| History of AMI   | -                | 24 (38%)         | 53 (56%)        | 59 (59%)         | 0.002*           |

BMI=body mass index; TC=Total cholesterol; TSH=Thyroid stimulating hormone; AMI=Acute myocardial infarction; *P < 0.05.

### miRNA microarrays and quantitative real-time PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and purified with a PureLink® RNA Mini Kit (Invitrogen). For microarrays, three samples were randomly chosen from the four groups and mRNA was amplified, labelled, and hybridized according to the instructions provided by CapitalBio Corporation. The data was read by a confocal LuxScan scanner (CapitalBio Corp, Beijing, China) and analyzed by SpotData Pro software (CapitalBio Corp). “Fold Change” and “Regulation” columns were added in Excel for estimation of gene expression and normalization. For qRT-PCR, first strand cDNA was synthesized according to the manufacturer’s instructions using PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Shiga, Japan). Reverse transcription was performed at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Relative expression levels of differentially expressed miRNAs obtained from the microarray analysis were obtained using SYBR Premix Ex Taq™ (Takara) on an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). All reactions were performed with at least three replicates. Relative quantification of miRNA and mRNA expression levels were calculated using the
Diagnostic value of miR-499 in paroxysmal AF

Table 2. The TaqMan stem-loop primers for reverse transcription PCR and the forward and reverse primers for real-time PCR

<table>
<thead>
<tr>
<th>Accession ID</th>
<th>Sequence</th>
<th>Stem loop primer</th>
<th>PCR-F</th>
<th>PCR-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Has-miR-1</td>
<td>UGGAAUGUAAGAAGUAGUAU</td>
<td>GTCGATCCAGTGCCTGAGCTGGAGCTGGCAAATTGCACTGGACATACGACATAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Has-mir-21</td>
<td>UACGUUAUCAGAGUAGAGUAU</td>
<td>GTCGTACCATCGGCTGGAGCTGGCAAATTGCACTGGATCGACATACGACATAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Has-mir-208</td>
<td>ATACAGACGACAAAGA</td>
<td>GTCGTACCATCGGCTGGAGCTGGAGATGCAATTGCACTGGACATACGACATAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Has-mir-499</td>
<td>ATACAGACGACAAAGA</td>
<td>GTCGTACCATCGGCTGGAGCTGGAGATGCAATTGCACTGGACATACGACATAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Has-mir-328</td>
<td>ATACAGACGACAAAGA</td>
<td>GTCGTACCATCGGCTGGAGCTGGAGATGCAATTGCACTGGACATACGACATAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Has-mir-133</td>
<td>ATACAGACGACAAAGA</td>
<td>GTCGTACCATCGGCTGGAGCTGGAGATGCAATTGCACTGGACATACGACATAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U6</td>
<td>AGCTGGTAAAGACTGGAGAGC</td>
<td>GTCGTACCATCGGCTGGAGCTGGAGATGCAATTGCACTGGACATACGACATAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>AGCTGGTAAAGACTGGAGAGC</td>
<td>GTCGTACCATCGGCTGGAGCTGGAGATGCAATTGCACTGGACATACGACATAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CACNA1C</td>
<td>TGGGTGTACAGGTGGGTGGT</td>
<td>GTCGTACCATCGGCTGGAGCTGGAGATGCAATTGCACTGGACATACGACATAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CACNB2</td>
<td>TGGGTGTACAGGTGGGTGGT</td>
<td>GTCGTACCATCGGCTGGAGCTGGAGATGCAATTGCACTGGACATACGACATAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2-ΔΔCt method normalized with respect to U6 and β-actin, respectively. Table 2 shows the primers used for qPCR.

miRNA target prediction

Computational prediction of putative targets for miR-499 was performed by searching miRNA databases including TargetScan, miRanda, miRWalk, and miRDB.

Cell culture and transfection

Human atrial myocytes HL-1 (GFO15, Shanghai Gefan Biotechnology, Co., LTD, China) were cultured in DMEM (Invitrogen) supplemented with 50 unit/ml penicillin/streptomycin, 10% calf serum, and 10% fetal calf serum (Gibco, CA, USA). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2.

miR-499 mimic and inhibitor were synthesized by RiboBio Biological Technology Co., Ltd (Guangzhou, China). The negative control was a scrambled 22-nucleotide miRNA. Transfections with miR-499 mimic, inhibitor, or their negative controls and luciferase reporter plasmid were carried out using Lipofectamine® 2000 (11668-029, Invitrogen) according to the manufacturer's instructions. Transfection efficiency was determined through fluorescence detection.

Western blot

HL-1 cells with miR-499 mimics or inhibitors were lysed with RIPA buffer (Beyotime, China) for 30 min on ice. The lysates were cleared by centrifugation at 10,000× g for 5 min and the BCA Protein Assay kit (Beyotime, China) was used for protein quantification. Equal amounts of protein were used for SDS-PAGE, transferred onto PVDF membranes, and blocked with 5% non-fat milk. The membranes were incubated with first antibodies included anti-Cav1.2 (1:1000, Abcam), Cavβ2 (1:500, Abcam), and β-actin (1:2000, Abcam) overnight at 4°C fol-
Diagnostic value of miR-499 in paroxysmal AF
lowed by incubation with secondary antibodies (Merck, 1:5000) for 1.5 h at room temperature. After extensive washes with TBST, the proteins were detected by chemiluminescence (Chem-Doc XRS, Bio-Rad).

**Luciferase reporter assay**

The luciferase assay was used to assess whether miR-499 targets the 3'-UTR of CACNA1C or CACNB2. HL-1 cells were transfected with the pMir-Target luciferase expression reporter vector carrying the 3’UTR of CACNA1C, CACNB2, or an empty control vector and the promoter-luciferase fusion plasmid. In brief, cells were cultured in 48-well plates and the Dual-Luciferase Reporter Assay System (Promega, E1980, Wisconsin, USA) was performed according to the manufacturer’s instructions.

**Calcium current recordings**

Voltage-gated Ca2+ channel function was evaluated by recording inward Ca2+ currents (ICa, L) in HL-1 cells using the whole-cell patch-clamp technique (AXON-200B, Molecular Devices, Inc., Sunnyvale, CA USA). HL-1 cells were stimulated on a ramp protocol with a velocity of 1.5 mV/sec and intracellular Ca2+ signals were analyzed by confocal microscopy in HL-1 cells loaded with pClamp10. The membrane capacitance (Cm) was calculated according to the equation Cm=I/(dV/dt) and the current value was the ratio of the current amplitude to the cell capacitance (pA/pF).

**Statistical analysis**

Data are mean ± standard deviation from three independent experiments. An unpaired t-test was used for two-group comparisons and a one-way ANOVA was used followed by a Fisher’s Least Significant Difference (LSD) t-test for more than two groups. ROC curves were constructed, and the area under the curve (AUC) was used to assess the diagnostic values of miRNAs. P < 0.05 was considered statistically
Diagnostic value of miR-499 in paroxysmal AF

**Results**

The changes in miRNAs in atrial fibrillation patients

miRNA signatures in AF patient plasma were obtained by comparing microRNA microarrays between the paroxysmal AF group, persistent AF group, permanent AF group, and healthy volunteers group. As illustrated in Figure 1, 10 miRNAs exhibited significantly altered expression levels (P < 0.05 and fold change ≥ 2). Of these, six miRNAs were significantly upregulated and four were suppressed. qPCR was performed to confirm this data. We hypothesized that miRNAs may contribute to atrial electrical remodeling and involvement in arrhythmogenesis. Therefore, we investigated the six upregulated miRNAs: miR-1, miR-21, miR-208, miR-499, miR-328, and miR-133. However, miR-1, miR-328, and miR-133 did not show upregulation in any subgroup of AF patients compared with healthy patients (Figure 2A, 2E, 2F). The expression levels of miR-21, miR-208, and miR-499 were consistent with the microarray findings (Figure 2B-D, P < 0.05 for all). Interestingly, only the miR-499 expression level gradually increased between AF groups suggesting that it may participate in the development of AF and could serve as a biomarker for early stage AF.

Diagnostic performance of miRNAs in patients with paroxysmal AF

Based on the above hypothesis, we constructed receiver operating characteristic curves to assess the diagnostic value of selected miRNAs in plasma from paroxysmal AF patients. The AUC value for miR-21 and miR-208 was 0.73 (95% CI, 0.63-0.81) and 0.74 (95% CI, 0.64-0.82), respectively (P < 0.001; Figure 3A, 3C). miR-499 had a greater power to diagnose paroxysmal AF with an AUC value of 0.83 (95% CI, 0.74-0.90), a cut-off value of 6.35, sensitivity 81.36%, and specificity 72.51%. Detailed information about the diagnostic performance of miRNAs is shown in Table 3.

miR-499 alters inward L-type calcium channel expression and function in human myocardial cells

Because the expression of miR-499 differed between AF subtypes, it was valuable to explore

**Table 3.** The receiver operating characteristic (ROC) analysis of miRNA in patients with paroxysmal AF

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>AUC</th>
<th>95% CI</th>
<th>P value</th>
<th>Youden</th>
<th>Cut-off</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21</td>
<td>0.73</td>
<td>0.63-0.81</td>
<td>&lt;0.001</td>
<td>0.44</td>
<td>2.53</td>
<td>61.02%</td>
<td>80.50%</td>
</tr>
<tr>
<td>miR-208</td>
<td>0.74</td>
<td>0.64-0.82</td>
<td>&lt;0.001</td>
<td>0.57</td>
<td>6.35</td>
<td>81.36%</td>
<td>72.51%</td>
</tr>
<tr>
<td>miR-499</td>
<td>0.83</td>
<td>0.74-0.90</td>
<td>&lt;0.001</td>
<td>0.56</td>
<td>4.74</td>
<td>75.22%</td>
<td>85.00%</td>
</tr>
</tbody>
</table>

Note. AUC, area under the receiver operating characteristic curve; CI, confidence interval; P-value, compared with AUC of 0.5.
whether miR-499 was involved in the pathogenesis of AF. We assumed that CACNA1C and CACNB2 (L-type Ca\(^{2+}\) channel subunits α1c and β2 coding genes) were the putative target genes of miR-499. The potential complementary sequence between miR-499 and the candidates is illustrated in Figure 4A. CACNA1C and CACNB2 mRNA amounts were evaluated by qPCR in HL-1 cells transfected with miR-499 mimic or inhibitor or matched negative control (NC). Interestingly, lower CACNA1C mRNA amounts were observed in the miR-499 mimic group compared with the other three groups (Figure 4B, \(P < 0.01\)). However, CACNB2 mRNA levels did not change between groups (Figure 4C). Subsequently, Cav1.2 and Caβ2 protein (products of CACNA1C and CACNB2 mRNA, respectively) levels were evaluated by western blot in HL-1 cells. The results showed that Cav1.2 protein levels decreased in the miR-499 overexpression group and increased when miR-499 was inhibited (Figure 4D, \(P < 0.01\)). In accordance with the qPCR results, there was no statistically significant change in Caβ2 protein expression (Figure 4D). We further used a standard 3'UTR luciferase reporter assay to validate the interaction between miR-499 and CACNA1C. miR-499 mimic induced a reduction in luciferase activity in cells co-transfected with CACNA1C 3'UTR regions, whereas transfection with the negative control miRNA (NC) or with the CACNB2 3'UTR region showed no effect (Figure 4E). These findings suggest that CACNA1C, but not CACNB2, is subject to regula-

Figure 4. miR-499 directly targets CACNA1C in HL-1 cells. A: Putative binding sites between miR-499 and CACNA1C. B, C: The relative expressions of CACNA1C and CACNB2 mRNA using qPCR. D: Western blot showing the altered expression of Cav1.2 and Cavβ2 proteins (encoded by CACNA1C and CACNB2 mRNA, respectively) in the different groups. E: Luciferase assays in HL-1 cells (transfected with miR-499 mimic, miR-499 inhibitor, or corresponding negative controls) for posttranscriptional excitation of CACNA1C and CACNB2.
Diagnostic value of miR-499 in paroxysmal AF

**Discussion**

Atrial fibrillation (AF) is independently associated with a two-fold increased risk of all-causes of mortality in women and a 1.5-fold increase in men [15, 16]. AF-related hospital admissions (the single most important determinant of cost) are twice as high as those in the general population [17]. Early detection of AF could dramatically reduce the risk by facilitating timely treatment with oral anticoagulants [18]. Likewise, the chance for curative treatment of AF also increases through early identification. The potential for microRNA to act as non-invasive molecular biomarkers of systemic disease is of great interest owing to their high level of stability in many different biofluids [19]. However, only a small set of miRNAs have been validated as altered in AF, and few studies provide the regulatory mechanism of atrial fibrosis in non-valvular paroxysmal AF [20].

In this work, we generated miRNA profiles of AF patients using microarray assays and validated the upregulated miRNAs through qPCR. Our
results show that miR-21, miR-208, and miR-499 were at higher expression levels in plasma from paroxysmal AF patients than healthy volunteers. A number of studies have reported that miR-499 is a cardiac-abundant miRNA under physiological conditions [21, 22]. miR-499 can inhibit cardiomyocyte apoptosis through its suppression of calcineurin-mediated dephosphorylation of dynamin-related protein-1 and p53 transcriptionally downregulates miR-499 expression [23]. miRNA-499 is a newly discovered member of miRNAs encoded by the myosin gene family and is located in an intron of the Myh7B (skeletal muscle and cardiac myosin) gene [24]. A recent study compared and analyzed the miR-499 expression profiles between non-ST-segment elevation myocardial infarction (NSTEMI) and congestive heart failure patients and found miR-499 was significantly increased in NSTEMI patients, suggesting circulating miR-499 is a sensitive biomarker of acute NSTEMI [25]. In agreement with this, Zhang et al. reported that miR-499 was significantly elevated in acute myocardial infarction (AMI) patients. Importantly, miR-499 was detectable in the plasma as early as 1 h after the onset of chest pain in AMI patients and was shown to substantially increase the diagnostic accuracy of creatine kinase-MB and troponin in the diagnosis of AMI [26]. Furthermore, researchers also identified significantly upregulated atrial miR-499 in patients with permanent AF and found that miR-499 targets the small conductance calcium-activated potassium channel 3, contributing to the electrical remodeling in AF [27]. In the present study, miR-499 was significantly upregulated in paroxysmal AF plasma samples compared with negative controls and showed excellent diagnostic performance of paroxysmal AF with the AUC value of 0.83 (95% CI: 0.74-0.90).

Furthermore, we identified CACNA1C as the downstream target of miR-499, which may explain how miR-499 is involved in AF development. Previous studies suggest that multiple miRNAs contribute to controlling arrhythmogenicity of the heart and different miRNAs are involved in different types of arrhythmias under different pathological conditions of the heart [28, 29]. Cardiomyocytes possess distinct calcium channels that are dynamically regulated during development [30, 31]. CACNA1C is not expressed in cardiomyocytes initially, but is one of the hallmarks for cardiac electrophysiological in later developmental stages [32]. Mutations in CACNA1C may cause life-threatening arrhythmias in humans [33, 34]. Reduction of the density of the L-type Ca^{2+} current (\(l_{\text{CaL}}\)) is a hallmark of the electrical remodeling in AF [35]. Recently, studies report that miRNA-223 could regulate the expression of CACNA1C gene and its encoded cardiac L-type calcium channel alpha 1C subunit (Cav1.2) in rat cardiomyocytes [36]. Here, computational analysis suggested that miR-499 regulates the CACNA1C gene, which we validated using a series of biological experiments. In addition, overexpression of miR-499 could reduce \(l_{\text{CaL}}\) density. Our findings suggest that miRNA-499 targets the CACNA1C gene and regulates its encoded L-type Ca^{2+} channel Cav1.2, thus contributing to the arrhythmia. The calcium channel beta 2 (CACNB2), another voltage-gated calcium channel, is required during embryogenesis for normal ventricular cardiomyocyte proliferation, cardiac looping, chamber morphogenesis, and cardiac contractility [37], and was assumed to be a target of miR-499. However, our experiments did not identify CACNB2 as a target of miR-499.

In addition, the expression level of plasma miR-21 and miR-208 were increased in AF patients, which was in line with other studies [11, 38]. These two miRNAs were able to properly diagnose paroxysmal AF patients according to our data.

However, this study has limitations. On the one hand, the miRNA profile was acquired from blood samples but not atrial samples as lots of patients didn’t receive cardiac surgery and the diagnostic value of selected miRNAs was not validated in another study group. On the other hand, we only highlight the status of miR-499 and may miss the importance of other miRNAs that are pertinent to the development of AF. Within these limitations, more studies need to be conducted to clarify the regulation mechanisms of miRNA in AF.

Conclusions

In summary, our data present the first demonstration that plasma miRNAs could serve as diagnostic biomarkers in paroxysmal AF. This study also establishes that miR-499 directly targets the CACNA1C gene and regulates the expression and function of Cav1.2. These find-
ings suggest that miR-499 could be a potential therapeutic target in paroxysmal AF.

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Disclosure of conflict of interest

None.

Address correspondence to: Yi Song, Department of Cardiovascular Surgery, The Second People’s Hospital of Yunnan Province, Kunming City, Yunnan Province, China. E-mail: marunweiynkm@163.com

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Diagnostic value of miR-499 in paroxysmal AF


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