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ORIGINAL RESEARCH

MicroRNAs as Novel Biomarkers for P2Y12 – Inhibitors Resistance Prediction

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Aim: The aim of this study is to assess 6 micro-RNAs: miR-126, miR-223, miR-150, miR-29, miR-34, miR-142 as potential biomarkers for P2Y12- inhibitors resistance prediction.

Methods: Eighty patients with an acute coronary syndrome undergoing percutaneous coronary intervention treated in a multidisciplinary hospital in Moscow with DAPT (either with ticagrelor, n=45, or clopidogrel, n=35) were enrolled. The carriership of 6 clinically relevant polymorphisms for ticagrelor and 17 for clopidogrel was detected. Expression levels of six prospective miRNAs were measured. The activity of CYP3A4 isoenzyme was measured as the ratio of the concentrations of cortisol and 6β-hydroxycortisol.

Results: The polymorphisms of the P2Y12-inhibitors ADME genes that demonstrated statistically significant connection with miRNA expression levels are as follows: P2Y12R (A>G, rs3732759) and miR-29 (p=0.017), miR-34 (p=0.003); CYP2C19*17 (C-806T, rs1224856) and miR-142 (p=0.012); PON1 (Q192R, rs662) and miR-29 (p=0.004), ABCG2 (G>T, rs2231142) and miR-34 (p=0.007). MiRNAs expression levels showed connection with the results of the platelet reactivity assessment by utilizing VerifyNow assay ("Instrumentation laboratory", MA, US). MiR-126 (β coefficient=−0.076, SE=0.032, p=0.021), miR-223 (β coefficient=−0.089, SE=0.041, p=0.032), miR-29 (β coefficient=−0.042, SE=0.018, p=0.026), miR-142 (β coefficient=−0.072, SE=0.026, p=0.008) have the potential to be used as biomarkers and may substitute platelet reactivity testing.

Conclusion: This study has revealed new biomarkers for P2Y12-inhibitors resistance testing: miR-29, miR-34, miR-126, miR-142, miR-223.

Keywords: biomarker, miRNA, polymorphism, acute coronary syndrome, pharmacogenomics

Plain Language Summary

- There is an unmet need for a novel biomarker for P2Y12 - Inhibitors Resistance Prediction
- The choice of an appropriate P2Y12-inhibitor as part of DAPT poses a challenge to cardiologists
- miR-29, miR-34, miR-126, miR-142 and miR-223 can be used as novel biomarkers
- These miRNAs may be included into future smart diagnostic tools

Introduction

Patients with an acute coronary syndrome [ACS] who undergo percutaneous coronary intervention [PCI] receive dual antiplatelet therapy [DAPT] as a standard of care.[1] DAPT usually includes aspirin and a P2Y12-inhibitor. The DAPT therapy works towards prevention of thrombotic complications: cardiovascular death, myocardial infarction, stent thrombosis.[2]

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Nowadays, 3 P2Y12-inhibitors are available per os: ticagrelor, clopidogrel, prasugrel. Clopidogrel is a prodrug, hence it needs to be metabolized in the liver, and there are some enzymes involved in its metabolism: CYP2C19, CYP2B6, CYP1A2, CYP3A4, CYP3A5, among them.[3] Not only does metabolism of clopidogrel involve a two-stage transformation in the liver, but also transporter P-glycoproteins to facilitate prodrug absorption through the intestinal cells. *ABCB1* gene is responsible for its regulation. All this contributes to a number of sites for a possible breakdown, which leads to altered response to clopidogrel. This high number of sites for a possible breakdown accounts for the number of patients who are resistant to clopidogrel, which can be as high as 35% of the population.[4] As observed in a number of observational and randomized trials, a personalized approach to antiplatelet therapy may lead towards a decrease in the number of complications.[5–8]

Ticagrelor and prasugrel are considered more potent P2Y12–inhibitors in terms of the number of thrombotic complications among patients with an acute coronary syndrome undergoing percutaneous coronary intervention.[9,10] Higher potency comes at a price of a higher number of bleeding complications, lower compliance due to high costs of the drugs. In addition, ticagrelor and prasugrel are not considered the drugs of choice when used as a combined antithrombotic treatment.[11]

The choice of an appropriate P2Y12-inhibitor to be used as part of DAPT poses a challenge to cardiologists as they need to know the patient's characteristics to favour a certain drug.[12]

There were some trials which assessed doubling and tripling the dose of clopidogrel for low responders. However, the current strategy for low responders is switching to another P2Y12 inhibitor like ticagrelor or prasugrel.[2,13–15]

Various approaches are used for selection of an adequate antiplatelet therapy: genetic testing is implemented, and platelet reactivity is measured using the VerifyNow P2Y12 Assay.[16–18] Currently, the Clinical Pharmacogenetics Implementation Consortium (CPIC) recommends genetic testing and genotype-directed treatment for high-risk patients.[2] Therefore, CYP2C19 poor metabolizers should be prescribed an alternative antiplatelet regimen.[19]

Although, both genetic testing and platelet reactivity measurements are implemented, there is an unmet need for newer biomarkers developed for a simple, quick, reliable and comprehensive prediction of insufficient response to clopidogrel.[20] And micro-RNAs are described in literature as such potential biomarkers.[21–25]

These micro-RNAs are small non-coding sequences of nucleotides which bind to mRNA sites and block transcription. This causes a decrease in the production of protein. The miRNA-mRNA mechanism is a fine instrument of gene expression regulation.[26] The miRNAs may regulate ADME genes and affect the effective drug concentration in blood.

Micro-RNAs can be biomarkers for clopidogrel resistance through the ADME genes involved in the metabolism of clopidogrel.[27]

In this article, we suggest six prospective microRNAs as potential biomarkers for guiding antiplatelet therapy among patients with ACS who have undergone PCI.

Materials and Methods

Patients

Eighty patients with an acute coronary syndrome undergoing percutaneous coronary intervention treated in a multidisciplinary hospital in Moscow were consecutively enrolled.

The study was approved by the Ethics Committee of Russian Medical Academy of Continuous Professional Education, Moscow, Russian Federation and conducted in accordance with the Declaration of Helsinki; all the patients gave written informed consent for participation.

Inclusion criteria: presence of an informed written consent, age above 18 years, ACS event less than 7 days. Exclusion criteria: pregnancy, lactation, active internal bleeding, liver cirrhosis with C Child-Pugh liver insufficiency; chronic renal disease, HIV-infection, alcohol/drug addiction; mitral valve stenosis moderate and severe, mechanical heart valves, severe psychiatric disorders, allergic reactions/drug intolerance. The study assessed the secondary outcomes (platelet reactivity units, miRNAs expression levels). As part of dual antiplatelet therapy (DAPT), the patients took aspirin 100 mg daily and either clopidogrel 75mg SID (n=35) or ticagrelor 90 mg BID (n=45).

Assessment of Platelet Activity

The assessment of residual platelet reactivity was performed by utilizing VerifyNow assay ("Instrumentation laboratory", MA, US). For assessment of platelet activity venous blood was used, drained on the 3rd day after the beginning of DAPT therapy in vacuum vials 2 mL with 3.2% sodium citrate. The level of aggregation of platelets

is measured in P2Y12 Reactivity Units or percentage of inhibition. The study was conducted within 1 hour after the whole venous blood sample was drained. The therapeutic range of P2Y12-inhibitors when measured with VerifyNow P2Y12 assay are as follows: PRU>208 – the risk of thrombotic events, PRU<95 – bleeding risk, 95<PRU<208 – adequate response.

Genotyping

Blood (2 mL each) for DNA analysis was sampled from the peripheral vein using ethylene diamine tetra acetate (EDTA) tubes (VACUETTE®, Greiner Bio-One, Austria). A panel of 18 SNPs was selected. The following 17 SNPs were selected for clopidogrel:ABCB1 (C3435T, rs1045642), CYP2C19*2 (681G > A, rs4244285), CYP2C19*3 (636G > A, rs4986893), CYP2C19*17 (C-806T, rs1224856), CYP3A4*22 (C>T, rs35599367), CYP3A4 (20239G>A, rs2242480), CYP3A5*3 (A6986G, rs776746), CYP4F2 (C > T, Val433Met, rs2108622), CES1 (A-33C, rs2244613), PON1 (Q192R, rs662), IGTB3 (rs5918), P2Y12 (rs2046934), P2Y12R (A>G, rs3732759), PEAR1 (C>T, rs41273215), PEAR1 (C>T, rs57731889), B4GALT2 (C>T, rs1061781), ABCG2 (G>T, rs2231142). The following 6 SNPs were selected for ticagrelor: ABCB1 (C3435T, rs1045642), CYP3A5*3 (A6986G, rs776746), CYP3A4*22 (C>T, rs35599367), CYP3A4 (20239G>A, rs2242480), P2Y12 (rs2046934), SLCO1B1 (T521C, rs4149056). Real-time polymerase chain reaction with commercially available assays («Sintol», Russia; Thermo Fisher Scientific, USA) was performed utilizing Real-Time CFX96 Touch amplifier (Bio-Rad Laboratories, Inc., USA).

MicroRNA Testing

Blood was collected into sterile EDTA tubes and centrifuged for 10 min at 2000g. The obtained supernatant was transferred to sterile 2 mL tubes and stored before use at −80 °C. Total RNA, including miRNA, was extracted using QIAzol lysis reagent and the miRNeasy Serum/Plasma kit (Qiagen, Hilden, Germany) according to the recommended protocol. QIAzol was added to 300 µL of plasma in a volume ratio of 3:1. After chloroform was added to the tube and centrifuged to separate phases, the aqueous phase was transferred to a new tube, and 1.5 times the volume of 100% ethanol was added. The solution containing RNA was loaded into the miRNeasy column and further washed according to the manufacturer's instructions. The final volume of elution was 20 µL. Concentration and purity of the obtained RNA were measured on the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, New York, USA). The extraction process was repeated for each sample until a sufficient amount of RNA was obtained for the next steps. Reverse transcription was carried out with the MiScript II RT Kit (Qiagen) according to the manufacturer's protocol. Expression levels of miR-142, miR-126, miR-223, miR-150, miR-29, miR-34 were estimated with quantitative reverse transcription polymerase-chain reaction (qRT-PCR) using the MiScript SYBR Green PCR Kit (Qiagen) and presynthesizedmiScript Primer Assay (Qiagen) primers. qRT-PCR was performed with three repetitions for the analyzed miRNA in the volume of the reaction mixture of 12 µL on the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, USA) according to the manufacturer's recommended program (15 min at 95 °C to activate the HotStarTaq DNA Polymerase and 40 three-step cycles (94 °C – 15 s, 55 °C – 30 s, 70 °C – 30 s)). Relative quantifications were calculated using the comparative Ct method (2 ΔΔCt).

Activity of CYP3A4 Isoenzyme

To assess the activity of CYP3A4, patients gave samples of morning urine with a volume of 5 mL, collected in tubes without preservative at the time of the blood draw. Samples were stored frozen at −20 °C. The activity of CYP3A4 was expressed as the ratio of the concentrations of cortisol and 6β-hydroxycortisol, the formation of which occurs under the influence of these isoenzymes. Cortisol and its metabolite were assessed by chromatography–mass spectrometry on an Agilent G1978B Multimode Source high performance liquid chromatograph for 6410 Triple Quad LC/MS (Agilent Technologies, Inc., USA).

Statistics

Data analysis was carried out in the statistical package IBM SPSS Statistics 20.0. All quantitative variables were tested for normal distribution by the Shapiro–Wilk criterion, resulting in abnormal data distribution, except for miR-34, miR-223 expression levels and Base PRU. For the subsequent analysis of quantitative variables between subgroups, nonparametric criteria (Mann-Whitney, Kruskal-Wallis) were applied. Independent Samples *t*-test and one-way ANOVA were utilized for miR-34, miR-223 and Base PRU variables. A p-value < 0.05 was considered significant. In order to establish the connection between micro-RNA expression levels and platelet reactivity, linear regression was carried out. For the regression analysis, the following variables were selected as the dependent variables: miR-142, miR-126, miR-223, miR-150, miR-

29, miR-34. There were no differences from Hardy-Weinberg equilibrium (Table 1).

Results

MicroRNAs and Genes

The polymorphisms of the P2Y12-inhibitors ADME genes that demonstrated statistically significant connection with miRNA expression levels are as follows (see Table 1): P2Y12R (A>G, rs3732759) and miR-29 (p=0.017), miR-34 (p=0.003); CYP2C19*17 (C-806T, rs1224856) and miR-142 (p=0.012); PON1 (Q192R, rs662) and miR-29 (p=0.004), ABCG2 (G>T, rs2231142) and miR-34 (p=0.007). Therefore, miR-29, miR-34, miR-142 can be used as biomarkers of altered function polymorphisms (see Figure 1).

MicroRNAs and Platelet Reactivity Assessment

MiRNAs expression levels showed connection with the results of the platelet reactivity assessment utilizing VerifyNow assay ("Instrumentation laboratory", MA, US)

Table 1 Genotyping Data and Connected miRNA Expression Levels

P2Y12 (rs2046934)	**GG**			**GA**			**AA**			**p-value**
	N	**M**	**SD**	**N**	**M**	**SD**	**N**	**M**	**SD**	
miR-34 expression	33	33.49	2.95	14	33.45	1.86	3	33.60	0.77	0.063
miR-126 expression	33	32.60	4.00	14	34.96	2.62	3	33.51	1.59	0.082
P2Y12R (A>G, rs3732759)										
miR-29 expression	3	38.10	3.27	9	33.35	0.64	15	32.89	1.26	0.017
miR-34 expression	3	38.25	3.01	9	32.72	2.11	15	32.95	2.23	0.003
CYP2C19*2 (681G > A, rs4244285)										
miR-126 expression	20	32.48	4.08	6	36.16	1.89	1	34.94	1.79	0.066
CYP2C19*17 (C-806T, rs1224856)	CC			CT			TT			
	N	M	SD	N	M	SD	N	M	SD	
miR-142 expression	14	35.96	2.66	9	33.13	2.64	4	37.66	1.57	0.012
ABCB1 (C3435T, rs1045642)										
miR-223 expression	17	31.20	4.85	28	31.70	4.12	17	30.54	5.13	0.080
PON1 (Q192R, rs662)	CT			TT						
	N	M	SD	N	M	SD				
miR-29 expression	13	32.68	1.29	14	34.49	2.38				0.004
PEAR1 (C>T, rs57731889)	CC			CT						
	N	M	SD	N	M	SD				
miR-34 expression	25	33.21	2.56	2	36.63	4.76				0.078
miR-223 expression	25	32.23	4.60	2	34.62	1.74				0.094
B4GALT2 (C>T, rs1061781)										
miR-34 expression	24	33.54	2.27	3	32.86	6.37				0.096
ABCG2 (G>T, rs2231142)	CC			CA						
	N	M	SD	N	M	SD				
miR-34 expression	22	33.42	3.08	5	33.68	0.60				0.007

Table 2 P-values of the Linear Regression Analysis Between miRNA Expression Levels and Results of the Platelet Reactivity Testing

	PRU	% Inhibition	Base
miR-126	0.021	0.009	0.011
miR-223	0.032	0.005	0.098
miR-29	0.026	0.026	0.002
miR-142	0.008	0.003	0.001

(see Table 2). MiR-126 (β coefficient=−0.076, SE=0.032, p=0.021), miR-223 (β coefficient=−0.089, SE=0.041, p=0.032), miR-29 (β coefficient=−0.042, SE=0.018, p=0.026), miR-142 (β coefficient=−0.072, SE=0.026, p=0.008) have the potential to be used as biomarkers and to substitute platelet reactivity testing.

Discussion

In this article, six miRNAs and eighteen gene polymorphisms and their connection to platelet reactivity have been assessed. There have been few publications which described the possible connection between miRNA expression levels and resistance to P2Y12-inhibitors.[24,28–33] On the basis of these publications six prospective miRNAs

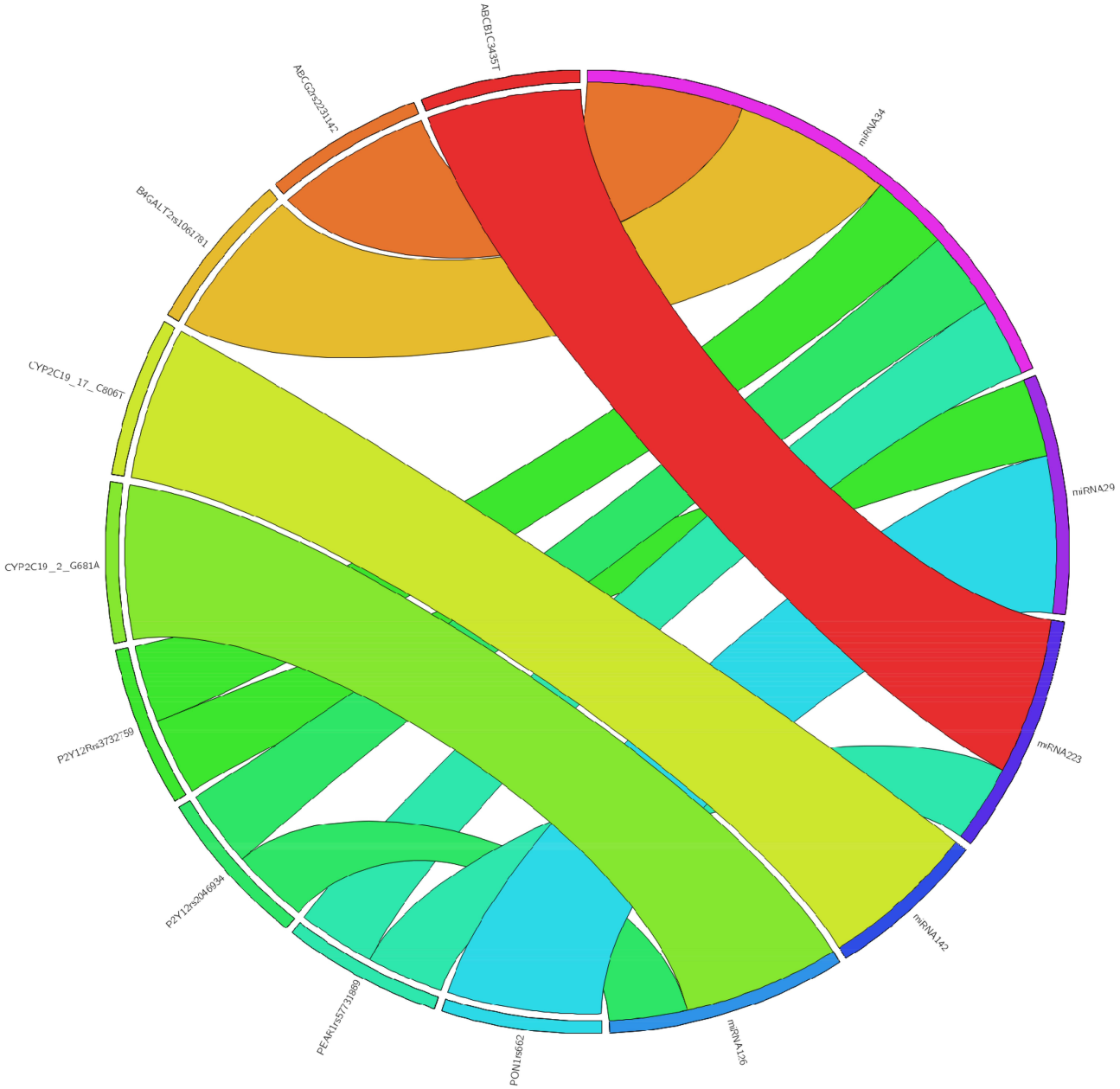

Figure 1 Connections between miRNAs and polymorphisms of the ADME genes for P2Y12-inhibitors.

have been selected. These are miR-29, miR-34, miR-126, miR-142, miR-150 and miR-223. The aforementioned miRNAs are also noted in the following databases: miRanda,[34] PITA,[35] miRTarBase,[36] Pharmaco-miR,[37] TargetscanHuman 7.2.[26] These miRNAs are also preselected in the commercially available assays.[38] All these facts supported the decision to test the connection between these miRNAs and platelet reactivity as well as loss-of-function genetic polymorphisms.

Recently published trials which assessed the genotype-based approach to prescription of a more potent P2Y12-inhibitor yielded interesting results.[16,17] The TAILOR-PCI study demonstrated a 34% reduction in the primary endpoint - a composite of cardiovascular death, MI, stroke, definite or probable stent thrombosis, and severe recurrent ischemia - for patients with genotype-guided approach (4.0% vs 5.9%; adjusted HR 0.66; 95% CI 0.43–1.02). In a post hoc analysis, the researchers found a significant absolute 2.1% benefit in using the genotype-guided strategy at 3 months (HR 0.21; P = 0.001).[16] While the TAILOR-PCI study was designed to show superiority of genotyping, another study POPular Genetics compared a genotyping strategy to no-genotyping strategy. It showed non-inferiority of genetic testing to standard care with respect to the primary composite endpoint of all-cause death, MI, definite stent thrombosis, stroke, and PLATO major bleeding (5.1% vs 5.9%; P for non-inferiority < 0.001). Moreover, genetic testing was superior to standard care for the endpoint of PLATO major and minor bleeding (9.8% vs 12.5%; P = 0.04).[17]

The high number of patients in the study, the number of polymorphisms and the well-established method of P2Y12-inhibitors effectiveness assessment enable miR-29, miR-34, miR-126, miR-142, miR-223 to be used as either biomarkers of platelet reactivity or loss-of-function genetic polymorphisms. Therefore, they can be used as biomarkers of the resistance to P2Y12-inhibitors.

Currently, the genotype-based strategy in prescription of P2Y12-inhibitors lowers the risk of high on-platelet reactivity and MACE.[39] Currently the genotype-based strategy in the prescription of P2Y12-inhibitors is only recommended in patients at high risk of thrombotic events and not suitable for all comers as the trials have shown the conflicting results.[40] There is an unmet need for a novel complex biomarker that can combine genetic profile and platelet laboratory parameters. MiR-29, miR-34, miR-126, miR-142, miR-223 are such biomarkers.

Limitations

The current study features 6 miRNAs and 80 patients. Although the number of miRNAs is large, the cohort is rather small for pharmacogenomics studies which may, in turn affect statistics and the possibility of false negatives.

Conclusion

This study has revealed new biomarkers of P2Y12-inhibitors resistance: miR-29, miR-34, miR-126, miR-142, miR-223. Vividly demonstrating a connection to on-treatment platelet reactivity, which in the case of miR-29, miR-34, miR-142 is also supported by pharmacogenomics, these novel biomarkers have a potential to be used for P2Y12-inhibitors resistance testing as part of future smart diagnostic tools.

Ethical Approval

All the patients were informed about the purposes of the study and consequently have signed their “consent of the patient”. All investigations conformed to the principles outlined in the Declaration of Helsinki and were performed with permission by the responsible Ethics Committee of Russian Medical Academy of Continuous Professional Education.

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Disclosure

The authors declare that they have no competing interests in this work.

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