Regulatory mechanism of miR-223 on platelet reactivity in ischemic stroke patients after clopidogrel treatment

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Running heads: miR-223 and colpidogrel HTPR.

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Abstract

Background: Recent studies have confirmed that microRNA-223 may participate in high-on clopidogrel treatment platelet reactivity. Clopidogrel requires hepatic enzyme metabolic activation to produce its metabolite with pharmacological activity, as a result, there are few researches about platelet reactivity. The aim of the current study is to explore the possible regulatory mechanism between microRNA-223 and platelet reactivity after clopidogrel treatment.

Methods: In this study, we established an experimental model of MEG-01 cells treated with clopidogrel and human liver microsomes incubation system, so that the effects of clopidogrel active metabolites on megakaryocytes and platelets can be simulated. The relation of microRNA-223, C/EBP α and P2Y12 was further investigated by both cell experiments and clinical studies.

Results: The ratio of platelet P2Y12 expression before and after treatment was significantly higher in Low Responders group (1.14604 vs 0.77097, p=0.031). After treatment at 200 ul/2 ml for 3 and 5 consecutive days, miR-223 expression in MEG-01 cells decreased by 47.3% and 32%, respectively ($p < 0.05$). P2Y12 mRNA expression was 193.4% higher after 3 consecutive days ($p < 0.001$), and significantly lower after 5 consecutive days than that in the negative control group ($p < 0.05$). P2Y12 and C/EBP α protein expression were significantly lower after 5 consecutive days ($p < 0.01$).

Conclusions: A negative feedback loop was carried out by clopidogrel active metabolite to recede its inhibition of P2Y12 signal pathway through P2Y12-PI3K/Akt-C/EBP α-miR-223 pathway, which may be excessively activated and play a role in the occurrence of high-on clopidogrel treatment platelet reactivity.

Keywords : ischemic stroke clopidogrel platelet reactivity microRNA-223 live microsomes

Introduction

Effective antiplatelet therapy plays an important role in the treatment and secondary prevention of acute ischemic stroke. Aspirin and clopidogrel are commonly used in antiplatelet therapy for patients with ischemic stroke. Despite the antiplatelet therapy, it is still unavoidable for some patients that ischemic vascular events relapse. There is a significant difference in response to antiplatelet drugs among different individuals, that is, variability of platelet response (VPR). For those with low drug response, the risk of thrombotic events may be increased. The former is generally considered to be low response to antiplatelet drugs or resistance to antiplatelet drugs. At present, it is commonly referred to as high on-treatment platelet reactivity (HTPR)[1]. To be exact, it means that antiplatelet drugs are not enough to inhibit platelet aggregation in vitro. Previous studies have shown that HTPR is a risk factor for adverse prognosis and ischemic events recurrence. The incidence of high platelet reactivity after clopidogrel treatment is 8%-61%[2-4]. The exploration of the underlying mechanism is of great importance for evaluating the drug efficacy and individualized treatment.

The mechanism of clopidogrel HTPR is not completely understood. It is generally believed that it is the result of multiple factors, including gene, cell and clinical aspects[5]. It has been recognized that many factors (including gene polymorphism[6-10], drug interaction[11-13], smoking[14, 15], inadequate intake and absorption[16-18], poor compliance, activation of other platelet pathways[19], increased platelet renewal[20], inherent platelet reactivity[10, 21-23], diabetes[24, 25], etc.) can lead to insufficient platelet inhibition. In addition, a variety of factors such as height and body mass index, renal function, decreased left ventricular ejection function, and inflammation may also play a role in the occurrence of clopidogrel HTPR.

In fact, many studies have confirmed that the elevated expression of platelet receptor P2Y12 may affect the regulation of platelet reactivity. Previous studies[26, 27] showed that platelet P2Y12 expression in patients with diabetes mellitus was significantly up-regulated. Hu et al.[28] confirmed that platelet P2Y12 receptor in patients with diabetes mellitus increased and activated continuously, which led to the occurrence of high platelet reactivity and weakened the efficacy of antiplatelet drugs. Some scholars[19] proposed that the up-regulation of P2Y12, P2Y1 and P2Y-independent signaling pathways may lead to incomplete platelet inhibition after clopidogrel treatment. Braun et al.[27] found that the residual platelet reactivity after clopidogrel treatment was partly attributed to the incomplete antagonistic effect of clopidogrel on P2Y12 receptor, while partly to the activation of P2Y1 receptor which might not be affected by clopidogrel. Additionally, enhanced P2Y12 expression was significantly correlated with clopidogrel HTPR determined by P-selectin.

Recently, more and more studies have showed that hsa-miR-223-3p (hereinafter referred to as the miR-223) may paticipate in the regulation of platelet reactivity. MiR-223 expression in platelets and plasma was significantly correlated with clopidogrel HTPR[29-31]. Landry et al.[32] explored the underlying machanism of platelet microRNAs through luciferase reporter assays in HEK293 cells and MEG-01 cells.

The results suggested that miR-223 can target P2Y12 and regulate P2Y12 expression in platelets by silencing P2Y12 mRNA through Ago2-microRNA-223 complex. Previous studies have shown that many transcription factors (including PU.1, NFI-1, C/EBP α, C/EBP β etc.) have been confirmed to regulate the transcription of miR-223 in megakaryocyts[33-35]. Moreover, inhibition of PI3K/Akt pathway in preadipocyte 3T3-L1 cells can lead to a decrease in the expression of C/EBP α. Based on the results of literature review, we speculate that C/EBP α may be involved in the regulation of miR-223 expression in megakaryocyts.

Bioinformatics analysis showed that mouse P2Y12 mRNA 3'UTR did not contain the binding site of microRNA-223, suggesting that microRNA-223 may not have a direct effect on mouse P2Y12 mRNA[36]. Additionally, after searching several databases such as TargetScan, TransmicroRNA and TarBase, no evidence was found that microRNA-223 could directly affect P2Y12 mRNA in either rats or mice. The difference of the regulatory machanism in mice and rats makes it difficult to study the effect of miR-223 on platelet reactivity after clopidogrel treatment in animal models. Besides, platelets cannot be cultured in vitro for a long time because they are cytoplasmic fragments of megakaryocytes, which do not contain nuclei and cannot replicate and divide.The researchers[37] found that miRNA expression spectrum in megakaryocyte (MEG-01) was highly consistent with that in platelets. Given that, a cell model could be established which can simulate the mechanism of clopidogrel treatment on megakaryocytes/platelets. In this study we explored the role of miR-223, C/EBP α and the specific mechanism in the occurrence of clopidogrel HTPR in patients with acute ischemic stroke.

Methods

Subjects

This study was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of The Second Hospital of Tianjin Medical University. All patients or their family members signed informed consent before the study. Patients admitted in the Department of Neurology of the Second Hospital of Tianjin Medical University from September 2018 to December 2018 were screened, who were diagnosed with acute cerebral infarction. Clinical information, such as past history, computed tomography (CT)/MRI, electrocardiogram (ECG) at admission and laboratory examination, was collected.

Patients meeting the inclusion criteria were enrolled in the study. Inclusion criteria : (1) admitted within one week from the time of onset; (2) Meeting the diagnostic criteria established by The Chinese Guidelines for the Diagnosis and Treatment of acute ischemic stroke in 2014. Exclusion criteria :(1) patients with intracranial hemorrhage, acute myocardial infarction, atrial fibrillation and severe valvular heart disease, thrombosis or diseases of the blood system; (2) Treated with antiplatelet

drugs, anticoagulants, thrombolytic drugs and other non-steroidal anti-inflammatory drugs within 2 weeks before onset; (3) Operation history within 6 months; (4) Those who meet the thrombolysis standard and plan to perform thrombolysis; (5) Severe infection, liver and kidney disease, active bleeding, malignant tumor or chronic infection. After admission, the enrolled patient was treated with 75 mg/d of clopidogrel manufactured by Shenzhen Salubris Pharmaceuticals Co., Ltd.

For all patients, platelet aggregation rate induced by adenosine diphosphate (ADP) was examined before or 7±2 days after treatment. According to the platelet aggregation rate, Relative inhibit platelet reactivity rate (Relative Inhibition, RI) was calculate[formula: RI = (baseline platelet aggregation rate － platelet aggregation rate after treatment)/baseline platelet aggregation rate×100%]. The extreme cases were screened by octiles according to RI.

Platelet Aggregation Rate

2.7ml of blood was collected in sodium citrate anticoagulation tubes (1:9, concentration tendency for 0.129mmol/L), and platelet aggregation rate was examined by flow cytometry (FCM) referring to the methods in previous literature. To activate the platelets, 5ul of whole blood was mixed with 5ul of ADP (final concentration 20 umol/L) for 5 miniutes at room temperature. Phosphate buffer saline (PBS) was used instead of ADP in the control group. 5ul PerCP monoclonal antibody (PerCP) was added and mixed gently. The mixture was incubated in dark at room temperature for 15 min for labeling. Then，1 mL of precooled (2-8℃) fix solution (1% PFA) was added. After fixation at 4℃ for more than 30 min, flow cytometry analysis was performed. The difference of the platelet aggregation proportion in the two groups reflected the platelet aggregation rate induced by ADP.

Platelet extraction and purification

The differential centrifugation method was used to separate platelets. 5 mL of blood was collected in EDTA anticoagulation tube and centrifuged at 180×g for 10 min. The upper platelet rich plasma (PRP) was pipetted into the 15 mL centrifuge tube to avoid disturbing the middle "buffy coat" and the lower red blood cell. The centrifugation was repeated to minimize the contamination of red blood cells and white blood cells. The PRP was centrifuged at 1000×g for 10 min. Discarding the supernatant, in the opalescent precipitates at the bottom there were platelets.

Add 2 ml of the prepared MACS separation buffer (AutoMACS Rinsing Buffer: MACS BSA Stock Solution = 20:1) into platelets, and gently wash the platelets. After centrifugation at 1000×g for 10 min, the supernatant was discarded again. Platelets

were resuspended in 200 ul of separation buffer. 20 ul CD45 MicroBeads were added and votexed. After 15 min of incubation at 4℃, wash the platelets in 2 ml of separation buffer, centrifuge at 1000×g for 10 min and discard the supernatant. Resuspend platelets in 3 ml of the separation buffer again. Place a new column in the MidiMACS Separator. Add 1ml of the suspension into the column and collect the sorted suspension through the colunm into a new 15 mL centrifuge tube. Repeat until all the suspension complete the separation. Repeat the centrifugation at 1000×g for 10 min, the purified platelets at the bottom were collected. After being resuspensed with 2 mL PBS, 1 ml suspension was taken into a Falon tube to detect the concentration and purity of platelets with a hemocyte analyzer.

Liver microsome incubation

The incubation system volume was 1ml, containing 0.1M phosphate buffer (pH=7.40), 0.5mg/ml microsome, 1mM nicotinamide adenine dinucleotide phosphate (NADPH), 10mM L-GSH. Vortex and incubate the system in 37℃ water bath for 5 minutes, then 1000×clopidogrel 1ul was added to the system to start the reaction. After incubation for 120 minutes, the incubation solution was filtered by a 0.2um filter for use. A portion of incubation solution without clopidogrel acted as the negative control.

Cell culture

Human megakaryocytic cell line (Meg-01) was cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum. Cells were treated in medium with incubation solution of liver microsomes (100ul/2ml, 200ul/2ml) for 3-5 days. For experiments with pharmacological inhibition of intracellular PI3K/Akt pathway, different concentration of inhibitor LY294002 (20uM, 50uM) was added. At the end of the treatment, cells were collected and processed for RNA and protein extraction.

Immunoblotting

Washed platelets and cells were prepared as described earlier and lysed in RIPA protein lysate with PMSF protease inhibitor. After centrifugation at 12000g for 5 minutes, the precipitate was discarded. Protein concentration of samples were determined by BCA Protein Assay Kit. Protein samples were subjected to immunoblotting.

Real-time Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from megakaryocytes with Trizol reagent (Invitrogen). The

integrity, quantity, and purity of RNA were examined using Picodrop Spectrophotometer. After RNA isolation, 1 μg of total RNA was reverse transcribed to cDNA and the relative expression levels of mRNAs and miRNAs were quantified by the real-time RT-PCR with SYBR Green I (Applied Biosystems). The threshold cycle (Ct) was determined and relative RNA level was calculated based on the Ct value and normalized to GAPDH or U6 level for each sample.

Adenovirus infection

Adenovirus vectors expressing miR-223-3p inhibitor or NC sequence were constructed (Ad-ZsGreen-hsa-miR-223-3p inhibitor, Ad-ZsGreen, Wuhan Boster Biology Co., LTD). MeG-01 cells in the logarithmic growth stage were prepared for infection when the cell confluence was 60%-80%. The optimal MOI value was determined by the pre-experiment. The corresponding volume of adenovirus stock solution or RPMI 1640 was added, with 3 replicates in each group. Place the megakaryocytes in the incubator. After 48h of infection, cells were collected and processed for RNA and protein extraction.

Statistical analysis

Image J software was used to detect the gray value of strip in Western blot. The RT-PCR data were analyzed by SDS software of ABI 7900HT fluorescence quantitative PCR instrument. The independent sample t test was used for the comparison between the two groups of all continuous variables, and the ANOVA variance analysis was used for the comparison between the two groups. Correlation analysis was performed using Person correlation coefficient analysis and double-tail test, and significance was defined as $p<0.05$. The statistical graph was drawn using Graphpad software.

Results

Screening of subjects and comparison of baseline clinical data

62 patients with acute ischemic stroke were enrolled in the study. The relative inhibition rate of platelet reactivity (RI) after clopidogrel treatment was calculated based on the ADP-induced platelet aggregation rate by flow cytometry at admission and after clopidogrel treatment for 7 +2 days. The process of platelet aggregation detection by flow cytometry is shown in Fig. 1. Referring to the previous research of our group, we screened the cases with extreme RI by octiles (LR, low responders, RI < 13.098%, HR, high responders, RI ≥ 52.276%). There were 4 cases in LR group and 5 cases in HR group. The baseline clinical data of the two groups are compared in Table 1. The results showed that there was no significant difference in platelet aggregation rate before and after treatment ($p > 0.05$), but RI in LR group was significantly lower than that in HR group. There was no significant difference in baseline clinical data between the two groups ($p > 0.05$).

Weakened change of platelet P2Y12 expression in low responders after clopidogrel treatment

Platelets were purified by magnetic beads from the blood samples of selected extreme cases before/after clopidogrel treatment. Platelet protein was extracted and P2Y12 expression was detected by Western blot. The correlation analysis showed that platelet P2Y12 expression was positively correlated with platelet aggregation rate both before and after treatment (r=0.814 before treatment, Fig. 2A; r=0.879 after treatment, Fig. 2B, P < 0.01). Similar to platelet reactivity, platelet P2Y12 expression varied greatly among individuals (Fig. 2C). There was no significant difference in P2Y12 expression between LR group and HR group whether before or after clopidogrel treatment. For each patient, no significant change of P2Y12 expression was found in LR group after treatment, while decreased P2Y12 pression was detected in HR group after treatment with no statistical significance (p=0.073, Fig. 2D).

Accordingly, it is speculated that there may be differences in the change of platelet P2Y12 expression after clopidogrel treatment between the two groups. Further, the ratio of platelet P2Y12 expression before and after treatment was defined as R [R=platelet P2Y12 expression after treatment/platelet P2Y12 expression before treatment]. Statistical analysis showed that R value in LR group was significantly higher than that in HR group (1.14604 vs 0.77097, p=0.031, Fig. 2E). The results suggested that platelet P2Y12 expression in LR group decreased more slightly than that in HR group after colpidogrel treatment. Figure 2F shows platelet P2Y12 expression in typical patients which is also in line with our deduction.

Establishment of cell model treated with incubation solution of clopidogrel and human liver microsome

According to the previous literature and preliminary experimental results, clopidogrel and human liver microsome was incubated together for the acitvation of clopidogrel. PBS of the same volume was incubated with human liver microsome as the negative control. Further, two different concentration was selected to explore the effect of

incubation solution on P2Y12 expression in MEG-01 cells - 100ul/2ml and

200ul/2ml. After mixing, the incubation system was cultured for 12 hours and then cells were collected to extract total protein. Immunoblotting results showed that P2Y12 expression in MEG-01 cells treated with clopidogrel incubation solution at both concentrations did not change significantly (p > 0.05, Fig. 3A, B).

We further explored the effect of incubation solution on P2Y12 expression in MEG-01 cells in the time dimension. According to the literature and previous results, the incubation system was cultured for different lengths of time at 200 ul/2 mL

concentration - 3 and 5 consecutive days. Immunoblotting results showed that P2Y12 expression decreased slightly after treatment with the clopidogrel incubation solution for 3 consecutive days, but no significant difference was observed (p > 0.05, figs. 3C, D). After treatment for 5 consecutive days, P2Y12 expression in the group treated with clopidogrel incubation solution was significantly decreased by 42.6% (p < 0.01, figs. 3E, F) compared with that of the control group (p < 0.01). From the above, these results suggested that MEG-01 cells treated with clopidogrel incubation solution at 200 ul/2 ml for 3-5 days could simulate the effects of clopidogrel active metabolites on megakaryocytes and platelets. It may provided a new proach for further investigation.

Decreased microRNA-223 expression and receded regulation of P2Y12 in megakaryocytes after clopidogrel treatment

Bioinformatics analysis and literature research show that hsa-microRNA-223-3p (hereinafter referred to as miR-223) can regulate P2Y12 expression. On the basis of the above experimental results, we further observed the expression of miR-223 in MEG-01 cells after treatment with clopidogrel incubation solution. After treatment at 200 ul/2 ml for 3 and 5 consecutive days, miR-223 expression in MEG-01 cells decreased by 47.3% and 32%, respectively (p < 0.05, Fig. 4A, B). P2Y12 mRNA expression was 193.4% higher after 3 consecutive days (p < 0.001, Fig. 4C), and significantly lower after 5 consecutive days than that in the negative control group (p < 0.05, see Fig. 4D). To further elucidate the regulation of miR-223 on P2Y12 expression, we constructed a virus vector to down-regulate miR-223 in MEG-01 cells and observed the consequence on P2Y12 expression. The results showed that miR-223 expression in the adenovirus inhibitor group decreased significantly (p < 0.001, Fig. 4E). Besides, P2Y12 mRNA and protein expression increased by 77.2% (p < 0.001, Fig. 4G) and 85.6% (p < 0.001, Fig. 4F, H) respectively, compared with the blank adenovirus group. These results prompted that treatment with clopidogrel might reduce miR-223 expression and recede the negative regulation of P2Y12 expression in megakaryocytes.

Decreased C/EBP α expression in megakaryocytes after clopidogrel treatment and P2Y12-PI3K/Akt-C/EBP α-miR-223 negative feedback loop

We further explored C/EBP α mRNA and protein expression in Meg-01 cells treated with clopidogrel incubation solution. C/EBP α mRNA expression in Meg-01 cells was significantly decreased after treatment with clopidogrel incubation solution for 3 or 5 consecutive days at 200 ul/2 ml (P < 0.01, Fig. 5A, B). Immunoblotting results showed that P2Y12 and C/EBP α protein expression were significantly lower after 5 consecutive days (p < 0.01, Fig. 5C, D). These results prompted that transcription factor C/EBP α might play a role in the regulation of miR-223 and P2Y12 expression after clopidogrel treatment.

Based on the above results, it is suggested that there may be a negative feedback loop

--P2Y12-PI3K/Akt-C/EBP α-miR-223, which regulates miR-223 and P2Y12 expression after clopidogrel treatment. To confirm this deduction, Meg-01 cells was treated with PI3K/Akt pathway inhibitors (LY294002) at different concertrations. The results showed that PI3K/Akt pathway activity was significantly inhibited and p-Akt protein expression was significantly decreased in MEG-01 cells treated with LY294002, which was correlated with inhibitor concentration (p < 0.05). C/EBP α expression was significantly decreased, but there was no significant correlation between protein expression and inhibitor concentration. MiR-223 expression decreased significantly in Meg-01 cells. P2Y12 mRNA expression increased significantly, while P2Y12 protein expression differed little (Figure 6). Combined with the above results, this study confirmed the existence of P2Y12-PI3K/Akt-C/EBPalpha-microRNA-223 negative feedback regulatory loop, which may be activited abnormally and contribute to low response to clopidogrel treatment (Figure 7).

Discussion

In this study we demonstrated that (1) decrease of platelet P2Y12 expression in low responder group was significantly slighter than that in the high responder group after clopidogrel treatment；(2) Meg-01 cells treated with clopidogrel incubation solution at 200 ul/2 ml for 3-5 days could simulate the effects of clopidogrel active metabolites on megakaryocytes and platelets; (3) there may be a negative feedback loop - P2Y12-PI3K/Akt-C/EBP α-miR-223, which regulates miR-223 and P2Y12 expression after clopidogrel treatment. Our results suggested that over-activation of the negative feedback regulation loop may be related to the occurrence of low response to clopidogrel treatment.

In this study, results showed that there were significant differences in platelet P2Y12 protein expression among individuals, and no significant differences were found between different groups, whether before or after colpidogrel treatment. However, platelet P2Y12 protein expression was indeed significantly correlated with the platelet reactivity aggregation rate, which was consistent with the previous research results of Braun et al. [38]. Braun et al. only detected the platelet P2Y12 protein expression and residual platelet reactivity after clopidogrel treatment, and did not discuss the relationship before treatment. Our results showed the significant correlation whether before or after treatment, which strongly supported that changes in P2Y12 protein expression may play an important role in platelet reactivity regulation. The different degree of reduction in P2Y12 protein expresison between the two groups may play an important role in the occurrence of HTPR after clopidogrel treatment. It was also found that P2Y12 protein expression in platelets decreased after clopidogrel treatment, which due to the small sample size, and the results showed no statistical difference. Previous studies on the pharmacokinetics of clopidogrel[39] showed that the

effect did not start immediately after oral administration. A significant decrease in platelet aggregation rate was usually observed after 2 consecutive days of oral administration, and the best effect was achieved 4-7 days later. In combination with our results, the decreased of P2Y12 protein expression in platelets may serve as an observation indicator for the stability of clopidogrel treatment effect.

Although platelets contain a variety of RNAs, these have been thought to be left over from the synthesis of megakaryocyte protein. Recent stydies showed that, these RNAs, once considered useless, appear to be involved in protein synthesis processes that occur in platelets. McRedmond et al.[40] combined analysis of platelet proteomics and genomics, and the results showed that the intracellular transcriptome of platelets was significantly consistent with the proteome. Landry et al.[32] further pointed out that platelets contain mRNA and cell elements which are necessary for protein synthesis such as endoplasmic reticulum, ribosomes and Golgi bodies, and there is indeed a set of functional miRNA regulating mRNA translation and protein synthesis. Now it is widely believed that platelets retain cytoplasmic mRNAs and maintain the functional integrity of the protein-translating machine inherited from megakarocytes. Once platelets are activated, protein resynthesis, or platelet protein resynthesis, can be initiated[41]. However, this protein synthesis process requires platelet activation to trigger, and only a few protein resynthesis processes have been reported, such as BCL-3, IL-1, PAI 1, dimpondin-1 (TSP-1) and SDF-1 etc[42]. No reports on the resynthesis of P2Y12 protein in activated platelets have been indexed. These results suggest that it seems to be more effective to focus studies of P2Y12 protein expression changes on megakaryocytes from which platelet RNAs and protein are inherited.

After treatment with clopidogrel incubation solution, megakaryocyte miR-223 expression in the upstream pathway of P2Y12 regulation significantly decreased. The results suggested that miR-223 expression in platelets might also be decreased after clopidogrel treatment. However, it needs magnetic bead separation to eliminate white blood cells pollution for RNA detecting in platelets. Thus blood samples of a larger volume was needed (at least 15 to 20 ml), which greatly limited the sample collection. At present there are few researches about the change of microrna expression in platelets. In fact, microRNAs can also be detected in plasma, and the sources mainly include three aspects: membrane vesicles (exosomes and microvesicles), lipoprotein and other nucleoprotein complexes. Microvesicles are the main carrier of microRNAs in plasma, and platelet is one of the main sources of microvesicles in blood plasma[43-45]. Previous study showed[46] that platelets might release miR-223 specifically after activitation. It is speculated that miR-223 expression in platelets is highly correlated with that in plasma. In fact, Willeit et al.[47] showed that circulating miR-223 could be significantly reduced after antiplatelet therapy, which exactly confirmed our previous speculation.

These results suggested that there may be a negative feedback loop to counter the inhibition of clopidogrel AM on platelet reactivity in megakaryocytes and platelets.

Some scholars proposed[48] that some unknown mutations in P2Y12 receptor might lead to spontaneous activation, which may contribute to the low response to clopidogrel. It may also play a role that clopidogrel has weak reverse excitatory activity, on this basis, it is speculated that the weak reverse excitation activity of clopidogrel inhibited the activation of the downstream signaling pathway of P2Y12 and triggered a negative feedback regulation pathway, thereby reducing miR-223 expression and receding the inhibition of P2Y12 expression. Our results confirmed that clopidogrel AM could weaken the inhibitory effect of miR-223 on P2Y12 through a negative feedback pathway of P2Y12-PI3K/Akt-C/EBPa-microRNA-223 in megakaryocytes. Combined with the previous results that P2Y12 protein expression in the low-response group decreased more slightly, it is speculated that low response to clopidogrel may be related to the over-activation of the negative feedback pathway of P2Y12-PI3K/Akt-C/EBPalpha-micro223. However, further investigation is required.

As a precursor drug, clopidogrel has to produce active metabolites through the activation of drug metabolizing enzymes in the liver to play a pharmacological role. In addition, Clopidogrel AM is very unstable. Although the active metabolite can be chemically synthesized in vitro at present, its cost is so high that it is difficult to be used in routine experiments. In fact, researchers[38, 49] have studied and reported the pharmacokinetics characteristics of clopidogrel through the liver microsomes metabolism experiments in vitro. In this study Meg-01 cells were treated with incubation solution of clopidogrel and liver microsomes, which means an alternative approach of less cost, easy operation, stable and reliable result. Although the results showed a high degree of consistency with our assumptions, there were some divergences. P2Y12 mRNA, a target of mir-223, increased significantly after 3 days of treatment. However, after 5 days of treatment, P2Y12 mRNA in the negative control group increased significantly and was significantly higher than that in the normal control group and clopidogrel incubation solution group. We speculate that liver microsomes in the incubation solution may have some toxic effects on cells and significantly increased P2Y12 mRNA expression through some unknown mechanism. More studies are required to improve the cell model for further investigation.

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Table 1 Comparison of baseline data between the low response group and the high response group

	Low responders (n=4)	high responders (n=5)	P
Demographic data			
Female	0	2(40%)	0.444
Age	69(64-70)	81(67-82)	0.325
Medical history			
Hypertension	4(100%)	2(40%)	0.167
Diabetes	0	1(20%)	1.000
Coronary heart disease	1(25%)	2(40%)	1.000
Symptomatic stroke	2(50%)	0	0.167
Smoking	3(75%)	1(20%)	0.206
NIHSS	5.75±4.5735	2.8±1.6432	0.293
Drug			
Proton pump inhibitor	2(50%)	3（60%）	1.000
Statins	4(100%)	2(40%)	0.167
Calcium channel blocker	2(50%)	2(40%)	1.000
Platelet aggregation rate			
Before treatment	7.5625±8.82540	12.2300±12.137	0.309
After treatment	9.9125±10.44405	4.6580±1.15003	0.389
Relative inhibition rate	-0.2047(-1.1227~-0.1551)	0.6252(0.6060~0.6429)	0.014
Laboratory examination			
WBC	6.650±1.8046	7.980±3.3011	0.496
RBC	4.3975±0.49789	4.6400±0.54795	0.515
Hb	142.250±8.3815	141.800±21.9363	0.968
PLT	229.250±35.6499	199.000±27.6767	0.193
MPV	9.125±0.9605	9.340±0.8385	0.730
PDW	15.950(15.900-16.200)	15.900(15.700-16.000)	0.381
Fbg	2.8950(2.5350-3.1750)	2.8200(2.1400-2.8300)	0.327
D-dimer	522.1800±241.68127	1153.5340±1083.33611	0.268
BUN	5.225±1.6153	7.400±5.1459	0.414
Cr	85.850(77.750-101.950)	73.100(62.900-79.500)	0.327
UA	357.700±135.8205	351.160±147.7130	0.947
TC	6.0900(4.7000-6.6150)	4.9800(4.8700-5.0800)	0.624
TG	2.4600(1.5550-2.5950)	1.6300(0.8300-2.3200)	0.462
HDL	1.2250±0.29034	1.1780±0.28394	0.814
LDL	3.7500(2.6350-4.5700)	3.0100(2.9300-3.1400)	0.624

ALT	20.225±12.4272	21.340±10.1859	0.886
AST	23.300(13.800-31.400)	16.900(16.700-17.600)	0.806

NIHSS，the National Institutes of Health Stroke Scale；WBC，white blood cell；

RBC，red blood cell；Hb，hemoglobin；PLT，platelet；MPV，mean platelet volume；

PDW，platelet distribution width；Fbg，fibrinogen；BUN，blood urea nitrogen；

Cr，creatinine；UA，urea acid；TC，total cholesterol；TG，total triglyceride；

HDL，high density lipoprotein；LDL，low density lipoprotein；ALT，alanine

aminotransferase；AST，aspartate aminotransferase.

Figure legends

Fig. 1. Platelet aggregation measured by flow cytometry

A and **B.** PBS control group; **C** and **D.** ADP induction group. The difference of the ratio of cells in the porta delineated in **D.** and **B.** means platelet aggregation rate of this sample.

Fig. 2 Changes of P2Y12 protein expression in platelets before/after clopidogrel treatment

A and **B.** The correlation analysis of platelet P2Y12 protein expression and platelet aggregation before(**A**) or after(**B**) clopidogrel treatment. **C** and **D.** Relative platelet P2Y12 protein expression in the two extreme groups before/after clopidogrel treatment. **E.** The R value in LR group was significantly higher than that in HR group. **F.** Platelet P2Y12 expression in typical patients. S is low responder, V is high responder, S'and V' represent clopidogrel treatment respectively.

Fig. 3 Effects of clopidogrel incubation solution treatment on P2Y12 expression in megakaryocytes

Effects of clopidogrel incubation solution treatment on P2Y12 expression in megakaryocytes at different concentrations(**A**, **B**) and time(**C-F**).

Fig. 4 Decreased microRNA-223 expression and receded regulation of P2Y12 in megakaryocytes after clopidogrel treatment.

A and **B.** Decreased microRNA-223 expression in megakaryocytes after clopidogrel treatment for 3、5 days. **C** and **D.** P2Y12 mRNA expression in megakaryocytes after clopidogrel treatment for 3、5 days. **E-F.** Decreased microRNA-223 expression could reced the negative regulation of P2Y12 in megakaryocytes.

Fig. 5 Decreased C/EBP α expression in megakaryocytes after clopidogrel treatment.

A and **B.** Decreased C/EBP α mRNA expression in megakaryocytes after clopidogrel treatment for 3、5 days. **C**，**D** and **E.** Decreased C/EBP α and P2Y12 protein expression in megakaryocytes after clopidogrel treatment for 3、5 days.

Fig. 6 Decreased C/EBP α and miR-223 expression and increased P2Y12 mRNA expression in megakaryocytes after PI3K/Akt pathway inhibitor treatment.

After PI3K/Akt pathway inhibitor (LY294002) treatment, p-Akt (**A**), C/EBP α (**C**, **D**, **E**) and miR-223 (**F**) expression was decreased, P2Y12 mRNA (**G**) expression was increased, but there was no sigificant change in P2Y12 protein expression (**B**).

Fig. 7 A schematic diagram illustrating the feedback circuit of the signaling pathway P2Y12-PI3K/Akt-C/EBP-miR-223 in megakaryocytes.
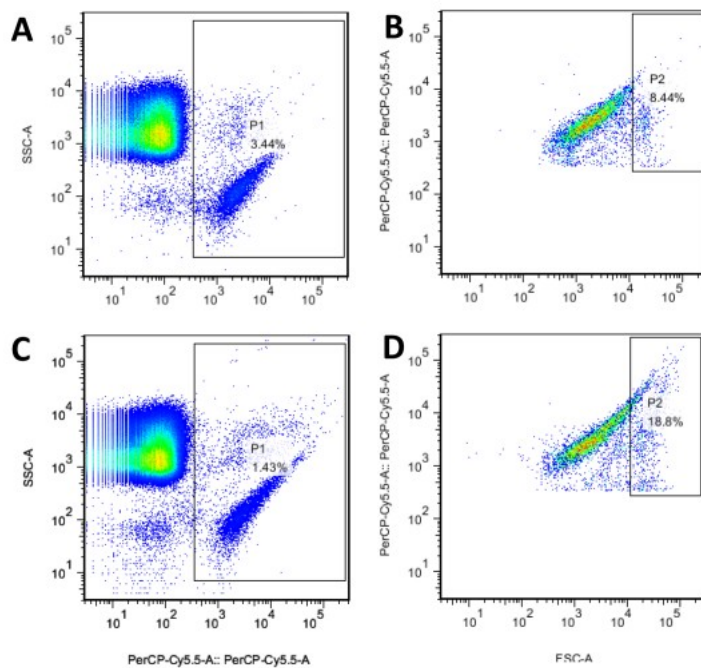
Fig. 1. Platelet aggregation measured by flow cytometry
A and B. PBS control group; **C and D.** ADP induction group. The difference of the ratio of cells in the porta delineated in **D.** and **B.** means platelet aggregation rate of this sample.
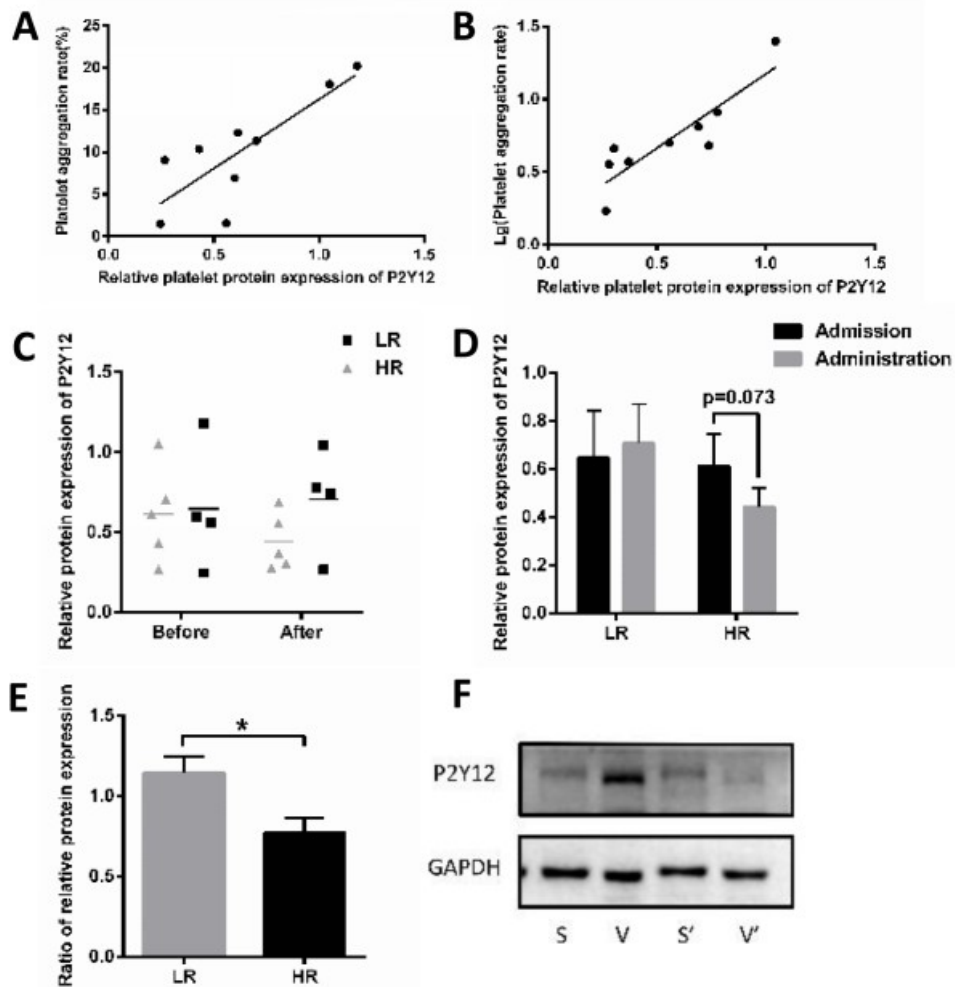
Fig. 2 Changes of P2Y12 protein expression in platelets before/after clopidogrel treatment

A and B. The correlation analysis of platelet P2Y12 protein expression and platelet aggregation before(**A**) or after(**B**) clopidogrel treatment. **C and D.** Relative platelet P2Y12 protein expression in the two extreme groups before/after clopidogrel treatment. **E.** The R value in LR group was significantly higher than that in HR group. **F.** Platelet P2Y12 expression in typical patients. S is low responder, V is high responder, S'and V' represent clopidogrel treatment respectively.

Fig. 3 Effects of clopidogrel incubation solution treatment on P2Y12 expression in megakaryocytes.
Effects of clopidogrel incubation solution treatment on P2Y12 expression in megakaryocytes at different concentrations(**A**, **B**) and time(**C-F**).
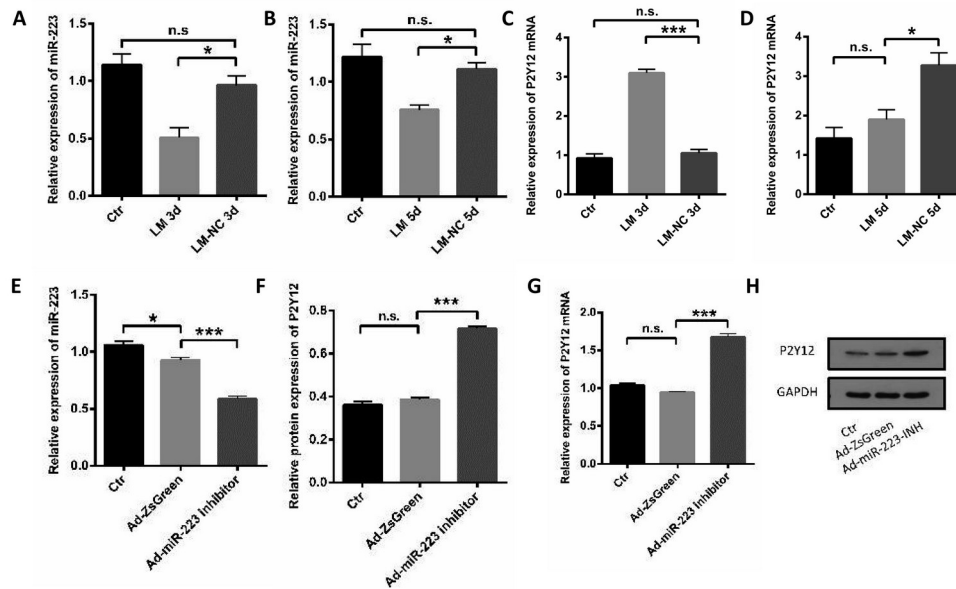
Fig. 4 Decreased microRNA-223 expression and receded regulation of P2Y12 in megakaryocytes after clopidogrel treatment.

A and B. Decreased microRNA-223 expression in megakaryocytes after clopidogrel treatment for 3、5 days. **C and D.** P2Y12 mRNA expression in megakaryocytes after clopidogrel treatment for 3、5 days. **E-H.** Decreased microRNA-223 expression could reced the negative regulation of P2Y12 in megakaryocytes.
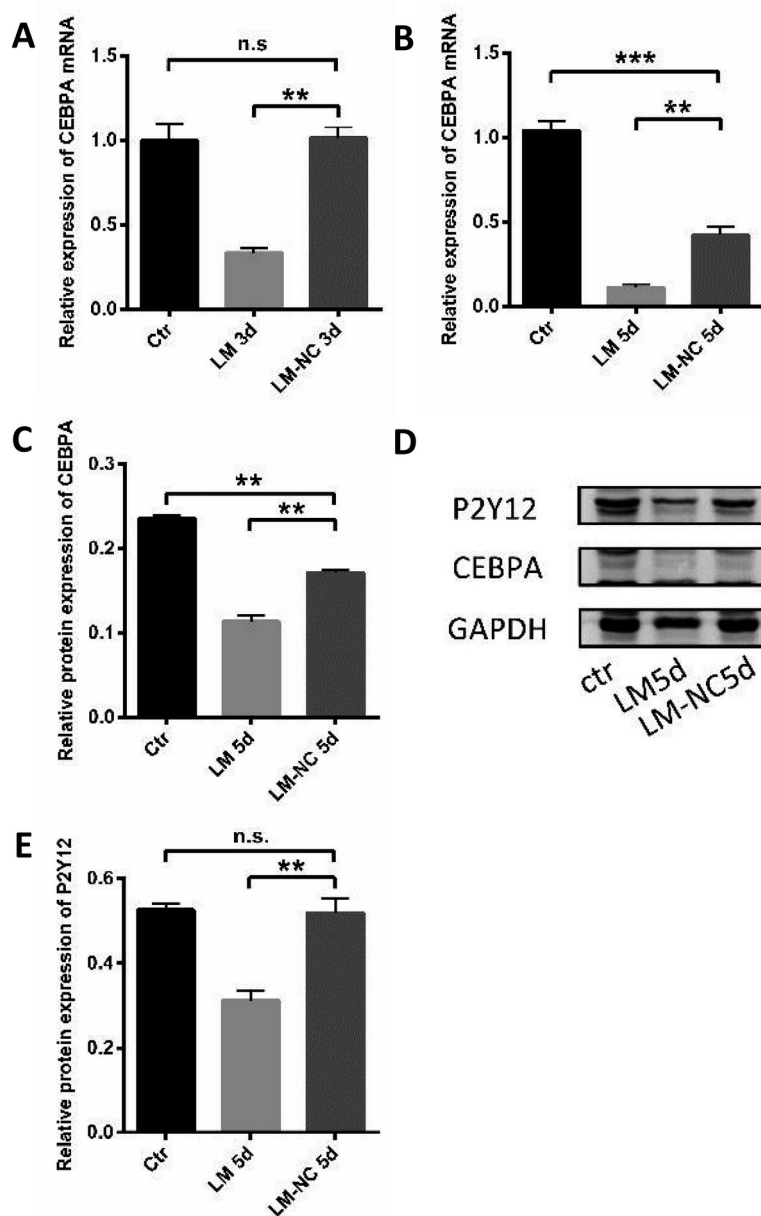
Fig. 5 Decreased C/EBP α expression in megakaryocytes after clopidogrel treatment.
A and B. Decreased C/EBP α mRNA expression in megakaryocytes after clopidogrel treatment for 3、5 days. **C , D and E.** Decreased C/EBP α and P2Y12 protein expression in megakaryocytes after clopidogrel treatment for 5 days.
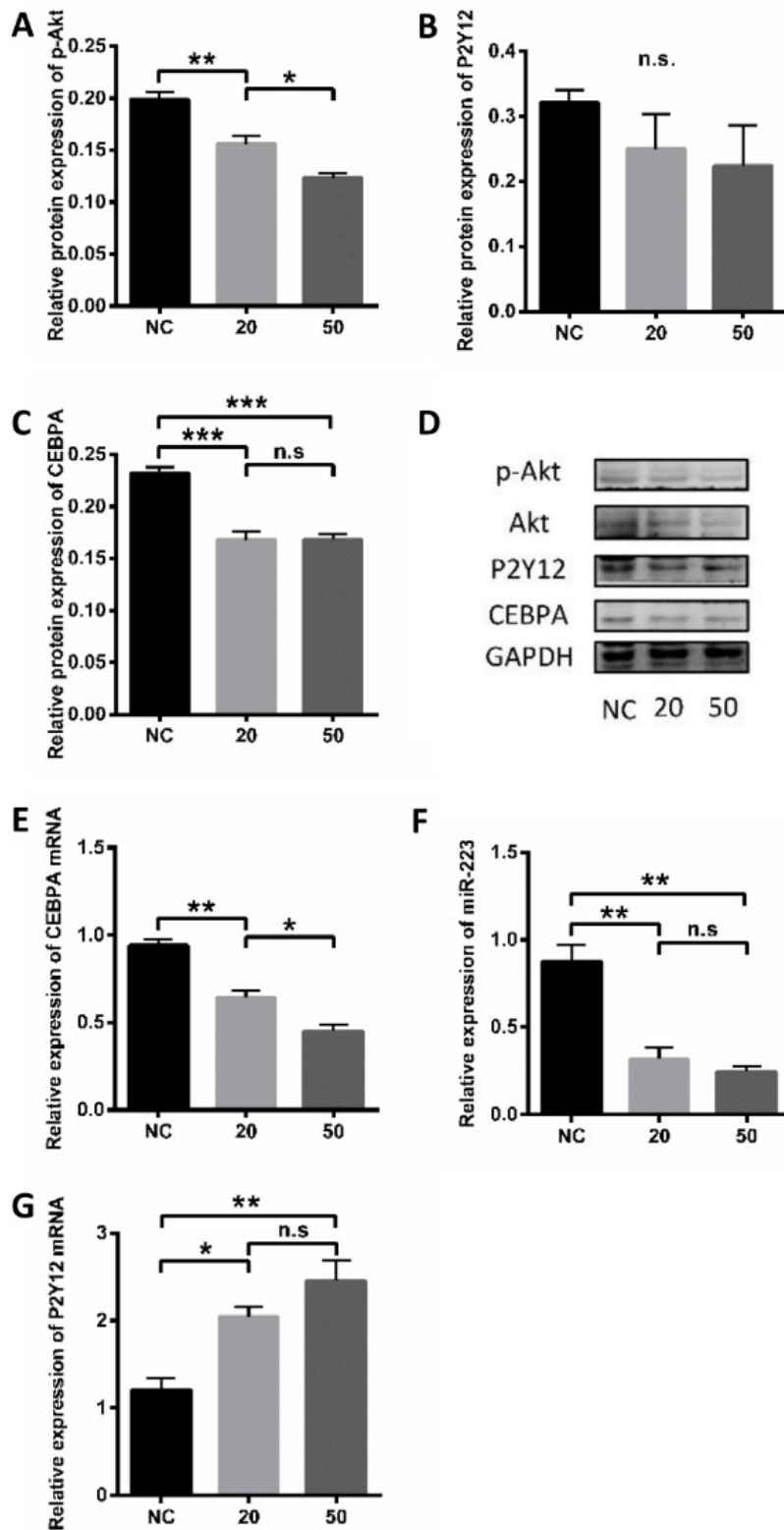
Fig. 6 Decreased C/EBP α and miR-223 expression and increased P2Y12 mRNA expression in megakaryocytes after PI3K/Akt pathway inhibitor treatment.
After PI3K/Akt pathway inhibitor (LY294002) treatment, p-Akt (**A**), C/EBP α (**C**, **D**, **E**) and miR-223 (**F**) expression was decreased, P2Y12 mRNA (**G**) expression was increased, but there was no sigificant change in P2Y12 protein expression (**B**).
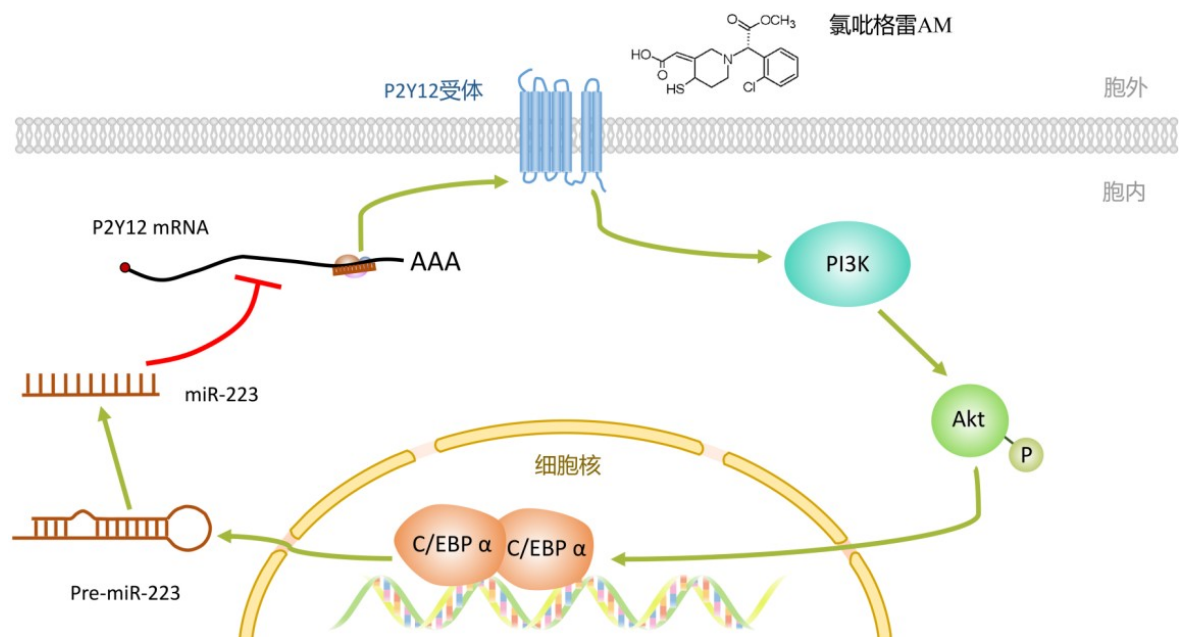
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