

Clinical observation of low-dose combination chemotherapy in refractory/recurrent paroxysmal nocturnal hemoglobinuria patients: a single-centre retrospective analysis

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Abstract

Objective We performed a retrospective analysis to investigate the clinical characteristics and therapeutic strategies of 20 refractory/recurrent PNH patients, including the clinical efficacy of chemotherapy treatment and survival. **Main Measures** The clinical data of 20 classic PNH patients that were refractory/recurrent or had glucocorticoid dependence in our hospital were analyzed, including clinical manifestations, laboratory examinations, treatment efficacy and survival. **Key Results** Seventeen patients had a marked improvement in anemia after chemotherapy, 14 patients acquired blood transfusion independence, and the Hb of 3 patients increased to normal levels. Although 6 patients still needed blood transfusion, the transfusion interval was significantly prolonged. The percentages of LDH, TBIL and RET, which are indicators of hemolysis, were significantly lower than those before chemotherapy. The dosage of adrenal glucocorticoids was reduced by more than half compared with that before chemotherapy. **Conclusions** Chemotherapy can reduce PNH clones, promote normal hematopoiesis, and control hemolytic attack. It is a promising and widely used therapeutic method.

1. Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal disease of hematopoietic stem cells caused by somatic cell mutations. The pathological defect of PNH is the abnormal synthesis of glycosyl phosphatidyl inositol (GPI) caused by a PIG-A gene mutation on the X chromosome, which leads to the loss of a group of anchor proteins anchored by GPI on the blood cell membrane, including CD55, CD59 and CD16. The main clinical manifestations are chronic intravascular hemolysis, bone marrow failure, and high-risk complications of thrombosis, etc.[1,2].

With the advent of eculizumab (a recombinant human anti-complement C5 monoclonal antibody), PNH treatment has entered the era of complement pathway inhibition. The efficacy and safety of eculizumab in the treatment of PNH have been confirmed[3-5]. It can reduce the need for blood transfusion, improve anemia, relieve symptoms related to complement-mediated chronic intravascular hemolysis (such as renal function damage), reduce pulmonary hypertension and severe thrombotic events, and ultimately improve quality of life and prolong survival. Moreover, the research and development of many new complement pathway inhibitors (ravulizumab, crovalimab, coversin, etc.)[6-8] have also achieved good therapeutic effects and overcome many shortcomings of eculizumab, which provides new hope for PNH treatment.

Unfortunately, none of the above monoclonal antibodies are currently on the market in China. In addition, these monoclonal antibodies are extremely expensive [9,10]. Therefore, in our country, glucocorticoids are still the first-line drug for controlling hemolytic attack in PNH with definite curative effects. However,

the long-term use of glucocorticoids can cause serious adverse reactions, such as hypertension, diabetes, and femoral head necrosis, and some PNH patients are prone to relapse after ineffective or reduced glucocorticoid treatment. Therefore, treatment of refractory/relapsed PNH that is ineffective or dependent on glucocorticoids has always been a difficult problem. In recent years, studies by Cooper et al[11], Lee et al[12] and domestic studies by Wu D et al[13,14] have confirmed that allo-hematopoietic stem cell transplantation (HSCT) has a definite curative effect in PNH patients with transplantation indications, and the prognosis of patients is improving. In 2016, the team of Professor Wu Depei in China reported 18 PNH patients who underwent haplotype-matched HSCT, and the expected 5-year disease-free survival rate was $80.5 \pm 10.2\%$ without relapsed cases [13]. The above research results provide hope for the radical cure of refractory/relapsed PNH, but the risk of HSCT is higher, the limited treatment experience and the transplant indications are currently inconclusive and difficult to promote.

Therefore, we designed several chemotherapy regimens to treat some refractory and relapsed PNH patients. As the same dose of chemotherapy is tolerated less well in PNH patients than in leukemia patients, to avoid the risk of excessive bone marrow suppression, we used a low dose and a short course of treatment. PNH is a benign clonal disease, and normal clones and PNH clones coexist in PNH patients. The current treatment involves killing a considerable number of PNH clones with chemotherapy and then using the strong ability to tolerate complement and faster recovery of normal clones compared to PNH clones to promote the gradual replacement of PNH clones with normal clones.

Twenty patients with PNH treated with chemotherapy from June 2010 to June 2020 were enrolled in our study. We analyzed the clinical characteristics of these PNH patients, including the clinical efficacy of chemotherapy, survival, chemotherapy-related adverse reactions and safety.

2. Methods and patients

2.1 Patients

Twenty classic PNH patients with refractory/recurrent or glucocorticoid dependent disease from June 2010 to June 2018 at Tianjin Medical University General Hospital were enrolled in our study. All patients who were enrolled in our study were treated with glucocorticoids and had serious side effects, such as diabetes, hypertension, gastrointestinal bleeding, etc. The characteristics and related risks of chemotherapy were clearly explained to patients, and patients provided written informed consent. Patients with active bacterial or fungal infections or a history of lung or heart disease were not included in this study. The clinical data of the patients were collected, including sex, age, clinical classification, clinical manifestations and laboratory results. PNH clones and FLAER were detected by flow cytometry in 20 patients (Fig. 1A and Fig. 1B). The PNH diagnostic criteria were based on the Chinese expert consensus on the diagnosis and treatment of PNH (2014) [15]. The study was in compliance with the Declaration of Helsinki, and the protocol was approved by the ethical committee of Tianjin Medical University General Hospital. Consent for research and publication was obtained from participants and/or their immediate family if certain participants had passed away.

2.2 Treatment and therapeutic index

All patients received glucocorticoid treatment after diagnosis to control hemolysis. The initial treatment included methylprednisolone intravenous infusion (1 mg/kg/d) and vitamin E (300 mg/day), and the dose of methylprednisolone was increased as appropriate if hemolytic crisis occurred. Some patients received blood cell transfusion if $Hb < 60$ g/L and/or $PLT < 10 \times 10^9/L$.

Chemotherapy regimens: low-dose DA or HA regimens, which included DNR (40 mg/d, d1; 20 mg/d, d2-3) and Ara-C (100 mg/d, d1-5 or d1-7) or HHT (2-3 mg/d, d1-5) and Ara-C (100 mg/d, d1-5), respectively. The chemotherapy regimens were administered intravenously every 4 weeks for one to three cycles. The concomitant administration of hematopoietic growth factors (EPO or G-CSF), immunosuppressive drugs, vitamin E, sodium bicarbonate, and other supportive therapies (including isolation wards, component blood support when necessary, anti-infectives when infection occurred and anticoagulation treatment) was also performed.

2.3 Clinical efficacy indicators

The two main endpoint evaluation indicators were the stability of hemoglobin levels, which was defined as the maintenance of hemoglobin levels above 60 g/l without blood transfusion within 4 to 8 weeks after chemotherapy), and the change in lactate dehydrogenase level from baseline to 12 weeks (fold increase compared with the normal value). Other prespecified measurements included the proportion of reticulocytes, total bilirubin level, proportion of PNH clone cells and dosage of corticosteroids after chemotherapy during treatment.

2.4 Follow-up

The median follow-up was 79 (33-129) months. Overall survival (OS) was calculated from the time of the initial diagnosis of symptomatic PNH to the time of death. The end of the follow-up was July 2018 or the date of loss or death.

2.5 Statistical analysis

SPSS 23.0 software was used for statistical analysis. The measurement data are expressed as the mean \pm standard deviation (\pm S). Differences in quantitative parameters between groups were assessed using a t test (for normally distributed data) or nonparametric test (for nonnormally distributed data). Categorical variables are presented as frequencies. OS estimated by the Kaplan-Meier method was compared using the log-rank test. P values lower than 0.05 were considered significant.

3. Results

3.1 Baseline characteristics of 20 PNH patients

Twenty patients with refractory/recurrent or glucocorticoid dependent classic PNH were enrolled in our study. The results of bone marrow cytology in 20 patients with PNH showed that 9 patients (9/20, 45.00%) had increased bone marrow viability, 11 patients (11/20, 55.00%) had obvious activity, and no decreased proliferation was found. There were 12 males and 8 females with ages ranging from 19-68 years, and the median age was 41 years. The course of disease from diagnosis to chemotherapy ranged from 0.08 to 16 years. All patients had been treated with corticosteroids before, but the clinical efficacy of corticosteroids was not as expected. Some patients had complications such as diabetes, hypertension, and gastric mucosal bleeding, which may be related to long-term corticosteroid use and high-dose corticosteroids. Some of the patients were also treated with androgens, erythropoietin and cyclosporine before chemotherapy. None of the patients had thrombotic complications or had received prophylactic anticoagulant therapy. The dose and cycle number of chemotherapy drugs were adjusted according to the severity of myelosuppression or hemolysis. The baseline characteristics of the patients are summarized in Table 1.

The clinical symptoms of the 20 PNH patients were summarized as follows: 17 (85.00%) cases of dizziness and fatigue, 11 (55.00%) cases of palpitation and shortness of breath, 3 (15.00%) cases of tinnitus, 18 (90.00%) cases of pale skin mucosa, 15 (75.00%) cases of yellow skin mucosa, and 16 (80.00%) cases of darkened urine (including 7 cases of soy sauce colored urine, 6 cases of strong tea colored urine, and 3 cases of dark yellow urine).

3.2 Efficacy evaluation

3.2.1 Improvement of anemia and transfusion dependence

The anemia of 17 patients (17/20, 85.00%) significantly improved. Fourteen patients (14/20, 70.00%) were able to stop blood transfusion, and 3 patients (3/20, 15.00%) had hemoglobin levels that recovered to normal. Among the 6 patients (6/20, 30.00%) who still needed blood transfusion, the interval time of blood transfusion was prolonged from 6 days (Hb 48 g/L) to 19 d (Hb 59 g/L) in 3 patients. Generally, anemia improved, and hemoglobin increased from 68.40 ± 14.79 g/L to 86.80 ± 24.32 g/L ($p = 0.0042$) (Fig. 2A1, 2A2). Among the 20 patients, 3 patients (3/20, 15.00%) had no significant improvement in anemia (HB fluctuated between 40-50 g/L) and still needed intermittent blood transfusion. Eculizumab will hopefully be available in China

in the near future. It is believed that this drug could improve the clinical symptoms of the two patients for whom chemotherapy was ineffective.

3.2.2 The hemolytic symptoms were controlled, and the hemolytic indexes were obviously improved .

The effect of chemotherapy on chronic intravascular hemolysis is characterized by a continuous decrease in LDH levels. The mean LDH levels decreased from 1592.0 ± 718.7 U/L at baseline to 390.0 ± 252.8 U/L at 8 weeks ($p = 0.002$) (Fig. 2C1, 2C2). In addition, TBIL and the percentage of reticulocytes were evaluated as indirect biochemical indicators of intravascular hemolysis. After chemotherapy, reticulocytes decreased from $10.20 \pm 7.01\%$ to $4.23 \pm 3.03\%$ ($p=0.0012$) (Fig. 2B1, 2B2). Of the 20 patients, 3 patients (3/20, 15.00%) had normal TBIL, and the remaining 17 patients had a significant decrease in TBIL from 41.42 ± 22.92 $\mu\text{mol/L}$ to 18.72 ± 9.08 $\mu\text{mol/L}$ ($p=0.003$) (Fig. 2D1, 2D2). Plasma free hemoglobin and haptoglobin levels were monitored in another 12 patients. The results showed that the serum free hemoglobin concentration was normal in 2 patients, and the decrease was significant in the other patients. Not all patients were tested for the above indicators, so they were not included in the analysis.

3.2.3 The proportion of PNH clones decreased

The ratio of CD59- peripheral blood granulocytes and erythrocytes before and after chemotherapy was detected by flow cytometry. The results showed that the proportion decreased after chemotherapy, but the difference was not statistically significant. The proportion of CD59- granulocytes and erythrocytes in 16 patients decreased from $75.05 \pm 18.33\%$ and $57.75 \pm 16.28\%$ to $66.53 \pm 20.69\%$ and $49.18 \pm 14.27\%$ ($p = 0.072$ and $p = 0.053$), respectively. However, there were still 3 patients (3/20, 15.00%) whose granulocyte and erythrocyte CD59- ratios did not decrease as expected. Consistent with the above results, anemia and blood transfusion in these 3 patients did not improve.

3.2.4 The dosage of corticosteroids decreased

After chemotherapy, the dosage of corticosteroids in all PNH patients decreased gradually, and no severe hemolytic symptoms occurred. The dose of corticosteroids decreased significantly from 62.38 ± 19.47 mg at baseline to 26.12 ± 11.80 mg at 6 weeks after chemotherapy and to 13.00 ± 6.75 mg at 8 weeks after chemotherapy ($p = 0.000$).

3.2.5 Hemolytic relapse time

Among the 17 PNH patients for whom chemotherapy was effective, 6 patients relapsed within 2 years, and 2 patients experienced recurrence twice within one year. In the remaining 11 patients, 7 patients did not relapse after 1 year of observation, and 4 patients did not recur after 2 years of observation.

3.3 Long-term follow-up and survival

Of the 20 patients, 1 patient was lost to follow-up, and one patient died. The median treatment time was 79 (33-129) months, and the OS 10 years after diagnosis was estimated to be 121 months. We analyzed the overall survival of 19 PNH patients in terms of age, Hb level and LDH elevation. The results showed that there was no significant difference in the effects of the above factors on survival (Fig. 3).

3.4 Safety

No deaths occurred during chemotherapy. The most common adverse reactions during chemotherapy were infection (14/20, 70.00%), nausea (12/20, 60%), vomiting (11/20, 55%) and fatigue (9/20, 45%). Almost all patients had myelosuppression after chemotherapy. Among them, 14 patients had agranulocytosis with fever, and their body temperature fluctuated between 38.5 and 39.8°C. The main infections were upper respiratory tract infection in 4 patients, pneumonia in 3 patients, sepsis in 2 patients, oral infection in 1 patient, perianal infection in 2 patients, skin herpes in 1 patient, suspected cholecystitis in 1 patient, and local infection in 2 patients. The above adverse reactions resolved after the recovery of hematopoietic reconstitution. The mean duration of myelosuppression was 24.28 (18-52) days, the minimum values of leukocytes and granulocytes

were 0-1.29, and the minimum values of platelets were $1-35 \times 10^9/L$. Two patients had platelet transfusion failure. No patient had complications with severe bleeding symptoms. During chemotherapy, patients were treated with heart- and liver-protecting treatments at the same time, and no serious complications occurred.

4. Discussion

PNH is one of the most common hemolytic anemias in northern China. According to the reported cases in papers. In our country, the traditional treatment for PNH still focus on "protecting" PNH clones, reducing complement attack and destruction, and reducing hemolysis. Glucocorticoids are still effective drugs for reducing hemolysis to control PNH. After years of clinical progress, the current use of glucocorticoids and hematopoietic therapy has allowed the remission rate of the disease reach more than 60%, but nearly 40% of patients are not sensitive to this treatment[16,17]. Even in patients who achieve remission after treatment, there is still more than a 10% possibility of relapse[18].

At the end of the 1970s, Soviet scholars reported that cyclophosphamide or 6-mercaptopurine achieved certain effects in the treatment of PNH[19]. In our previous study[20], we found that the expression rates of G-CSFR and SCFR on $CD34^+CD59^+$ cells of PNH patients were significantly lower than those on $CD34^+CD59^+$ cells, while there was no significant difference in the expression of G-CSFR and SCFR between $CD34^+CD59^+$ cells of PNH patients and healthy controls. These results suggest that the response of mutated hematopoietic stem/progenitor cells from PNH patients to stimulation with G-CSF and SCF may be reduced due to the insufficient expression of G-CSF and SCFR. In a follow-up study[20], we confirmed that the MFI of p-stat5 in $CD34^+CD59^+$ cells was significantly higher than that in $CD34^+CD59^+$ cells before and after G-CSF or SCF stimulation based on protein phosphorylation flow cytometry, and the MFI of phosphorylated STAT5 in $CD34^+CD59^+$ cells of PNH patients was significantly higher than that in $CD34^+CD59^+$ cells of PNH patients after G-CSF or SCF stimulation. However, the proliferation of abnormal clones was not significant, which widened the gap between normal hematopoietic cells and abnormal PNH cells and restored normal hematopoiesis.

Chemotherapy can kill both PNH clones and normal clones, but normal clones have faster proliferation rates and higher expression of CD114 and CD117 on their surface than PNH clones. After G-CSF stimulation, normal clones are more reactive than PNH clones, so normal clones are dominant, and the symptoms of patients disappear or are reduced. PNH patients have GPI-AP deletions other than CD55 and CD59. Our previous research results[21-23] have proven that chemotherapy can effectively reduce the PNH clonal load, control hemolysis, improve anemia, and greatly reduce the level of corticosteroids and is thus a promising treatment. In this study, we retrospectively analyzed the efficacy and safety of reduced-dose combined chemotherapy in 20 patients with refractory/relapsed PNH.

All patients enrolled in the study had refractory/relapsed PNH, 1 mg/kg/d prednisone was ineffective for more than 1 month, or adrenal glucocorticoid treatment was effective, but relapse occurred soon after a dose reduction. Among the 20 patients with PNH, 17 had a marked improvement in anemia after chemotherapy, 14 patients stopped blood transfusion, and Hb in 3 patients rose to normal levels. Although 6 patients did not stop blood transfusion, the transfusion interval was significantly prolonged. The percentages of LDH, TBIL and RET, which are indicators of hemolysis, were significantly lower than those before chemotherapy. The above results confirmed that after chemotherapy with reduced-dose DA or HA regimens, the hemolysis index of PNH patients was significantly improved, PNH clones were reduced, and the dosage of adrenal glucocorticoids was reduced by more than half compared with that before chemotherapy.

Our study also found that the 10-year overall survival of patients from diagnosis to the end of follow-up was 78.29%. We used multivariate regression to analyze the effects of sex, age, LDH elevation, and PNH clone ratio on survival. The results showed that the above factors had no statistically significant impact on OS, which may be related to the number of samples in each group in our study. In future research, it will be necessary to increase the number of samples and conduct an in-depth analysis of the above factors.

Of the 17 patients with PNH for whom chemotherapy was effective, 6 patients relapsed within 2 years, and 2 patients relapsed twice within 1 year, which suggests that low-dose chemotherapy did not eradicate PNH

clones. Of the remaining 9 patients, 7 patients did not relapse after observation for 1 year, and 4 patients did not relapse after observation for 2 years. The above results indicate that reduced-dose chemotherapy cannot eradicate PNH clones, but it can inhibit PNH clones to a great extent. The long-term efficacy of low-dose chemotherapy needs to be further observed with an expanded sample size.

The development and launch of eculizumab and new complement pathway inhibitory drugs have brought new hope for the treatment of PNH[24-27]. Since the above drugs have not yet been marketed in our country, new treatment methods urgently need to be identified. Our results confirm that reduced-dose chemotherapy combined with hematopoietic growth factors, component blood infusion, and symptomatic supportive therapy can effectively reduce PNH clones, reduce hemolytic attacks, and stabilize the disease. This is currently a promising and widely used therapeutic method.

5. Conclusion

As a traditional treatment for PNH, corticosteroids are still the first-line drug for PNH patients in China. However, how to treat refractory/relapsed PNH for patients in whom corticosteroids are ineffective or who are dependent on corticosteroids has always been a difficult problem for clinicians. For patients with refractory/relapsed PNH, low-dose combined chemotherapy is an effective choice, which can reduce the number of PNH clones and allow normal clones to expand to promote normal hematopoiesis and control hemolytic attack. This is a promising and widely used therapeutic method at present.

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Figure legends

Fig 1 A show the proportion of CD59-negative granulocytes and erythroid cells detected by flow cytometry; 1B shows FLAER detection in the peripheral blood of PNH patients by flow cytometry.

Fig 2 The trend of Hb, RET%, LDH and TBIL level changes in 0 to 8 weeks before and after chemotherapy. A1/A2 and B1/B2 show the average levels of Hb and reticulocyte percentages before and after chemotherapy from baseline (0 weeks) to 8 weeks after chemotherapy, respectively. Hb and RET% increased significantly from before chemotherapy to 8 weeks after chemotherapy. C1/C2 and D1/D2 show the average levels of LDH and TBIL before and after chemotherapy from baseline (0 weeks) to 8 weeks after chemotherapy, reflecting the degree of intravascular hemolysis. After chemotherapy, lactate dehydrogenase (LDH) levels in all PNH patients decreased to slightly higher than the upper limit of the normal range in 8 weeks, and the LDH level in 15 patients dropped to the normal range.

Fig 3 Survival rate in the cohort of PNH patients. The overall survival (OS, %) of all enrolled patients over 10 years is shown in **A**. There was no significant difference in the effects of age, Hb level and LDH elevation on survival (**B**, **C**, and **D**).

Conflicts of Interest

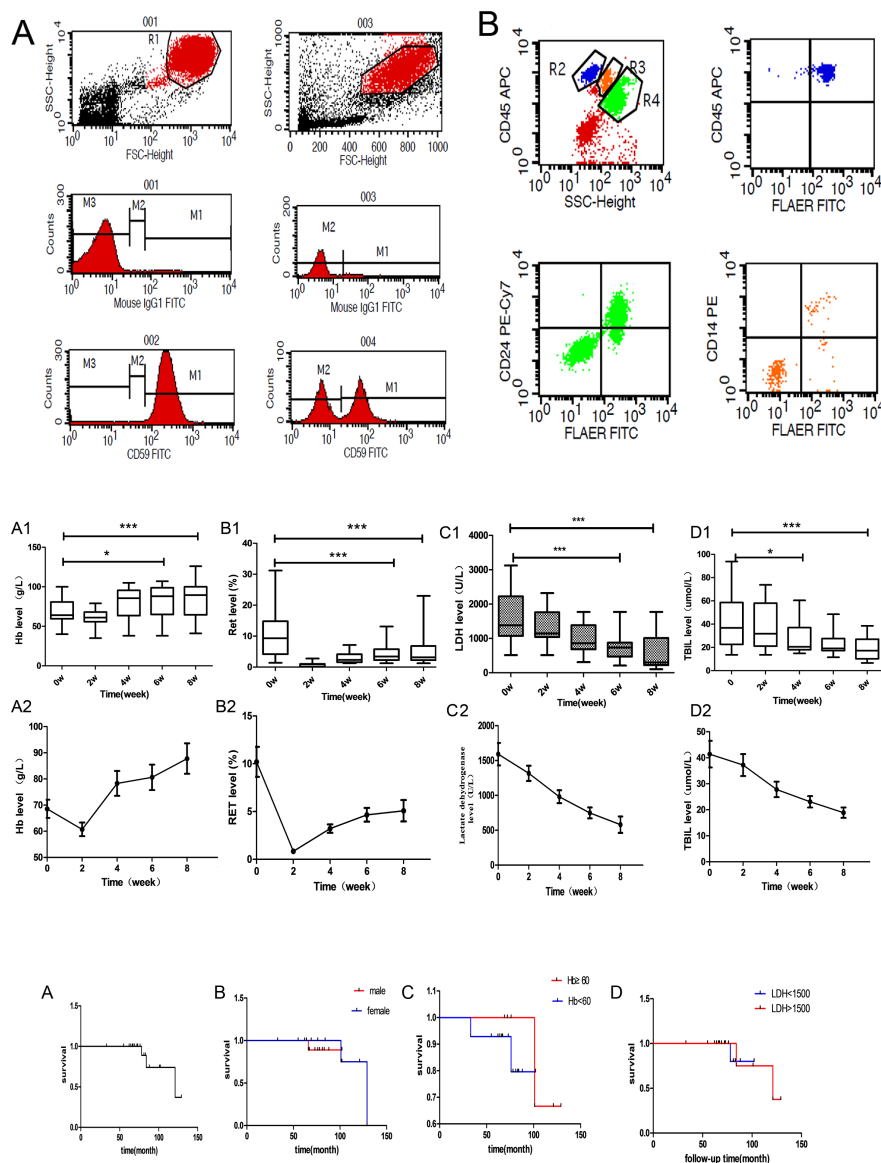
The manuscript has been read and approved for submission by all qualified authors, and there are no conflicts of interest.

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Authorship

Liyan Li and Hui Liu performed the experiments, analyzed the data and wrote the article. Honglei Wang, Zhaoyun Liu, Yingying Chen, Chunyan Liu, Xiaoyu Zhao contributed to the experimental work and the collection of patients' features. Lijuan Li, Huaquan Wang, Zonghong Shao contributed essential reagents or tools, Rong Fu designed the research and revised the manuscript.



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