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Impact of GGCX polymorphisms on warfarin dose requirements in atrial fibrillation patients

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Background/aim: Warfarin is a common anticoagulant with large interindividual differences and a narrow therapeutic range. The polymorphisms of gamma-glutamyl carboxylase (GGCX) are important genetic factors for warfarin dose requirements.

Materials and methods: Polymerase chain reaction and direct sequencing methods were used to detect the GGCX rs699664 genotype in 215 atrial fibrillation (AF) patients with warfarin administration. The effects on warfarin dose by different genotypes were analyzed. A warfarin dosing algorithm was developed based on age, height, CYP2C9, VKORC1, and GGCX genotype.

Results: In 215 AF patients, there were 104 cases of wild-type GG genotype (48.4%), 92 cases of GA genotype (42.8%), and 19 cases of AA genotype (8.8%). Patients with the GGCX rs699664 A allele (GA or AA genotypes) needed higher warfarin doses than those with the GG genotype ($P < 0.05$). A warfarin dosing algorithm showed that age, height, CYP2C9, VKORC1, and GGCX genotype were the best variables for estimating warfarin dose ($R2 = 41.2\%$). Another independent cohort of 60 AF patients showed a significant linear correlation between predicted warfarin maintenance dose and actual dose ($R = 0.660$, $P < 0.01$).

Conclusion: AF patients with the GA and AA genotypes in GGCX rs699664 required significantly higher warfarin doses. GGCX rs699664 is a potential predictor for the warfarin dose of AF patients.

Key words: Atrial fibrillation, gamma-glutamyl carboxylase, warfarin, genetic polymorphisms

1. Introduction

Atrial fibrillation (AF) is the most common clinical cardiac arrhythmia with high morbidity and mortality (1). The incidence of AF is 1%–2% in the general population and it is increased with age, heart failure, and hypertension (2–4). AF patients have increased risk of complications, such as strokes and systemic thromboembolism (5), which can be reduced by anticoagulant therapy such as warfarin (6).

Warfarin is the most commonly used oral anticoagulant drug and it has long been applied in the prevention of various thromboembolic disorders, such as prosthetic heart valves, deep venous thrombosis, pulmonary embolism, and recurrent stroke (7). Due to interindividual differences in drug responses, warfarin has a narrow therapeutic range and inadequate or excessive warfarin may result in thromboembolism or bleeding (8). Therefore, it is essential to monitor and control the warfarin dose within this therapeutic window in clinical application.

Because of the interindividual variability in response to warfarin, it is difficult to make accurate dose predictions. Warfarin dose requirements can be influenced be many factors, such as age, body size, environment, interacting medications, and genetic polymorphisms, thereby contributing to the interindividual variability of warfarin dose response (9–11). Among these, genetic polymorphisms, and especially single nucleotide polymorphisms (SNPs), can affect the stable warfarin dose, and these polymorphisms modulate warfarin's pharmacodynamics and pharmacokinetics, including variants in genes coding for cytochrome P450 2C9 (CYP2C9) and vitamin K epoxide reductase complex 1 (VKORC1) (12,13). Therefore, to reduce the risk of overanticoagulation and bleeding complications, many warfarin dosing algorithms have been developed to predict warfarin dose (14,15). A clinical trial confirmed that using the warfarin dose predicted by pharmacogenetics is superior to the standard warfarin dose in establishing and maintaining anticoagulation therapy, thereby reducing the risk of adverse events (16).

Gamma-glutamyl carboxylase (GGCX) is an enzyme that affects the metabolism of warfarin by catalyzing the

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biosynthesis of vitamin K-dependent clotting factors. Warfarin exerts its anticoagulant effects by inhibiting the regeneration of a reduced form of vitamin K, which is essential for γ-carboxylation and activation of vitamin K-dependent clotting factors (factors II, VII, IX, and X) (17). It has been reported that genetic polymorphisms in the GGCX gene can influence the variability of warfarin dose response in the general population (18–20). In this study, we aimed to investigate the contribution of GGCX polymorphism rs699664 to warfarin dose requirement in AF patients and to develop and evaluate a warfarin dosing algorithm based on the GGCX genotype in AF patients.

2. Materials and methods

2.1. Study population

The study population consisted of 275 AF patients from January 2014 to March 2016 at Shanghai Seventh People's Hospital. All the patients were Han Chinese individuals who lived in Shanghai in eastern China. The inclusion criteria for study subjects were as follows: 1) patients were ≥18 years old and had received a stable maintenance dose of warfarin therapy for at least 3 months; 2) a balanced diet was followed in each case and smoking and drinking were forbidden; 3) patients did not take any medications that might interfere with the pharmacokinetics or pharmacodynamics of warfarin; 4) patients had no hepatic or renal impairments according to laboratory tests; 5) during the warfarin therapy, patients showed no hemorrhage or thrombosis complications. Clinical data were collected from each AF patients on age, sex, height, body weight, left atrial size, ejection fraction, and maintenance dose of warfarin. On arrival at the hospital, plasma and blood samples were obtained. All 275 AF patients were divided into a discovery cohort (215 cases) and a replication cohort (60 cases). The discovery cohort was used to investigate the effect of GGCX genotype on warfarin dose and produce the dose algorithm for estimating the warfarin dose. The replication cohort was used to evaluate the feasibility of the warfarin dosing algorithm in AF patients. All patients provided written informed consent for study participation. This study was approved by the Ethics Committee of Shanghai Seventh People's Hospital. All study procedures were conducted in accordance with the Declaration of Helsinki.

2.2. GGCX genotyping

Peripheral venous blood samples were obtained from AF patients and genomic DNA was extracted from leukocytes with a DNA extraction kit (QIAGEN, Crawley, UK). The polymorphisms of CYP2C9 (rs1057910), VKORC1 (rs9923231), and GGCX (rs699664) were determined by polymerase chain reaction (PCR) and direct sequencing. PCR primers were designed using Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) and were synthesized by Shanghai Sangon Biological Engineering Co., Ltd. (Table 1). PCR amplification was performed in a C1000 Touch Thermal Cycler PCR instrument (Bio-Rad, Hercules, CA, USA) in a final volume of 20 μL, containing 0.25 μM primer, 0.1 mM deoxynucleoside triphosphate (dNTP), 0.625 U Taq polymerase (Molzyme, Bremen, Germany), and 0.5 μg genomic DNA. Thermocycling consisted of initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 40 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s, with a final extension of 72 °C for 10 min. The amplified PCR products were purified and sequenced by Shanghai Sangon Biological Engineering Co., Ltd.

2.3. Statistical analysis

Continuous variables were expressed as medians and interquartile ranges. Categorical variables were expressed as frequencies and percentages. The statistical analysis was performed with SPSS 19.0 (IBM Corp., Armonk, NY, USA). The Wilcoxon–Mann–Whitney test was used to compare continuous data and the chi-square test or Fisher's exact test was used to compare categorical data. Multivariate linear regression was performed to develop a novel warfarin dosing algorithm. Spearman's rank correlation test was performed to analyze the correlation between actual warfarin dose and warfarin dose predicted by the algorithm. $P < 0.05$ was considered statistically significant.

Table 1. PCR primers for CYP2C9, VKORC1, and GGCX.

Variant	Primers (5' to 3')
CYP2C9 rs1057910	Forward: CACGAGGTCCAGAGATACA
	Reverse: GGAATGAGATAGTTTCTGAATTTAAT
VKORC1 rs9923231	GCCAGCAGGAGAGGGAAATA
	AGTTTGGACTACAGGTGCCT
GGCX rs699664	AGTGGCCTCGGAAGCTGGT
	ACACAGGAAACACTGGGCTGAG

3. Results

3.1. Patient characteristics

The discovery cohort consisted of 215 AF patients with stable control of anticoagulation and was used to investigate the association between GGCX genotype and warfarin dose, and the replication cohort consisted of 60 patients to confirm the efficacy of the warfarin dosing algorithm. There were no statistically significant differences between the discovery cohort and replication cohort in age, sex, height, weight, left atrial size, ejection fraction, INR, or warfarin dose requirements.

3.2. GGCX genotyping

All 215 samples were genotyped for GGCX (rs699664), as this polymorphism was previously reported to affect warfarin dose requirements. There were 104 (48.4%) subjects with the homozygous GG genotype, 92 (42.8%) subjects with the heterozygous GA genotype, and 19 (8.8%) subjects with the homozygous AA genotype (Table 2). The observed genotype frequency of GGCX (rs699664) showed no deviation from Hardy–Weinberg equilibrium.

3.3. Associations of warfarin dose with GGCX genotype

The median warfarin daily dose requirement was 3.0 ± 0.79 mg in heterozygous GGCX GA patients, which was significantly higher than that in homozygous wild-type GGCX GG patients (2.7 ± 0.77 mg, $P < 0.05$). Furthermore, the median warfarin daily dose requirement was 3.7 ± 0.87 mg in patients with the GGCX AA genotype, which was significantly higher than that in patients with the GG or GA genotype ($P < 0.05$) (Figure 1). We also divided the discovery cohort into a low warfarin group and a high warfarin group according to the median warfarin dose (2.9 mg/day). The high warfarin group showed a significantly higher frequency of GA and AA genotypes as well as younger age and taller height compared with the low warfarin group (Table 2).

Table 2. Clinical demographics of the discovery cohort and replication cohort.

Characteristics	Discovery cohort (n = 215)	Discovery cohort (<2.9 mg) (n = 99)	Discovery cohort (≥2.9 mg) (n = 116)	Replication cohort (n = 60)
Age (years) †	56 (47–66)	59 (52–69)	52 (43–62) *	54 (45–63)
Sex #	136 (63.3%)	60 (60.6%)	76 (65.5%)	35 (58.3%)
Height (cm) †	170 (161–175)	170 (159–173)	172 (162–176) *	168 (158–175)
Weight (kg) †	66 (54–72)	66 (55–71)	67 (54–73)	64 (57–72)
Left atrial size (mm) †	46 (43–51)	47 (41–52)	46 (43–51)	48 (42–57)
Ejection fraction (%) †	49.3 (44.8–57.4)	51.4 (44.8–61.1)	49.3 (44.9–57.0)	51.1 (47.0–57.4)
INR †	2.8 (2.2–3.2)	2.6 (1.8–3.2)	2.8 (2.6–3.2)	2.7 (2.2–3.0)
Warfarin dose (mg) †	2.9 (2.5–3.3)	2.5 (2.3–2.6)	3.3 (3.1–3.7)	2.9 (2.4–3.2)
CYP2C9 #				
*1*1	198 (92.1%)	85 (85.9%)	113 (97.4%)*	54 (90.0%)
*1*3	17 (7.9%)	14 (14.1%)	3 (2.6%)	6 (10.0%)
VKORC1 #				
AA	158 (73.5%)	87 (87.9%)	71 (61.2%)*	49 (81.7%)
AG	52 (24.2%)	11 (11.1%)	41 (35.3%)	10 (16.7%)
GG	5 (2.3%)	1 (1.0%)	4 (3.4%)	1 (1.7%)
GGCX #				
GG	104 (48.4%)	59 (59.6%)	45 (38.8%)*	28 (46.7%)
GA	92 (42.8%)	37 (37.4%)	55 (47.4%)	26 (43.3%)
AA	19 (8.8%)	3 (3.0%)	16 (13.8%)	6 (10.0%)

Categorical variables are expressed as frequency (%) and analyzed by chi-square test; † continuous variables are expressed as medians (25th to 75th percentiles) and analyzed by Mann–Whitney U test.
*Compared with the discovery cohort group, the difference is significant ($P < 0.05$).

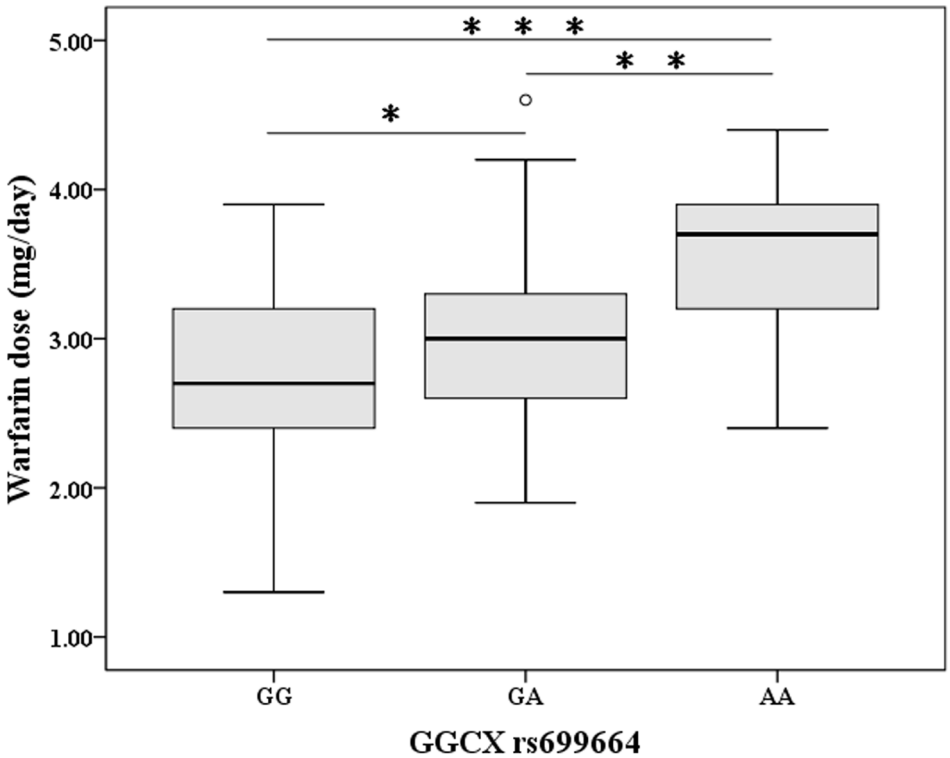

Figure 1. Warfarin dose in different GGCX genotypes. In box plots, bold black line indicates the median per group, the box represents interquartile ranges (25%–75%) of the values, and horizontal lines indicate minimum and maximum values; open circles indicate outlier values. The Wilcoxon–Mann–Whitney test was performed. *P < 0.05, **P < 0.01, ***P < 0.001.

3.4. Warfarin dosing algorithm

We performed multivariate linear regression analysis to detect variables that influence the warfarin dose. Results showed that age, height, CYP2C9, VKORC1, and GGCX genotype could develop models for estimating the warfarin dose (adjusted $R^2 = 0.412$) (Table 3). To verify the accuracy of this algorithm, we used Spearman's rank correlation test in the replication cohort of 60 AF patients and showed a significant correlation between the calculated warfarin dose using the warfarin dosing algorithm and actual dose ($R = 0.660$; $P < 0.001$) (Figure 2).

4. Discussion

In this study, we investigated the genotype of GGCX rs699664 in a discovery cohort of 215 AF patients, and the results showed that there were 104 (48.4%) cases of

Table 3. Regression equation for modeling warfarin daily dose requirements based on age, height, and genotypes.

X variables	Standardized coefficient	Adjusted R^2	P
Age	–0.008	0.029	0.002
Height	0.009	0.011	0.029
CYP2C9	–0.620	0.070	<0.001
VKORC1	0.439	0.172	<0.001
GGCX	0.257	0.130	<0.001

Regression equation: dose = 1.726 – 0.008 (age) + 0.009 (height) – 0.620 (CYP2C9) + 0.439 (VKORC1) + 0.257 (GGCX). Adjusted $R^2 = 0.412$.

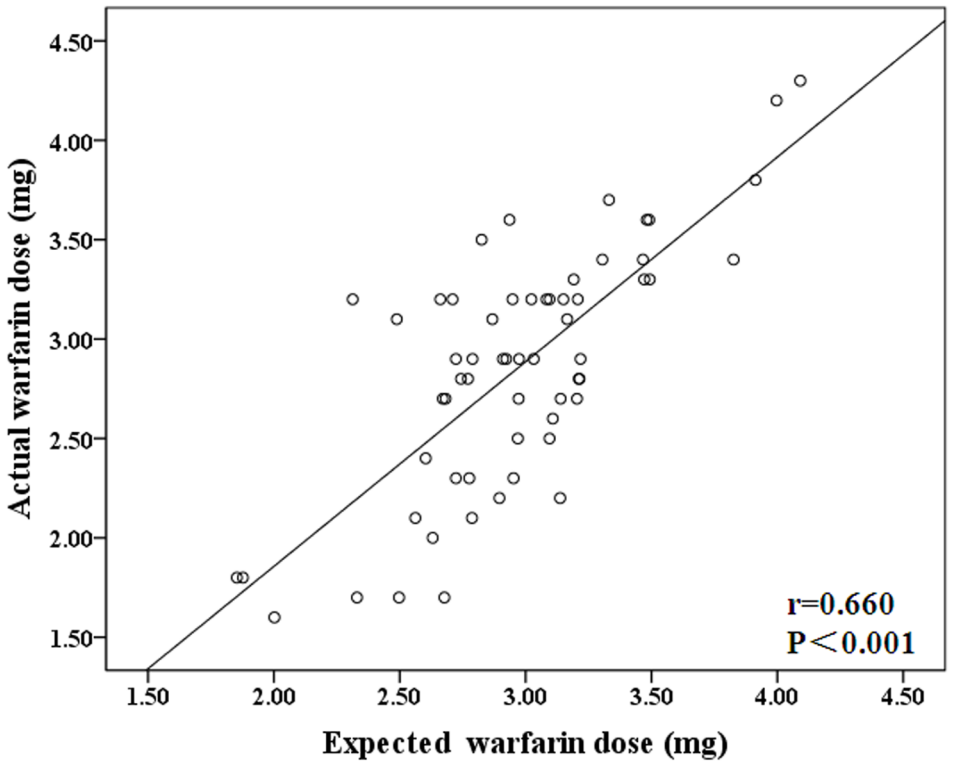

Figure 2. Correlation of calculated warfarin dose using warfarin dosing algorithm and actual dose in the replication cohort of AF patients. Each patient within the replication cohort is represented by a circle.

wild-type homozygous GG genotype, 92 (42.8%) cases of heterozygous GA genotype, and 19 (8.8%) cases of homozygous AA genotype. Patients carrying the GGCX rs699664 A allele (GA or AA genotype) had significantly higher warfarin dose requirements than those with the GG genotype ($P < 0.05$). A multivariate linear regression model was used to develop a warfarin dosing algorithm and showed that age, height, CYP2C9, VKORC1, and GGCX genotype were the best variables for estimating warfarin dose ($R^2 = 41.2\%$). Another independent replication cohort of 60 AF patients showed a significant linear correlation between actual warfarin dose and warfarin maintenance dose as predicted by the warfarin dosing algorithm ($R = 0.660$, $P<0.01$).

GGCX is a vitamin K-dependent carboxylase and could promote the biosynthesis of vitamin K-dependent clotting factors (21,22). GGCX resides in the endoplasmic reticulum membrane and oxidizes reduced vitamin K to vitamin K-2,3-epoxide though carboxylation of the gamma carbon on glutamic acids, thus producing functional vitamin K-dependent clotting factors II, VII, IX, and X (23,24). GGCX-knockout mice die from massive hemorrhaging at birth (25). Patients with defective GGCX have elevated INR values (26). The GGCX gene is located on human chromosome 2p12 and consists of 15 exons (27,28). Over the past decades, extensive studies have investigated the association between GGCX polymorphisms and warfarin maintenance dose requirements (17–20). The common GGCX polymorphisms include GGCX rs11676382, rs12714145, rs10654848, and rs699664. GGCX rs11676382 (C>G) SNP is located in intron 14 and correlated with lower warfarin dose requirements in Caucasians (20,29). The GGCX rs12714145 (3261G>A) SNP is located in intron 2 and subjects with the AA genotype have significantly higher warfarin dose requirements compared with the GG genotype in Chinese patients (30). The GGCX rs10654848 microsatellite (CAA repeats) is located in intron 6 and correlated with higher warfarin dose requirements in Caucasians and African Americans (31,32). GGCX rs699664 is defined by a G-to-A nucleotide substitution (8016G>A), thus leading to a nonsynonymous substitution and a change from arginine to glutamine at amino acid position 325 in exon 8. It is correlated with higher warfarin dose requirements in Japanese and Chinese patients (33,34). However, rs699664 was not associated with warfarin dose in Caucasians or African Americans (31,32), indicating the ethnic differences in warfarin metabolism. In this study, we found that the GGCX rs699664 polymorphism was associated with higher warfarin dose requirements in

Chinese AF patients, suggesting that it may be a potential predictor for warfarin maintenance dose.

We found a significant association of warfarin dose with CYP2C9 and VKORC1 genotype, as evidenced by differences in the frequencies of CYP2C9 rs1057910 and VKORC1 rs9923231 between the low warfarin dose group and high warfarin dose group. CYP2C9 is one isoform of the cytochrome P450 complex and metabolizes approximately 15% of the clinically used drugs (35). Among the various variants of CYP2C9 being identified, CYP2C9*3 is the most common allele (rs1057910). Compared with the wild-type CYP2C9*1, CYP2C9*3 is defined by an A-to-C nucleotide substitution (1075 A>C), thus leading to a nonsynonymous substitution and a change from leucine to isoleucine at amino acid position 359 in exon 7. The CYP2C9*3 allele could reduce the activity of CYP2C9 and decrease the metabolism and clearance capability of warfarin, thus enhancing the sensitivity to warfarin and reducing warfarin dose requirements. Our previous report demonstrated that AF patients with the CYP2C9 *1*3 genotype had lower stable warfarin daily doses compared with patients with wild-type CYP2C9 *1*1 (36). VKORC1 is responsible for the biosynthesis of vitamin K-dependent coagulation factors (factors II, VII, IX, and X) through transformation of epoxide (vitamin K-2,3-epoxide) to a reduced form of vitamin K and gamma-carboxylation of the vitamin K-dependent coagulation factors (37). Warfarin inhibits VKORC1-catalyzed gamma-carboxylation of coagulation factors, thus preventing the regeneration of the reduced form of vitamin K (38). Mutations in the VKORC1 gene can lead to warfarin resistance, which might enhance warfarin dose requirements (39,40). VKORC1 rs9923231 (-1639 G>A) is the most common SNP of the VKORC1 gene. Compared with Caucasian populations, Chinese populations have higher A allele rates and the AA genotype frequency is above 83% (41). In this study, the VKORC1 rs9923231 AA genotype frequency was significantly lower in the high warfarin group compared with the low warfarin group. Our results are in accordance with another report finding that a higher warfarin dose is needed in subjects with the GG genotype than subjects with the AG or AA genotype (42).

In this study, we found that there was a significant association between GGCX rs699664 polymorphism and sensitivity to warfarin in Chinese AF patients, suggesting that it may be a useful predictor of warfarin maintenance dose. Therefore, we developed a dosing algorithm by multivariate linear regression analysis and showed that variables such as age, height, CYP2C9, VKORC1, and GGCX genotype can be used to estimate the warfarin maintenance dose. To evaluate the accuracy of this dosing algorithm, we applied Spearman's rank correlation test to an independent replication cohort of 60 AF patients and showed a significant correlation between the actual warfarin dose and the warfarin dose predicted by the dosing algorithm ($R = 0.660$; $P < 0.001$). This indicates that GGCX rs699664 participates in warfarin dose variation and it could be added to the warfarin dosing algorithm following CYP2C9 and VKORC1.

Though we found that the CYP2C9 rs1057910, VKORC1 rs9923231, and GGCX rs699664 genotypes were correlated to warfarin dose requirements, there are some limitations in our study. The sample size is small and all of the AF patients included in the study were limited to Han Chinese individuals of East China. Therefore, further studies with a larger sample size and different populations are required to confirm the results. Furthermore, the mechanism by which GGCX rs699664 influences the warfarin dose requirements remains unknown. Based on higher warfarin dose requirements in subjects with the AA genotype, it seems plausible that the A allele may enhance GGCX enzyme activity and promote carboxylation of clotting factors, thereby leading to warfarin resistance and presumably enhancing warfarin dose requirements. However, the detailed mechanism remains to be investigated.

In conclusion, this study provides evidence that patients with the GGCX rs699664 AA genotype required higher warfarin doses in the Chinese AF patient population. The GGCX rs699664 genotype might help clinicians to guide warfarin dosage by a new warfarin dosing algorithm in AF patients. However, further studies are needed to confirm this study with a larger sample size and different populations.

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