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ORIGINAL ARTICLE

The relationship between on-clopidogrel platelet reactivity, genotype, and post-percutaneous coronary intervention outcomes in Chinese patients

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Abstract

Background. High on-clopidogrel platelet reactivity reflects a poor response to clopidogrel and is associated with ischemic events, which has been attributed to several factors such as demographic, clinical characteristics and a polymorphism of *CYP2C19*. Some new platelet assays monitoring on-clopidogrel platelet reactivity are currently available in China, but their relevance to the *CYP2C19* genotype and post-percutaneous coronary intervention outcomes remain to be elucidated. ***Methods.*** Patients were prospectively included if they had a successful percutaneous coronary intervention (PCI) for acute coronary syndrome (ACS) and received clopidogrel and aspirin. *CYP2C19* loss-of function genotype, adenosine diphosphate (ADP)-induced maximum platelet aggregation rate (MPA_{ADP}) measured by light transmittance aggregometry, ADP-induced platelet-fibrin clot strength (MA_{ADP}) measured by thrombelastography, platelet reactivity index (PRI) measured by vasodilator-stimulated phosphoprotein phosphorylation (VASP), and the occurrence of 6-month major adverse cardiovascular events (MACE) were assessed in 178 patients. ***Results.*** High on-treatment platelet reactivity prevalence defined by $MPA_{ADP} > 46.0\%$, $MA_{ADP} > 47$ mm and $PRI > 50.0\%$ was 27.0%, 24.2%, and 61.2%, respectively. ADP-specific assays (VASP PRI) differed according to *CYP2C19* genotype, with a significant gene–dose effect (PMs > IMs > EMs, $p < 0.05$). Multivariate analysis showed $MPA_{ADP} > 46.0\%$ and $MA_{ADP} > 47$ mm to be independent predictors of MACE at 6 months. ***Conclusions.*** *CYP2C19* loss-of function genotypes with the **2* and/or **3* allele are highly prevalent in the Chinese population and are associated with higher residual platelet reactivity. High on-treatment platelet reactivity defined by MPA_{ADP} or MA_{ADP} predicts an increased risk of MACE for ACS patients undergoing PCI.

Key Words: *Acute coronary syndrome, clopidogrel,* CYP2C19*, platelet function assay*

Introduction

Dual antiplatelet therapy using aspirin and clopidogrel is the common management strategy for patients undergoing percutaneous coronary intervention (PCI) [1]. Active metabolite of clopidogrel irreversibly binds and inhibits the adenosine diphosphate (ADP) receptor P2Y12 on the surface of platelets, however platelet response to clopidogrel treatment is highly variable for clinical, cellular and genetic factors [2]. In reality, despite apparently adequate antiplatelet treatment approximately 10% of patients experience recurrent ischaemic events. Several studies have demonstrated a strong association between high on-treatment platelet reactivity (HTPR) after a clopidogrel loading dose, as measured by various platelet assays, and post-PCI thrombotic events such as cardiovascular death, myocardial infarction, or stent thrombosis [3]. The platelet assays available in China include platelet aggregation tests such as light transmittance aggregometry (LTA), vasodilator-stimulated phosphoprotein (VASP) analysis, and thromboelastography (TEG). The elevated ADP-induced maximum platelet aggregation rate (MPA_{ADP}) determined by LTA indicates the low response to clopidogrel [2]; VASP is measured by whole blood flow cytometry and provides result as platelet reactivity index (PRI), which specifically links to P2Y12 ADP receptor inhibition [4]; ADP-induced platelet-fibrin clot strength (MA_{ADP}) is measured by TEG in way of adding ADP and fibrinogen activator into whole blood, and reflects the activities of platelet and fibrinogen [5]. These parameters have

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been demonstrated successively to be risk factors for post-PCI ischemic events in Western populations [4–7].

The highly polymorphic cytochrome P450 (CYP) system of the liver plays a key role in variable formation of the active metabolite of clopidogrel [2]. Specifically, carriers of the **2* loss-of-function allele of *CYP2C19* generate less amounts of the active metabolite of clopidogrel, a two-step oxidative process required for transformation of clopidogrel to its labile active metabolite, in which *CYP2C19* contributes substantially to each step resulting in a decreased antiplatelet effect [8]. Several studies using a candidate gene approach have identified associations between reduced-function variants of the *CYP2C19* isozyme and on-treatment platelet reactivity while receiving clopidogrel [9–13]. However the influence of the *CYP2C19* genotype on clinical outcomes after PCI in clopidogrel-treated patients was not supported in a recent multi-center study [14] and systematic review [15]. These *CYP2C19* loss-of-function genotypes are more frequent in Asian than Caucasian populations [16–19], so this polymorphism and related on-treatment platelet reactivity may have different effects on post-PCI outcomes in Chinese patients.

In the current study, we used *CYP2C19* genotyping with the above-mentioned platelet assays to compare and determine the value of these indices for predicting major adverse cardiovascular events (MACE) after PCI in Chinese acute coronary syndrome (ACS) patients.

Methods

Study population

Between December 2012 and October 2013, a total of 207 patients admitted for ACS who underwent successful PCI and received clopidogrel therapy at Tongji Hospital (Wuhan, China) were prospectively included in this trial. This study was approved by the Ethics Committee of Tongji Hospital, and written informed consent was obtained from all patients or their family members. Major exclusion criteria were a bleeding diathesis or a history of gastrointestinal bleeding, hemorrhagic stroke, surgery within the past month, anticoagulant therapy, a platelet count $< 100 \times 10^9$/L, and age $<$ 18 years or $>$ 75 years.

A total of 185 patients were already on maintenance therapy of a 75 mg daily dose of clopidogrel at the time of PCI; therefore, the remaining 22 patients were loaded with 300 mg clopidogrel prior to PCI. On the day of PCI, patients also received 100 mg of aspirin. Aspirin (100 mg daily) and clopidogrel (75 mg daily) were prescribed for all patients following PCI.

In 171 patients not treated with eptifibatide, blood samples were obtained 18–24 h post-PCI, whereas samples were collected 5 days post-PCI in those patients treated with eptifibatide to reduce the antiplatelet influence of the drug. Blood samples were drawn by venipuncture into two Vacutainer® tubes (Becton-Dickinson, Franklin Lakes, NJ, USA) containing 3.2% trisodium citrate and one Vacutainer® tube containing 40 USP lithium heparin.

Platelet function assays

To measure MPA_{ADP}, platelet-rich plasma was prepared by centrifugation at 120 *g* for 10 min at room temperature. Samples with platelet counts $> 400 \times 10^9$/L were adjusted to 250×10^9/L to standardize the aggregation study by adding homologous platelet-poor plasma obtained by centrifugation of the blood at 1500 *g* for 10 min. ADP (5.0 μmol/L)-induced platelet aggregation was monitored turbidimetrically at 37°C using an aggregometer (AggRAM, Helena Laboratories Inc, Beaumont, TX, USA). The MPA_{ADP} response was quantified as the maximum extent of aggregation. HTPR was defined as 5 μM ADP-induced MPA $>$ 46.0% [2].

The VASP phosphorylation analysis was performed within 24 h of blood collection by an experienced investigator using Platelet VASP kits (Diagnostica Stago, Asnières, France) according to the manufacturer's instructions. Flow cytometric analysis was performed using a FACS caliber cytometer (Becton Dickinson). Briefly, citrated blood samples were incubated with prostaglandin E1 (PGE1) or with PGE1 and 10 μM ADP for 10 min and fixed with paraformaldehyde, after which the platelets were permeabilized with non-ionic detergent. The platelet population was identified on its forward and side scatter distributions, and 10,000 platelet events were gated and analyzed for mean fluorescence intensity (MFI). A PRI VASP index was calculated from the MFI of samples incubated with PGE1 or PGE1 and ADP according to the formula: $PRI = [(MFI_{(PGE1)} - MFI_{(PGE1+ADP)}) / MFI_{(PGE1)}] \times 100$, and expressed as a percentage of platelet reactivity. PRI $>$ 50.0% was suggested to represent HTPR according to the consensus document [2,4].

Platelet-fibrin clot strength measurements were performed using the TEG® Hemostasis System (Haemoscope Corporation, Niles, IL, USA). The TEG® Hemostasis Analyzer with automated analytical software provides viscoelastic quantitative and qualitative measurements of the physical properties of a clot. Briefly, a stationary pin is suspended into an oscillating cup containing the entire blood sample. As the blood clots, it links the pin to the cup. Clot strength is determined by measuring the amplitude of the rotation of the pin, which increases propor-

tionally with clot strength. Maximum amplitude represents maximum clot strength, expressed as the MA parameter. MA_{ADP} was obtained by adding ActivatorF™ (to replace thrombin in converting fibrinogen into fibrin) and ADP into the heparinized sample, and reflects the activities of platelet and fibrinogen. $MA_{ADP} > 47$ mm has been considered a good predictor of long-term ischemic events, so we adopted it as a criterion of HTPR [5].

CYP2C19 *genotyping*

Microarray technology was used for *CYP2C19* genotyping. *CYP2C19* polymorphisms were analyzed for the nonfunctional alleles **2* and **3* by identifying two polymorphic positions: 681G > A in exon 5 and 636G > A in exon 4, respectively. PCR and subsequent melting curve analysis were performed using the Lightcycler® device (Roche Applied Science, Mannheim, Germany) and associated software. Control samples confirmed by sequencing were included in each run. The microarray assay was performed using the BR-526-6 automated hybridization system, BE2.0 biochip reader, and related reagents (Baio Technology, Shanghai, China). The following genotypes were determined: *CYP2C19*1/*1* (extensive metabolizer: EM), *CYP2C19*1/*2* (intermediate metabolizer: IM), *CYP2C19*1/*3* (IM), *CYP2C19*2/*2* (poor metabolizer: PM), *CYP2C19*2/*3* (PM), and *CYP2C19*3/*3* (PM).

Clinical endpoints

Patients were followed for the occurrence of adverse events during index hospitalization and for up to 6 months because normally clopidogrel cannot be administered for as long as 1 year to all patients in China. Patients were contacted either by telephone or through an appointment to determine the event occurrence and compliance with antiplatelet drug therapy. The primary clinical endpoint was the composite of cardiovascular death, non-fatal myocardial infarction, stent thrombosis, and unplanned revascularization. cardiovascular death was considered to be any death with a demonstrable cardiovascular cause or any death not clearly attributable to a non-cardiovascular cause; myocardial infarction was defined as ischemic symptoms with electrocardiographic abnormalities, and upper limits of normal troponin [20]; stent thrombosis was defined as definite stent thrombosis according to the Academic Research Consortium [21]; revascularization included either PCI or coronary artery bypass grafting. Independent physicians blinded to laboratory data adjudicated events after reviewing source documents.

Statistical analysis

A normal distribution of laboratory assessments was confirmed by the Kolmogorov-Smirnov test. Continuous variables were expressed as means ± SD or as medians [interquartile ranges], as appropriate, while categorical variables were reported as frequencies and percentages. Comparisons between continuous variables were performed using the Student's *t*-test or Mann-Whitney test. Comparisons between categorical variables were evaluated using Fisher's exact test or Pearson's Chi-squared test, where appropriate. Categorical variables including all baseline characteristics, platelet reactivity, and *CYP2C19* genotype were evaluated by univariate analysis for their ability to predict MACE within 6 months. Variables with $p < 0.10$ were then entered into the multivariate logistic regression analysis providing odds ratios (OR) and 95% confidence intervals (95% CI). *P*-values < 0.05 were considered statistically significant, and statistical analyses were performed using SPSSv18.0 software (SPSS Inc., Chicago, IL, USA).

Results

Relationship between platelet function parameters and CYP2C19 *genotype*

The prevalence of HTPR determined by LTA ($MPA_{ADP} > 46.0\%$), TEG ($MA_{ADP} > 47$ mm), and VASP (PRI > 50.0%) was 27.0%, 24.2%, and 61.2%, respectively. Patients were divided into three groups according to their *CYP2C19* genotype: 94 (45.4%) EMs (*CYP2C19*1/*1*), 84 (40.6%) IMs (*CYP2C19*1/*2* and *CYP2C19*1/*3*), and 29 (14.0%) PMs (*CYP2C19*2/*2*, *CYP2C19*2/*3*, and *CYP2C19*3/*3*). The relationship between the *CYP2C19* genotype and various platelet reactivity tests was then analyzed (Figure 1). Only VASP PRI increased according to the degree of *CYP2C19* loss-of-function genotype [PMs (73.2 ± 8.9%) > IMs (60.4 ± 13.7%) > EMs (49.0 ± 17.5%)], indicating an obvious gene-dose effect. MPA_{ADP} in PMs (46.0 ± 23.0%) was significantly higher than in IMs (30.6 ± 17.2%) and EMs (32.1 ± 19.3%), although there was no significant difference between the latter two. MA_{ADP} in PMs (51.9 ± 13.0 mm) was significantly higher than in EMs (37.7 ± 13.7 mm), but no significant difference was found between IMs (44.5 ± 12.1 mm) and PMs or IMs and EMs.

Association between patient characteristics, laboratory parameters, and 6-month outcome

The characteristics and outcomes of 178 patients were followed for 6 months (Table I); the remaining 29 patients did not adhere to their antiplatelet regime or were lost to follow-up. MACE occurred in a total

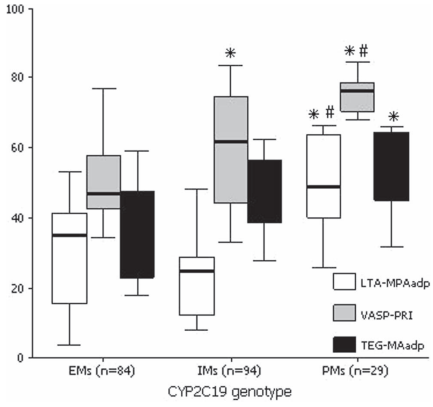

Figure 1. Effect of *CYP2C19* genotype on platelet reactivity as assessed by LTA-MPA_{ADP}, VASP-PRI, and TEG-MA_{ADP}. Box and whisker plots show median (horizontal lines), 25th and 75th percentiles (box), and 10th and 90th percentiles (error bars). EMs, extensive metabolizers; IMs, intermediate metabolizers; PMs, poor metabolizers; LTA, light transmittance aggregometry; VASP, platelet vasodilator-stimulated phosphoprotein; TEG, thromboelastography. $^{*}p < 0.05$ compared with EMs; $^{\#}p < 0.05$ compared with IMs.

of 21 patients (11.8%) during the 6-month follow-up period. There were five cases of cardiovascular death, 13 cases of non-fatal myocardial infarction, and three cases of a repeat PCI. Patients with MACE had a lower angiotensin-converting enzyme inhibitor use and higher on-treatment platelet reactivity, as determined by MPA_{ADP} and MA_{ADP}, compared with patients without MACE ($p < 0.05$).

Platelet function parameters as well as patient and procedural characteristics with $p < 0.10$ (PMs of *CYP2C19* genotype, diabetes, and use of angiotensin-converting enzyme inhibitors and statins) from the univariate analysis were examined in a multivariate logistic regression model to identify independent predictors of MACE at the 6-month time-point (Table I). When adjusted for patient and procedural covariates, HTPR defined by $MPA_{ADP} >$ 46.0% or $MA_{ADP} >$ 47 mm was independently and significantly associated with MACE, with ORs of 5.99 (95% CI 2.00–17.96) and 4.72 (95% CI 1.27–19.67), respectively (Table II). With the exception of angiotensin-converting enzyme inhibitor use, all other variables were excluded from the model. The positive and negative predictive values of HTPR defined by these platelet function parameters and *CYP2C19* loss-of-function genotypes for MACE are shown in Table III.

Discussion

To the best of our knowledge, this is the first study to simultaneously evaluate the efficiency of various platelet function assays and *CYP2C19* genotyping in Chinese post-PCI patients. According to the cut-off values of HTPR suggested by previous studies, we found that patients with MACE had a significantly higher MPA_{ADP} and MA_{ADP} ($p < 0.05$) than those without, and that both $MPA_{ADP} > 46.0\%$ and $MA_{ADP} > 47$ mm were independent predictors of MACE at the 6-month time-point ($p = 0.001$ and

Table I. Patient characteristics according to the presence of MACE.

	Total group ($n = 178$)	MACE group ($n = 21$)	Non-MACE group ($n = 157$)	p
Age, year	61.8 ± 9.1	62.5 ± 8.8	61.1 ± 9.4	0.390
Sex (F/M)	56/122	4/17	52/105	0.241
Risk factors/ Previous history, *n* (%)				
Smoking	78 (43.8)	7 (33.3)	71 (45.2)	0.344
Diabetes	26 (14.6)	6 (28.6)	20 (12.7)	0.097
Hyperlipidemia	10 (5.6)	2 (9.5)	8 (5.1)	0.334
Hypertension	104 (58.4)	12 (57.1)	92 (58.6)	0.783
Previous mycardial infarction	4 (2.2)	1 (4.8)	3 (1.9)	0.398
Previous stroke	10 (5.9)	1 (4.8)	9 (5.7)	1.000
Baseline medications, *n* (%)				
Statin	159 (89.3)	16 (76.2)	143 (91.0)	0.092
Proton-pump inhibitors	103 (58.0)	14 (66.7)	89 (56.7)	0.430
ACE inhibitors	109 (61.2)	6 (28.6)	103 (66.0)	0.002
Calcium-channel blockers	10 (5.3)	1 (4.8)	9 (5.7)	0.963
β blockers	139 (78.1)	14 (66.7)	125 (79.6)	0.214
MPA_{ADP} (%)	32.4 ± 18.9	52.9 ± 19.2	29.4 ± 18.7	0.002
VASP-PRI (%)	59.7 ± 17.9	65.6 ± 22.2	57.6 ± 17.8	0.328
MA_{ADP} (mm)	34.5 ± 16.6	43.5 ± 20.6	33.0 ± 15.2	0.021
CYP2C19 genotype, *n* (%)				
EM	82 (46.1)	7 (33.3)	75 (47.8)	0.213
IM	70 (39.3)	8 (38.1)	62 (39.5)	0.665
PM	26 (14.6)	6 (28.6)	20 (13.2)	0.054

MACE, major adverse cardiovascular events; ACE, angiotensin-converting enzyme; MPA_{ADP}, adenosine diphosphate (ADP)-induced maximum platelet aggregation rate; MA_{ADP}, ADP-induced platelet-fibrin clot strength; VASP-PRI, platelet reactivity index measured by vasodilator-stimulated phosphoprotein phosphorylation; EM, extensive metabolizer; IM, intermediate metabolizer; PM, poor metabolizer.

Table II. Multivariate predictors of MACE at 6-months follow-up.

	Multivariate analysis	
	Odds ratio (95% CI)	p
$MA_{ADP} >$ 47 mm	4.72 (1.27–19.67)	0.013
Angiotensin-converting enzyme inhibitors	3.33 (1.13–22.40)	0.045
$MPA_{ADP} > 46\%$	5.99 (2.00–17.96)	0.001
Angiotensin-converting enzyme inhibitors	4.10 (1.27–13.18)	0.018

MACE, major adverse cardiovascular events. MPA_{ADP}, adenosine diphosphate (ADP)-induced maximum platelet aggregation rate; MA_{ADP}, ADP-induced platelet-fibrin clot strength.

Table III. Positive and negative predictive values of platelet parameters.

	Positive predictive values	Negative predictive values
CYP2C19 poor metabolizer	23.1%	90.1%
$MPA_{ADP} > 46\%$	33.3%	97.6%
VASP-PRI > 50%	11.6%	95.2%
$MA_{ADP} > 47$ mm	31.6%	91.7%

MPA_{ADP}, adenosine diphosphate (ADP)-induced maximum platelet aggregation rate; MA_{ADP}, ADP-induced platelet-fibrin clot strength; VASP-PRI, platelet reactivity index measured by vasodilator-stimulated phosphoprotein phosphorylation.

0.013, respectively). The high prevalence of *CYP2C19* loss-of-function genotypes and HTPR defined by VASP PRI > 50.0% were found in Chinese patients, which seemed to impair their specificity of predicting ischemic events, despite VASP PRI showing a good correlation with *CYP2C19* genotypes.

Previous findings suggested that polymorphisms affect the enzymatic activity of the CYP system, especially that of CYP2C19, and play important roles in the pharmacokinetic and pharmacodynamic effects of clopidogrel [9–11]. The prevalence of *CYP2C19* loss-of-function genotypes (IM + PM, 54.6%) identified in our study is similar to that observed in Japanese patients (60.0%) [22], and higher than that seen in Caucasians (28.0%) [16]. Although various degrees of correlation were observed between the *CYP2C19* genotype and each platelet function parameter in our study, *CYP2C19* polymorphisms together showed a poor predictive value for long-term MACE occurrence. Previous prospective studies [12,23] have observed that despite a statistically robust association of *CYP2C19*2* carrier status with HTPR, reduced-function *CYP2C19* allele carriage accounts for only 4.6–7.0% of the variability in on treatment platelet reactivity in the long-term phase of therapy at 6 months, possible explanation might be that medication, patient characteristics and other genomic conditions could play roles as well. Hence in patients who have undergone platelet function testing and are thereby determined to be at higher risk of ischemic events, additional *CYP2C19* genotyping seems providing little help, along with our observation, these findings seem not support the routine use of *CYP2C19* genotyping in Chinese populations.

LTA is the gold standard for evaluating platelet function, and, as such, is the most commonly used [2]. However, because of the complex sample preparation and lack of standardization for ADP-induced aggregometry, several new platelet function assays available in China have been used to evaluate on-treatment platelet reactivity and predict the outcome of patients receiving clopidogrel therapy. One of these is the whole blood assay that measures VASP phosphorylation. It is a reproducible, standardized flow cytometric analysis that does not appear to be affected by P2Y1 receptor inhibition or aspirin treatment, in comparison with LTA, samples for VASP analysis can be stored longer after collection and be convenient for transporting to a central lab. VASP analysis has also been shown to be highly specific for P2Y12 ADP receptor inhibition, and therefore for the effect of clopidogrel [24]. In our study, however, VASP PRI was not correlated with MACE, and 61.2% of patients had a PRI > 50.0% so could be considered 'poor responders'. The high proportion of HTPR defined by VASP PRI is consistent with previous studies [6,25], and may be partially attributed to the high prevalence of *CYP2C19* loss-of-function genotypes in the Chinese population. It is also likely to decrease the specificity of VASP for determining the 'true' HTPR after a clopidogrel loading dose. Judge et al. previously showed that the VASP assay is particularly discriminating of levels of $P2Y_{12}$ receptor blockade between 60.0–100.0% of PRI [26], while Bonello et al. observed that on-treatment platelet reactivity measured by VASP predicts both ischemic and bleeding events at a 1-year follow-up in patients receiving prasugrel [7]. This latter study used a 53.5% cut-off value to define HTPR, after which only 22.3% of patients were considered to have an insufficient on-treatment platelet reactivity inhibition after a prasugrel loading dose; this also emphasizes the important influence of a proportion of HTPR on the predictive value of VASP.

A study by Gurbel et al. indicated that MA_{ADP} is a strong predictor of long-term post-PCI ischemic event occurrence in the presence of P2Y12 inhibition, and that the MA_{ADP} cut-off of > 47 mm is well matched with the consensus-based HTPR cut-off of 5 μM ADP-induced MPA < 46.0% and VerifyNow P2Y12 assay (P2Y12 reaction units ≥ 235) [5]. Several other clinical studies also proposed that MA_{ADP} may be useful to predict the risk of serious bleeding or the need for transfusion in PCI patients [27,28]. In our study, higher MA_{ADP} was independently associated with MACE at the 6-month time-point. Moreover, the additional advantages associated with TEG, including the fact that it is a whole blood assay, that sample preparation is easy, and that results are typically obtained more quickly than with LTA and VASP, provide support for the future use of TEG in a variety of settings.

This study has a number of limitations that should be taken into account when examining the results. First, it was a small, single-center observational study, so our findings should be confirmed in an adequately powered trial. Second, because no related prospective studies of large sample sizes have been conducted in Chinese ACS patients as yet, the cut-off values used to define HTPR derive from studies in Western populations which may not be good fit for Chinese patients.

In conclusion, *CYP2C19* loss-of function genotypes with the **2* and/or **3* allele are highly prevalent in the Chinese population and appear to be associated with higher residual platelet reactivity. MPA_{ADP} measured by LTA and MA_{ADP} measured by TEG were shown to be the best parameters to predict the occurrence of MACE in Chinese ACS patients undergoing PCI.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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