Original Article

Relevance of Polymorphism of Glutathione S-Transferase Genes (GSTM1 and GSTT1) in Diabetic Retinopathy and Diabetic Nephropathy

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Abstract

Introduction: The causative factors of diabetic retinopathy and nephropathy are genetic as well as environmental. The Glutathione S-transferase gene family is involved in redox balance to reduce oxidative stress, which is an important factor associated with these major diabetic complications. The objective of this study was to investigate the association between the polymorphism of glutathione S-transferase genes (GSTM1 and GSTT1) and type 2 diabetes mellitus (T2DM) patients with diabetic retinopathy (DR) and diabetic nephropathy (DN). **Methods:** In this cross-sectional study, GSTM1 and GST1 gene polymorphisms were studied in T2DM patients with three groups of 125 cases each: the 1st group DM without DN and DR, the 2nd group with DR, and the 3rd group with DN from December 2019 to January 2023. Polymerase chain reaction (PCR) was performed on DNA. GSTM1 and GSTT1 genotyping was conducted using gel electrophoresis. Statistical analysis was performed using SPSS software. **Results:** Compared to the DM group, in the DR group, the GSTT1 null and GSTM1 null genotypes were more prevalent and showed 2.68-folds (OR = 2.68; 95% CI = 1.60–4.48; $P < 0.001$) and 2.5-folds (OR = 2.50; 95% CI = 1.50–4.18; $P < 0.001$) increased risk of developing DR respectively. In the DN group, the GSTM1 null genotype was more prevalent, with a 1.97-fold increased risk of developing DR (OR = 1.97, 95% CI = 1.19–3.26; $P = 0.008$) when compared to the DM group. However, no significant difference was found in the GSTT1 null genotype between the DN and DM groups. **Conclusion:** The significant association between null genotypes of both GSTT1 and GSTM1 with DR and only the GSTM1 null genotype with DN suggests their roles as risk factors.

Keywords: Diabetic nephropathy, diabetic retinopathy, genetic polymorphism, GSTM1, GSTT1, type 2 diabetes mellitus

Introduction

Among multifactorial diseases, type 2 diabetes mellitus (T2DM) is considered a serious public health problem worldwide due to the rise in the number of patients, along with an increase in medical and socioeconomic costs for the treatment of the disease and its associated complications, such as diabetic retinopathy (DR), diabetic nephropathy (DN), etc.[1] With 77 million people with diabetes, India is facing a global diabetic epidemic after China.[1] According to Indian studies, the prevalence of DR varied from 4.8%[2] to 21.7%.[3] The prevalence of DN spanned from 0.9%[4] to 62.3%.[5]

Oxidative stress is one of the most common and significant factors involved in the pathogenesis of diabetes and its complications.[6] Studies have shown that malfunctions in genes that interact with antioxidant defence mechanisms against oxidative stress may contribute to the etiology of diabetic complications.[7,8] The metabolic antioxidant glutathione (GSH), along with the enzyme glutathione S-transferase (GST), protects cells against damage caused by free radicals and environmental toxins.[9] GST has various isoforms, of which GSTT1 and GSTM1 play significant roles in detoxification and are vastly studied.[10,11]

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Earlier researchers studied GSTT1 and GSTM1 gene polymorphisms separately in the pathogenesis of DR or DN and obtained inconsistent results.[10-12] However, only a few studies have been conducted on DR and DN simultaneously in the Indian population. Therefore, this study investigated GSTT1 and GSTM1 gene polymorphisms in patients with DR and DN.

Materials and Methods

Selection of study population

The present study was done in the Department of Biochemistry, Ophthalmology, Medicine, and Molecular Biology and Genetic Laboratory in a tertiary care hospital and a medical research institute from December 2019 to January 2023. This was a cross-sectional observational study. Studies by Ines Cilenšek *et al*.,[13] and Yu Yang *et al*.,[14] were used for sample size calculation. A total of 375 patients with type 2 diabetes mellitus with an age of more than 35 years were included in three groups with 125 patients in each group. The first group (DM) included patients without DN and DR, the second group (DR) contained patients with DR, and the third group (DN) included patients with DN.

Inclusion criteria: For the first group - known cases of T2DM based on the criteria suggested by the American Diabetes Association,[15] DM for >10 years on treatment without DN and DR. For the second group - patients with DR with no evidence of DN. For the third group - patients with DN diagnosed by the Department of Nephrology, with no evidence of moderate or severe non-proliferative DR (NPDR) or proliferative DR (PDR).

Exclusion criteria: Age<35 years, Type 1 diabetic patients or any other type of diabetes, patients with liver disease, oncologic disease, thyroid disorders or other endocrine diseases, pregnant and lactating females, persons using antioxidant medications, tobacco users, and smokers. Patients with mild NPDR, retinal vascular occlusions, glaucoma, uveitis, or mature cataracts.

After an explanation of the study, patients who accepted to participate in the study were selected consecutively according to the selection criteria from the outpatient department. Information about general characteristics like age, sex, etc., history, and clinical examination findings was filled in a form for every individual after taking the interview and from their medical records.

A Full ophthalmic examination of patients was conducted. Diabetic retinopathy grading was performed using direct and indirect ophthalmoscopes by Ophthalmologists according to the ETDRS scoring system.[16] In 1st group, DM patients were selected based on no DR on fundus examination and urinary albumin excretion <30 µg/mg of creatinine. In 2nd group, DR patients were selected based on moderate NPDR/severe NPDR/PDR on fundus examination and urinary albumin excretion <30 µg/mg of creatinine. In 3rd group, DN patients diagnosed by the Department of Nephrology with albuminuria >300 µg/mg of creatinine,[17] along with no evidence of moderate, severe NPDR, or PDR were selected.

Exclusion criteria were applied as these conditions might aggravate the diabetic complications mainly DR. Even though DN patients develop DR in the long term or vice versa, we have restricted our study to these two complications separately with either one presenting earlier to understand whether specific gene polymorphism is responsible for affecting specific organs initially.

Blood sampling and DNA extraction

A fasting venous blood sample (2 ml) was collected from each patient, 1 ml in a fluoride oxalate vacutainer tube, and 1 ml in an EDTA vacutainer tube. Plasma was used for the estimation of fasting plasma glucose (FPG). Auto analyzer was used for measurement of FPG in mg/dl by enzymatic colorimetric method using glucose oxidase peroxidase test. Glycosylated haemoglobin (HbA1c) in percentage was estimated by an immunoturbidimetric test. Isolation and purification of whole genomic DNA from the blood samples was carried out by using the HiPurA Blood Genomic DNA Miniprep Purification kit.[18] Agarose gel electrophoresis using 1% ethidium bromide was used to check the quality and quantity of purified DNA. This DNA was used for genotyping by polymerase chain reaction (PCR).

Genotyping of GSTT1 and GSTM1

Polymerase chain reaction was carried out using a PCR machine for GSTT1 and GSTM1. A reaction mixture of 20 µl containing dNTP, forward and reverse primers, Taq DNA polymerase, buffer, distilled water, and purified genomic DNA as a template was used for PCR.

Primer sequences,[19,20] PCR conditions, and PCR products for GSTT1 and GSTM1 are shown in Table 1. The amplification products of PCR for both genes were analysed by using agarose gel with 1.5% ethidium bromide. They were visualized under a UV transilluminator and photographs were taken by gel documentation system. The presence of the gene was established by the visible band of gene fragment at the desired level i.e. band at 480 bp for GSTT1 and band at 625 bp for GSTM1. The absence of a band at that level suggested a null genotype as shown in Figures 1 and 2.

Statistical analysis

SPSS software (SPSS Statistics, Version 20, IBM Corp., Chicago, Illinois, USA) was used for analysing the data. The Chi-square test and ANOVA test were used for comparison of the groups. A logistic regression analysis was done to study the association by Odds ratio (OR) with a 95% confidence interval (CI). The difference between the values reflected statistical significance when the *P* value was ≤0.05.

Ethical aspect

This study was approved by the Institutional Ethics Committee. Ref. No. KIMSDU/IFC/03/2019 Protocol no. 0575/2018–2019. Dated 09/07/2019. Written informed consent was

Table 1: Primer sequences, PCR conditions, and PCR products for GSTT1 and GSTM1

Gene	Forward primer (FP) Reverse primer (RP)	PCR amplification conditions	PCR product	Remark
GSTT1	FP: 5'- TTC CTT ACT GGT CCT CAC ATC TC-3' RP: 5'- TCA CCG GAT CAT GGC CAG CA- 3'	95°C- 5 min, 30 cycles of 95°C- 30 sec, 60°C- 30 sec, 72°C- 30 sec, 72°C- 5 min,	480 bp	Positive – Band at 480 bp Null – No band at 480 bp
GSTM1	FP: 5'- CAA ATT CTG GAT TGT AGC AGA TCA TGC-3' RP: 5'- CAC AGC TCC TGA TTA TGA CAG AAG CC-3'	95°C- 5 min, 30 cycles of 95°C- 30 sec, 56°C- 30 sec, 72°C- 30 sec, 72°C- 5 min	625 bp	Positive – Band at 625 bp Null – No band at 625 bp

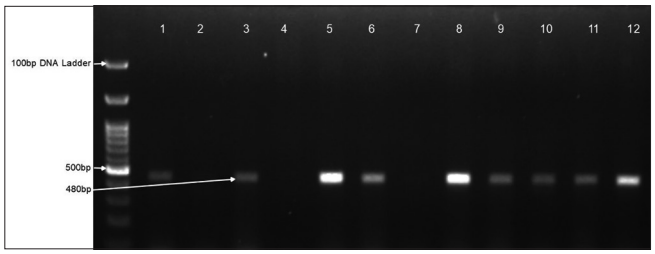

Figure 1: Gel electrophoresis showing PCR products of GSTT1. Lane 1,3,5,6, 8-12 GSTT1 positive, Lane 2,4,7-GSTT1 null genotype

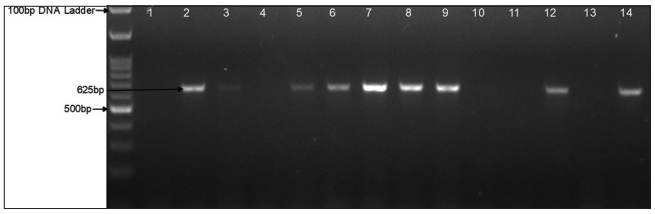

Figure 2: Gel electrophoresis showing PCR products of GSTM1. Lane 2,3,5-9,12,14 – GSTM1 positive, Lane 1,4,10,11,13-GSTM1 null genotype

obtained for participation in the study and use of the patient data for research and educational purposes. The procedures in the study follow the guidelines laid down in the Declaration of Helsinki (1964).

Results

General characteristics and biochemical parameters

General characteristics and biochemical parameters in the three groups as defined earlier with a total of 375 cases are displayed in Table 2. The distribution of gender among the groups was not statistically significant ($P = 0.456$). The DM group had a significantly higher mean age and duration of diabetes than other groups ($P < 0.001$). According to the ANOVA test, when compared to the DR and DN groups, the DM group had significantly low fasting plasma glucose levels ($P = 0.024$). In addition, HbA1c levels were significantly higher ($P < 0.001$) in the DR group than in other groups.

Genotype distribution of GSTT1 and GSTM1

The genotype distribution of GSTT1 and GSTM1 in groups is shown in Table 2. GSTT1 null genotype was observed more frequently in the DR group (66.4%). The GSTM1 null genotype was considerably higher among both DR (65.6%) and DN (60%) groups.

GST genotypes were arranged into four subgroups depending upon double combination: both GSTT1 and GSTM1 positive, both GSTT1 and GSTM1 null, GSTT1 positive and GSTM1 null, and GSTT1 null and GSTM1 positive. Among the double genotype combination, both GSTT1 and GSTM1 null combination was found to be higher among the DR group (47.2%) ($P = 0.003$) while GSTT1 positive and GSTM1 null was more frequent among the DN group (29.6%) ($P = 0.009$).

Genotype distribution in the DR and DN groups compared to the DM group

The genotype distribution in the DR and DN groups compared to the DM group with the odds ratios are shown in Table 3. The univariate analysis showed that genotypes GSTT1 null (OR = 2.68; 95% CI = 1.60–4.48; $P < 0.001$), GSTM1 null (OR = 2.50; 95% CI = 1.50–4.18; $P < 0.001$, and both GSTT1 and GSTM1 null (OR = 4.65; 95% CI = 2.39–9.07; $P < 0.001$) were associated with DR. Genotypes GSTM1 null (OR = 1.97; 95% CI = 1.19–3.26; $P = 0.008$) and GSTT1 positive and GSTM1 null were associated with DN but GSTT1 null genotype was not associated with DN ($P = 0.117$).

Multiple logistic regression analysis

The multiple logistic regression analysis was done using the variables showing significance ($P \leq 0.05$) to study the probable effect of these factors on DR and DN development [Table 4]. We excluded the age and duration of diabetes in this analysis because we had selected patients in the DM group with more than 10 years of duration.

After the adjustment of HbA1c and FPG, the carriers of both GSTT1 and GSTM1 null genotype showed a 2.16-fold (OR = 2.16; 95% CI = 1.26–3.72; $P = 0.005$) higher risk for DR. [Table 4] After the adjustment, the carriers of GSTM1 null genotype showed a 1.91-fold (OR = 1.91; 95% CI = 1.028–3.55; $P = 0.040$) and GSTT1 positive and GSTM1 null genotype variant showed a 2.13-fold (OR = 2.13; 95% CI = 1.015–4.47; $P = 0.046$) higher risk for DN [Table 4].

Discussion

The research is going on to understand the risk factors and pathophysiology of diabetes mellitus and its complications from genetic and environmental points of view. Oxidative stress induced by chronic hyperglycaemia is known to

Table 2: General characteristics, biochemical parameters, and genotype distribution of GSTT1 and GSTM1 in groups

Variables	Group 1 (DM) (N=125)	Group 2 (DR) (N=125)	Group 3 (DN) (N=125)	*P*
Gender				
Male	76 (60.8%)	85 (68%)	83 (66.4%)	0.456
Female	49 (39.2%)	40 (32%)	42 (33.6%)	
Age (y)	65.78±8.12[b,c]	57.63±8.43[a,c]	60.97±8.68[a,b]	<0.001*
Duration of diabetes (y)	19.36±7.38[b,c]	8.92±6.89[a,c]	12.51±7.19[a,b]	<0.001*
FPG (mg/dl)	199.62±89.44[b,c]	221.80±84.12[a]	227.36±80.3[a]	0.024*
HbA1c (%)	7.68±1.68[b]	8.55±1.69[a,c]	7.77±2.11[b]	<0.001*
GSTT1				
Positive	72 (57.6%)	42 (33.6%)	84 (67.2%)	<0.001*
Null	53 (42.4%)	83 (66.4%)	41 (32.8%)	
GSTM1				
Positive	71 (56.8%)	43 (34.4%)	50 (40%)	<0.001*
Null	54 (43.2%)	82 (65.6%)	75 (60%)	
Genotype combinations				
GSTT1 and GSTM1 positive	54 (43.2%)	19 (15.2%)	47 (37.6%)	<0.001*
GSTT1 positive and GSTM1 null	18 (14.4%)	23 (18.4%)	37 (29.6%)	0.009*
GSTT1 null and GSTM1 positive	17 (13.6%)	24 (19.2%)	3 (2.4%)	<0.001*
GSTT1 and GSM1 null	36 (28.8%)	59 (47.2%)	38 (30.4%)	0.003*

Data were stated in terms of percentage and mean±SD. FPG=fasting plasma glucose, HbA1c=glycosylated haemoglobin, y=years [a]– significantly different from group 1 (*P*<0.05), [b]– significantly different from group 2 (*P*<0.05) and [c]– significantly different from group 3 (*P*<0.05)

**P*<0.05

Table 3: Genotypes distribution in DR and DN groups compared with the DM group with the odds ratios

	Odds ratio	95% confidence interval	*P*
DR vs DM			
GSTT1 positive	1 (reference)		
GSTT1 null	2.685	1.60–4.48	<0.001*
GSTM1 positive	1 (reference)		
GSTM1 null	2.50	1.50–4.18	<0.001*
GSTT1 positive/GSTM1 positive	1 (reference)		
GSTT1 null/GSTM1 null	4.65	2.39–9.077	<0.001*
GSTT1 positive/GSTM1 null	3.63	1.61–8.15	0.002*
GSTT1 null/GSTM1 positive	4.01	1.78–9.03	0.001*
DN vs DM			
GSTT1 positive	1 (reference)		
GSTT1 null	0.663	0.396–1.11	0.117
GSTM1 positive	1 (reference)		
GSTM1 null	1.97	1.19–3.26	0.008*
GSTT1 positive/GSTM1 positive	1 (reference)		
GSTT1 null/GSTM1 null	1.213	0.665–2.211	0.529
GSTT1 positive/GSTM1 null	2.36	1.19–4.68	0.014*
GSTT1 null/GSTM1 positive	0.203	0.056–0.735	0.015*

**P*<0.05

play a major role in the commencement and progression of diabetes.[6] Nearly all molecules in the body are affected by enhanced oxidative stress, leading to cellular dysfunction. GST is one of the gene families engaged in the metabolism of hydrophobic and electrophilic substrates and reactive oxygen species (ROS) produced during oxidative stress. Many genes in the GST family encode phase II detoxifying enzymes. In mammals, eight classes of GSTs are discovered depending on the homology and substrate specificity. They are alpha (GSTA), mu (GSTM), theta (GSTT), Pi (GSTP), zeta (GSTZ), sigma (GSTS), Kappa (GSTK) and omega (GSTO).[21] The most common polymorphisms in both GSTM1 and GSTT1 are due to partial deletion of gene locus on respective chromosomes, which results in lowered or inactive enzyme activity.[22] Homozygous deletion or null genotype is one of the common gene polymorphisms due to deletion of both the alleles so it does not express enzyme. This leads to a total absence of enzymes produced by it. This may affect the metabolism of free radicals which results in susceptibility to diabetic complications due to increased oxidative stress.[6,7] Therefore, the role of polymorphism of these genes has created great interest in the researchers. In the present study, we tried to investigate the association of GSTT1 and GSTM1 gene polymorphisms with the development of DR and DN.

There is an inconsistency in the period between the detection of diabetes and the development of DN.[23] Also, after diagnosis of DM, the prevalence of DR is low in the first 10 years with occasional progression.[24] So, in the DM group, we included patients with more than 10 years of duration as controls. This explains the older age of the DM group than the DR and DN groups.

In the present study, GSTT1 null and GSTM1 null genotypes were frequently observed in cases with DR compared to the DM group (66.4%% vs 42.4%, $P = 0.000$ and 65.6% vs 43.2%, $P = 0.000$ respectively). The significant association between the GSTT1 null, GSTM1 null and both GSTT1 and GSTM1 null

Table 4: Adjusted odds ratio for risk factors for DR and DN

	Odds ratio	95% confidence interval	*P*
Risk factors for DR			
HbA1c	1.31	1.10–1.56	0.002*
FPL	1.001	0.99–1.004	0.451
Both GSTT1 and GSTM1 null	2.16	1.26–3.72	0.005*
Risk factors for DN			
HbA1c	0.95	0.83–1.10	0.517
FPL	1.006	1.002–1.009	0.001*
GSTM1 null	1.91	1.028–3.55	0.040*
GSTT1 positive and GSTM1 null	2.13	1.015–4.47	0.046*

*P<0.05

genotypes with DR suggests that they increase the risk of DR in patients with T2DM. Logistic regression analysis displayed that in addition to genetic factors, HbA1c was an independent risk factor for DR. In diabetic patients, HbA1c is considered the gold standard indicator for glycaemic control. Glycaemic control in DM is one of the important factors associated with the development of retinopathy.[2]

Increased risk of DR was detected in association with GSTT1 null genotype in Slovene[13] and Bangladeshi[25] populations. The connection between the GSTM1 null genotype and the risk of DR was seen in the Iranian population.[12] Some studies showed that the presence of the GSTM1 null genotype lowers the risk of DR.[13,26] No significant relationship between GSTT1 null genotype and DR was found in certain studies.[12,26] Tasdika TE *et al*.[25] revealed no significant association between GSTM1 null genotype and DR. The meta-analysis done by Li Sun *et al*.[10] with five studies including 3563 subjects showed that both GSTT1 and GSTM1 null genotypes were linked with a significantly elevated risk of DR. When categorized according to type of DM, a significantly higher DR risk was noticed in T2DM. Similar results in our study could indicate the fact that defective cellular metabolism with a decrease in antioxidant capacity due to the presence of non-functioning null genotypes increases free radicals and oxidative stress leading to an increased risk of DR.

In the present study, the GSTM1 null genotype was frequently observed in cases with DN compared to the DM group (60% vs 43.2%, $P = 0.000$) while the GSTT1 null genotype was evenly distributed among both groups (32.8% vs 42.4%, $P = 0.117$). Moreover, the association between the GSTM1 null genotype and DN remained significant after adjusting for FPG and HBA1c. This suggests that the presence of the GSTM1 null genotype increases the risk of nephropathy in patients with T2DM and the GSTT1 null genotype was not associated with the DN. Even though double genotype combination GSTT1 positive and GSTM1 null genotype variants showed a 2.13-fold higher risk for DN, the significance is mild ($P = 0.046$). This might be due to a different expression of GSTT1 and GSTM1 genes in various populations so people might react differently towards detoxification. This may explain the differences in risk for the same disease in different populations.[27] However, further studies with larger sample sizes might be needed to confirm the association of double genotype combination with DN.

Various studies observed that the GSTT1 null genotype was associated with diabetic chronic kidney disease (CKD) and end-stage renal disease (ESRD) irrespective of the GSTM1 status.[14,20] S. K. Datta *et al*.[20] found that the presence of GSTM1 positive and GSTT1 null and both GSTM1 and GSTT1 null was significantly higher among patients with diabetic CKD. In a group of Saudi Arabian patients, both GSTT1 and GSTM1 null genotypes were associated with the risk of DN.[8] In a meta-analysis by Jan Orlewski *et al*.,[11] GSTM1 null genotype was found to be associated with enhanced risk of DN along with both GSTT1 and GSTM1 null combination genotype. While doing subgroup analysis, a significant relationship was detected between GSTM1 null genotype and DN mainly in the Asian population but not in Caucasians. In only type 2 DM patients no association was found between the GSTT1 and GSTM1 null genotypes and DN. In the study by Hashemi-Soteh MB *et al*. in Egypt,[28] the GSTM1 null genotype conferred a statistically significant increased risk of nephropathy in T2DM but no association was found between GSTT1 and GSTM1 null genotypes and the risk of diabetic retinopathy. A meta-analysis study conducted by Nath S *et al*.[29] included 17 studies to evaluate the role of GSTM1 and GSTT1 null polymorphism in the development of T2DM-related complications. They showed that GSTT1 null and both GSTM1 and GSTT1 null genotypes were associated with enhanced risk of development of T2DM-related complications but the same was not true for GSTM1 null genotype.

Thus, several studies on DR and DN worldwide revealed conflicting outcomes regarding the association between them and GSTT1 and GSTM1 genotypes. The possible explanation for these contrasting results may include variations in the ethnicity of the study population, environmental factors, and sample size. To the best of our knowledge, ours is the first study from rural Maharashtra to reveal the correlation between GSTT1 and GSTM1 with DR and DN risk. Since this was a cross-sectional study, we could not comment on previous possible periods of poor glycaemic control in DN patients. Also, samples in double combination gene variants were less so it limits the risk assessment attributable to the population.

Conclusion

Among, the study population, GSTT1 null, GSTM1 null, and both GSTT1 and GSTM1 null genotypes are risk factors for developing DR while genotypes GSTM1 null and combined GSTT1 positive and GSTM1 null are risk factors for developing DN. GSTT1 null genotype is not associated with the risk of DN. Along with genetic factors, high HbA1c is an independent risk factor for DR. Prospective gene studies with large sample sizes are needed to confirm the interaction between environmental and genetic factors to enlighten the risk of DR and DN.

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Author contributions

Concept and design by VSP, KDD, AVS; Clinical studies by VSP, PSP; Experimental Studies by VSP, KDD, SKP; Data acquisition by VSP, KDD, SKP, PSP; Data analysis by VSP, KDD; Statistical analysis by VSP, SKP; Manuscript preparation by VSP, SKP; Manuscript editing and review by all. All authors read and approved the final manuscript.

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Conflicts of interest

There are no conflicts of interest.

Data availability statement

Derived data supporting the findings of this study are available from the corresponding author on reasonable request.

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