Association of *GRP78* promoter polymorphisms and serum GRP78 level with risk of asthenozoospermia

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Abstract

Purpose The aim of this study was undertaken to investigate the association of 78-kDa glucose-regulated protein (GRP78) gene promoter polymorphisms with risk of asthenozoospermia (AZS) men. In addition, we performed association analysis between GRP78 promoter mutations and serum GRP78 level in asthenozoospermia.

Methods The study population comprised 400 subjects with AZS patients and 400 healthy controls. We assessed GRP78 rs3216733, rs17840761, and rs17840762 polymorphisms by using Snapshot SNP genotyping assays; serum GRP78 level was measured by enzyme-linked immunosorbent assay (ELISA). Semen quality was assessed by computer-assisted semen analysis.

Results We found that rs3216733 was associated with increased risk of AZS (Gd vs. dd: adjusted OR = 1.42, 95% CI, 1.06–1.93, $P = 0.020$; Gd/GG vs. dd: adjusted OR = 1.43, 95% CI, 1.08–1.91, $P = 0.013$; G vs. d adjusted OR = 1.26, 95% CI, 1.03–1.56, $P = 0.027$). The haplotype analyses showed the frequency of G-C-C haplotype was significantly higher in AZS ($P = 0.026$). The percentage of progressive motility sperm was lower in the asthenozoospermic men with Gd and Gd/GG genotypes than dd genotype ($P = 0.003$). Moreover, the serum GRP78 levels were significantly lower in rs3216733 Gd/GG genotypes compared with the dd genotype ($P < 0.001$).

Conclusion Our findings suggest that rs3216733 Gd/GG genotypes contribute to poor sperm motility, probably by decreasing the level of GRP78.

Keywords *GRP78/HSPA5* gene · Promoter polymorphisms · Asthenozoospermia · Single nucleotide polymorphism

Introduction

Human infertility is a universal and difficult problem which affects about 15% of couples in the world. Among the many aspects, the male factor is considered to play a critical role in infertility [1, 2]. Up to now, the etiology of infertile men is complex, exhibiting a high incidence of anatomic defects, molecular genetics disorders, urogenital tract infections, endocrine disturbances, genetic variations, or environmental toxins

[3–9]. Semen quality analysis is an important reference data for infertile man's evaluation as it provides information on sperm morphology, concentration, and motility [10]. Asthenozoospermia, characterized by poor sperm motility, is considered as an important factor of infertile men [11]. The exact pathogenesis of asthenozoospermia (AZS) is unclear. Relevant studies reported that the etiology of AZS is of functional defects (epididymitis, varicocele, orchitis) or an array of biochemical and genetic factors (genes variation, chromosomal abnormalities, Y microdeletions) [12–14]. Recently, the relationship between gene variation and AZS became a hot topic. Zhang (2016) analyzed 104 idiopathic asthenozoospermia and found a significant difference in the missense mutation frequency in the tektin-t gene and predicted that this mutation may be a possible risk for AZS [2]. Zhang (2016) reported that the incidence of the missense PATE1 gene mutation increases in AZS patients (72.22%) compared with the controls (52.83%) [15].

GRP78 (78 kDa glucose-regulated protein), also known as heat shock 70 kDa protein 5 (HSPA5) or immunoglobulin

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heavy chain-binding protein (BiP), is a member of the heat-shock protein 70 family and an important molecular chaperone of the endoplasmic reticulum (ER), involved in the folding and assembly of proteins [16]. Previous studies showed that in addition to being observed in human epididymal epithelium and testis spermatogenic cells, GRP78 protein persists in the neck region of ejaculated spermatozoa [17]. GRP78 protein modulates the binding interaction of sperm and zona pellucida through a calcium-dependent pathway, which can affect capacitation of human sperm [18, 19]. However, no studies have been reported on whether the GRP78 protein and its gene polymorphisms are involved in sperm motility. In this study, we investigated *GRP78* promoter mutations and associated these with human sperm motility in asthenozoospermia.

Materials and methods

Subjects

In this case-control study, we recruited 400 subjects with AZS from the department of Reproduction and Genetics at the Affiliated Hospital of Youjiang Medical University for Nationalities (Guangxi, China). The patients with sperm morphology ($>4.0\%$), sperm concentration ($\geq 15 \times 10^6$ spermatozoa/ml), leukocytospermia ($<1 \times 10^6$ /ml), sperm viability ($\geq 40\%$), forward progressive motility ($<32\%$), and negative for anti-sperm antibodies, who had been unable to accomplish pregnancy for at least 18 months of unprotected sexual intercourse, were participated in this study. Exclusion criteria

included endocrine hypogonadism, Y chromosome microdeletions, karyotype anomalies, obstruction of the vas deferens, varicocele, cryptorchidism, orchitis, epididymitis and history of genital tract infection, and drug treatment. The 400 controls were recruited from the general population in the area of south China (Guangxi). The age-matched controls were fertility men who had fathered at least one child within the previous 2 years without using assisted reproduction. The routine sperm parameters in the control groups were normal following the World Health Organization (WHO) manuals (5th edition). Each subject was given a questionnaire to record information about his lifestyle, reproductive history, alcohol and cigarette smoking habits, medical history, and exposures. We could eliminate as much as possible the subjects of systemic diseases (hypertension, hypercholesterolemia, and diabetes mellitus), autoimmune diseases, and cancers.

All subjects carried out three complete semen analyses by a computer-assisted sperm analysis system (SCA5.0; Spain) after 2–7 days of sexual abstinence. Detailed characteristics are shown in Table 1. The study received the approval of the ethics committee of the Affiliated Hospital of Youjiang Medical University for Nationalities, and the informed consent was obtained from each subject. The study protocol followed the ethical principles of medical research involving human subjects in the Helsinki declaration.

SNP selection, DNA extraction and genotyping

The selection criteria of SNPs in *GRP78* are as below: Firstly, we conducted a search for all SNPs of the promoter in the *GRP78* gene from the international HapMap project databank

Table 1 Demographic characteristics and semen parameters of study subjects

Sample parameters	Asthenozoospermic group ($N=400$)	Control group ($N=400$)	P value
Age (years)	33.03 ± 6.8	32.29 ± 6.5	0.119
pH value of semen	7.35 ± 0.14	7.34 ± 0.15	0.831
Ejaculate volume (mL)	3.47 ± 1.39	3.25 ± 1.38	0.423
Sperm concentration ($\times 10^6$ /mL)	49.9 ± 20.7	50.3 ± 25.9	0.838
Progressive motility (%)	20.09 ± 8.18	57.16 ± 13.45	<0.001
Curvilinear velocity ($\mu\text{m/s}$)	32.03 ± 7.09	51.40 ± 8.88	<0.001
Straight linear velocity ($\mu\text{m/s}$)	12.71 ± 4.71	23.57 ± 5.66	<0.001
Linearity (%)	38.93 ± 9.03	45.86 ± 9.10	<0.001
Smoking			
Yes	121 (30.3)	137 (34.3)	0.226
No	279 (69.7)	263 (65.7)	
Drinking			
Yes	195 (48.8)	181 (45.2)	0.321
No	205 (51.2)	219 (54.8)	
Sleeping status (day)			
≤ 8 h	212 (53.0)	193 (48.2)	0.179
> 8 h	188 (47.0)	207 (51.8)	

(www.hapmap.org/) and the National Center for Biotechnology Information (NCBI). According to the above result, we selected SNPs that the frequency of the minor allele (MAF) outweighs 5% within Han Chinese. Secondly, Haploview software was used to pick tagging SNPs ($r^2 > 0.08$) that can represent other SNPs in the same region of *GPR78* gene. Thirdly, we examined previous papers that were executed to study the association between diseases and polymorphism in *GRP78* gene promoter. Finally, we selected three eligible SNPs for genotyping (rs3216733, rs17840761, and rs17840762). Genomic DNA was extracted from blood leukocytes by using a whole-blood genome DNA isolation kit (Tianjin Inc., Beijing, China). Genotyping reactions were carried out using the multiple single nucleotide primer extension technique. The primers for PCR amplification were designed using online primer 3.0 (<http://primer3.ut.ee/>) (Table 2). All the resultant band lengths of the PCR products in this study have a length of 369 bp.

ELISA

Venous blood from the patients and healthy controls was clotted for 2 h at room temperature or overnight at 4 °C by serum separator tube before centrifugation for 20 min at approximately 1000×g and collected the supernatant at −80 °C until use. The concentration of serum GRP78 levels was measured by enzyme-linked immunosorbent assay (ELISA) kits (LifeSpan BioSciences, Inc., USA; Catalog No. LS-F6280) following reagent manufacturer's instructions. The optical density (OD) of the well is measured at a wavelength of 450 nm ± 2 nm (RT-6000, China). Each sample was analyzed in duplicate. The concentration of serum GRP78 levels was performed by a standard curve constructed with the kit's standards.

Statistical analysis

The distribution of genotypes Hardy-Weinberg equilibrium was calculated by the chi-squared test between case and

control subjects. The unconditional logistic regression was applied to estimate Odds ratios (ORs) and corresponding 95% confidence intervals (CIs). The online SHesis software (<http://analysis.bio-x.cn/myAnalysis.php>) was used to analyze the haplotype analysis. Differences of variables were assessed by an unpaired *t* test. Statistical analysis of all data was performed using SPSS 17.0 software package (SPSS Inc., Chicago, USA). $P < 0.05$ was considered as statistically significant.

Results

Clinical characteristics of the study subjects

Table 1 compares the demographic and clinical characteristics of the study population. There was no significant difference between the AZS patients and controls regarding age, smoking status, alcohol consumption, sleeping status, and some semen parameters (pH, volume, concentration). However, the sperm motion kinetic parameters of the patients with AZS were significantly lower than those in the controls (the progressive motility, curvilinear velocity, straight linear velocity, and linearity, all $P < 0.001$).

Polymorphisms of *GRP78* promoter and the risk of AZS

All of the SNPs have three genotypes respectively (Fig. 1). The genotype and allele frequencies for the *GRP78* promoter polymorphism between asthenozoospermia and controls are shown in Table 3. The frequencies of the dd, Gd, GG genotypes and d, G alleles of rs3216733 polymorphism in the controls are 46.5%, 43.7%, 9.8% and 68.4%, 31.6% as against 38.0%, 49.8%, 12.2% and 62.9%, 37.1% in asthenozoospermia respectively. The frequencies of the CC, CT, TT genotypes and C, T alleles of rs17840761 polymorphism in the controls are 27.3%, 45.2%, 27.5% and 49.9%, 50.1% as against 28.0%, 47.5%, 24.5% and 51.8%, 48.2% in

Table 2 The primer sequences for the promoter region of the *GRP78* gene

SNP ID	Primer sequences
rs3216733	F: 5'-CCCCTCCGCAATAAACGTCACCTG-3'
	R: 5'-GCATCTAAGCTGCGACTGGTCTAC-3'
	E: 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTCAATGAATCAGCTGGGGGGG-3'
rs17840761	F: 5'-CCCCTCCGCAATAAACGTCACCTG-3'
	R: 5'-GCATCTAAGCTGCGACTGGTCTAC-3'
	E: 5'-CCCTAGGGGGTCGGAGTAG-3'
rs17840762	F: 5'-CCCCTCCGCAATAAACGTCACCTG-3'
	R: 5'-GCATCTAAGCTGCGACTGGTCTAC-3'
	E: 5'-TTTTTTTTTTTTTTTTTTTTTTTGTGAGTGACCCCCGGGGCTG-3'

F forward primer, R reverse primer, E extension primer

Fig. 1 Sequencing map of genotypes for *GRP78* gene polymorphisms. Sequencing map showed dd, Gd, and GG genotypes for rs3216733; CC, CT, and TT genotypes for rs17840761; CC, CT, and TT genotypes for rs17840762, respectively. (From top to bottom)

asthenozoospermia respectively. The frequencies of the CC, CT, TT genotypes and C, T alleles of rs17840762 polymorphism in the controls are 74.3%, 23.0%, 2.7% and 85.7%, 14.3% as against 75.0%, 23.5%, 1.5% and 86.8%, 13.2% in asthenozoospermia respectively. The Hardy-Weinberg equilibrium *P* value in two groups was >0.05. In rs3216733, the Gd and Gd/GG genotypes were associated with increased risk of AZS (Gd vs. dd: adjusted OR = 1.42, 95% CI, 1.06–1.93, *P* = 0.020; Gd/GG vs. dd: adjusted OR = 1.43, 95% CI, 1.08–1.91, *P* = 0.013). Compared with the rs3216733 d allele, the rs3216733 G allele was significantly associated with an increased risk of AZS (OR = 1.26, 95% CI, 1.03–1.56, *P* = 0.027). The genotype and allele of rs17840761 and rs17840762 polymorphisms showed no significant differences in the two study groups (*P* > 0.05). By haplotype analyses, the G-C-C haplotype was 33.9 and 29.2% in both cases and controls, respectively. Compared with the controls, the G-C-C haplotype was significantly increased risk of AZS (*P* = 0.026) (Table 4).

Sperm motion kinetics in asthenozoospermic and controls

We compared the association of sperm motion kinetic parameters (progressive motility, curvilinear velocity, straight linear velocity, and linearity) in the AZS patients and controls with different rs3216733 genotypes (Table 5). In the AZS patients, the sperm kinetic parameters were significantly lower in the Gd genotype vs. dd genotype with respect to progressive motility (*P* = 0.003), curvilinear velocity (*P* = 0.009), straight linear velocity (*P* = 0.004), and linearity (*P* = 0.010). Moreover, the patients with the Gd/GG genotypes had significantly lower sperm motion kinetics than the dd genotype with respect to progressive motility (*P* = 0.003), curvilinear velocity (*P* = 0.028), straight linear velocity (*P* = 0.019), and linearity (*P* = 0.014). Nevertheless, we failed to find the association of sperm motion kinetic parameters in controls with different rs3216733 genotypes (*P* > 0.05).

Association of polymorphisms and serum GRP78 level

The concentration of GRP78 in the serum of AZS patients (0.479 ± 0.104 ng/mL) was significantly lower than that in controls (0.661 ± 0.225 ng/mL, *P* < 0.001; Fig. 2a). Besides,

Table 3 Genotype and allele frequencies for the *GRP78* promoter polymorphism between asthenozoospermia and controls

Genotype/ allele	Asthenozoospermic group (%)	Control group (%)	OR (95% CI)*	<i>P</i> value*
rs3216733				
dd	152 (38.0)	186 (46.5)	1.00	
Gd	199 (49.8)	175 (43.7)	1.42 (1.06–1.93)	0.020
GG	49 (12.2)	39 (9.8)	1.45 (0.91–2.35)	0.120
Gd/GG	248 (62.0)	214 (53.5)	1.43 (1.08–1.91)	0.013
d	503 (62.9)	547 (68.4)	1.00	
G	297 (37.1)	253 (31.6)	1.26 (1.03–1.56)	0.027
rs17840761				
CC	112 (28.0)	109 (27.3)	1.00	
CT	190 (47.5)	181 (45.2)	1.05 (0.75–1.46)	0.797
TT	98 (24.5)	110 (27.5)	0.86 (0.59–1.27)	0.447
CT/TT	288 (72.0)	291 (72.7)	0.98 (0.71–1.34)	0.880
C	414 (51.8)	399 (49.9)	1.00	
T	386 (48.2)	401 (50.1)	0.93 (0.76–1.13)	0.443
rs17840762				
C/C	300 (75.0)	297 (74.3)	1.00	
C/T	94 (23.5)	92 (23.0)	1.03 (0.74–1.44)	0.864
T/T	6 (1.5)	11 (2.7)	0.51 (0.19–1.41)	0.195
CT/TT	100 (25.0)	103 (25.7)	0.97 (0.70–1.34)	0.866
C	394 (86.8)	686 (85.7)	1.00	
T	106 (13.2)	114 (14.3)	0.92 (0.69–1.23)	0.583

SNPs single nucleotide polymorphisms, *d* deleted, OR odds ratio, CI confidence interval, dd deletion

*Adjusted for age, sex, smoking, drinking, and sleeping statuses by the logistic regression model

Table 4 Haplotype distributions for the *GRP78* promoter polymorphism in asthenozoospermia and controls

Haplotype	Asthenozoospermic group (freq) 2n = 800 (%)	Control group (freq) 2n = 800 (%)	OR(95% CI)	P value
d-T-C*	363 (45.4)	384 (48.1)	0.93 (0.76–1.13)	0.446
G-C-C*	271 (33.9)	234 (29.2)	1.27 (1.03–1.57)	0.026
d-C-T*	91 (11.4)	111 (13.9)	0.81 (0.60–1.09)	0.158
d-C-C*	45 (5.6)	51 (6.4)	0.88 (0.58–1.33)	0.543
G-T-C	15 (1.8)	17 (2.1)	0.90 (0.44–1.83)	0.769
G-C-T	7 (0.9)	3 (0.3)	–	–
G-T-T	4 (0.5)	0 (0.0)	–	–
d-T-T	3 (0.4)	0 (0.0)	–	–

Major haplotypes—Haplotype frequencies less than 1% were not included in the statistical analysis

OR odds ratio, CI confidence interval

we also found that patients carrying rs3216733 Gd/GG genotypes (0.414 ± 0.069 ng/mL) had lower levels of GRP78 than carrying dd genotype (0.558 ± 0.082 ng/mL, $P < 0.001$; Fig.

2b). However, there was no relationship between serum GRP78 level and rs17840761, rs17840762 ($P > 0.05$, data not shown).

Table 5 Comparison of sperm motion kinetics parameters in the polymorphism of rs3216733 among asthenozoospermic men and healthy controls

Genotypes	Sperm motion kinetics parameters			
	Progressive motility (%)	Curvilinear Velocity, $\mu\text{m/s}$	Straight Linear Velocity, $\mu\text{m/s}$	Linearity, %
Case subjects				
dd ($n = 152$)	21.56 ± 6.56	33.03 ± 6.01	13.43 ± 4.13	40.34 ± 8.92
Gd ($n = 199$)	19.06 ± 9.31	31.14 ± 7.39	12.00 ± 4.85	37.82 ± 9.05
<i>t</i> value	2.948	2.633	2.898	2.595
<i>P</i> value	0.003	0.009	0.004	0.010
dd ($n = 152$)	21.56 ± 6.56	33.03 ± 6.01	13.43 ± 4.13	40.34 ± 8.92
GG($n = 49$)	19.78 ± 7.05	32.55 ± 8.51	13.42 ± 5.43	39.01 ± 8.90
<i>t</i> value	1.618	0.431	−0.002	0.906
<i>P</i> value	0.107	0.667	0.998	0.336
dd ($n = 152$)	21.56 ± 6.56	33.03 ± 6.01	13.43 ± 4.13	40.34 ± 8.92
Gd/GG ($n = 248$)	19.21 ± 8.91	31.42 ± 7.64	12.28 ± 4.99	38.06 ± 9.01
<i>t</i> value	3.035	2.208	2.363	2.465
<i>P</i> value	0.003	0.028	0.019	0.014
Control subjects				
dd ($n = 186$)	57.72 ± 13.54	51.48 ± 9.61	23.41 ± 5.72	45.51 ± 8.66
Gd ($n = 175$)	56.45 ± 13.69	51.57 ± 8.16	23.85 ± 5.70	46.12 ± 9.42
<i>t</i> value	0.882	−0.099	−0.746	−0.645
<i>P</i> value	0.378	0.921	0.456	0.520
dd ($n = 186$)	57.72 ± 13.54	51.48 ± 9.61	23.41 ± 5.72	45.51 ± 8.66
GG($n = 39$)	57.69 ± 12.04	50.28 ± 8.57	23.11 ± 5.30	46.42 ± 9.82
<i>t</i> value	0.011	0.718	0.298	−0.584
<i>P</i> value	0.991	0.473	0.766	0.560
dd ($n = 186$)	57.72 ± 13.54	51.48 ± 9.61	23.41 ± 5.72	45.51 ± 8.66
Gd/GG ($n = 214$)	56.68 ± 13.89	51.34 ± 8.23	23.71 ± 5.62	46.17 ± 9.47
<i>t</i> value	0.770	0.158	−0.550	−0.732
<i>P</i> value	0.442	0.874	0.582	0.465

Fig. 2 ELISA detection of GRP78 expression. **a** Serum level of GRP78 in patients with asthenozoospermia and controls. **b** The serum GRP78 level of rs3216733 Gd/GG genotype and dd genotype in asthenozoospermia patients

Discussion

In our study, we analyze the possible association of *GRP78* gene polymorphisms (rs3216733, rs17840761 and rs17840762) with the risk of developing AZS. The results indicated that rs3216733 was associated with an increased risk of AZS in Gd genotype, Gd/GG genotype, G allele, and G-C-C haplotype. Moreover, we found that there was a significant correlation between the rs3216733 polymorphism and sperm motion kinetics in AZS patients. Patients with Gd and Gd/GG genotype had significantly lower values of progressive motility, curvilinear velocity, straight linear velocity, and linearity than patients with a dd genotype. Interestingly, the serum GRP78 levels were significantly lower in individuals with rs3216733 Gd/GG genotype than carrying dd genotype. These findings suggest that Gd/GG genotype in rs3216733 polymorphism may exert influences on the sperm motility and serum GRP78 expression in asthenozoospermia.

The *GRP78* protein, located on chromosome 9q33-34 in humans, plays a key role in protein transport, folding, and assembly [20]. The core-promoter region of *GRP78* has described in 2.1 kB upstream of exon 1 including rs3216733 (−180 bp), rs17840762 (−370 bp), and rs17840761 (−378 bp), which involved in the binding of some transcription factors [21]. A functional study showed that rs391957/rs3216733 polymorphism alters basal promoter activity [22]. We found similar findings regarding the association of the *GRP78* promoter polymorphism with the risk of several common diseases when we looked up the relevant reports of *GRP78* polymorphism in recent years. Winder (2011) showed that gastric adenocarcinoma (GA) patients with rs391957 CT or TT genotypes were associated with an increased the risk of tumor recurrence and death than those with CC genotype [23]. Zhu (2013) reported that the rs391957 allele G was associated with higher levels of GRP78 mRNA and protein in hepatocellular

carcinoma (HCC) and probably regulated the expression of GRP78 by providing an Ets-2 binding site [24]. Jia (2015) indicated that rs391957 polymorphism was an independent risk factor in the pathogenesis of type 2 diabetic peripheral neuropathy [25]. These reports support our hypothesis that rs3216733 is associated with the risk of AZS. In addition, Lobo (2015) found sperm GRP78 phosphorylation occurred spatial reorganization during the maturation of the epididymis, and compared with normozoosperm, phosphorylated GRP78 decreased over 2-fold in asthenozoosperm [26]. Shen (2013) showed that the GRP78 protein is differentially expressed between asthenozoosperm and normal individuals, suggesting that post-translational cutting and splicing play an important role in sperm motility [27]. Consistent with these results, we found that AZS patients with Gd/GG genotype have significantly lower values of sperm motion kinetics parameters, probably by the Gd/GG genotype that plays a key role in downregulating the expression of serum GRP78.

Previously, it was reported that the polymorphisms of rs17840761 and rs17840762 were not associated with gastric and cancer, chronic HBV infection, and Parkinson's disease [23, 28, 29]. Obviously, these results are consistent with our results that the polymorphisms of rs17840761 and rs17840762 are not associated with the risk of AZS. However, we found that the G-C-C haplotype was significantly increased risk of AZS ($P = 0.026$) by haplotype analysis. Some possibilities should be considered to explain these issues. SNPs are the preferred marker for elucidating the relationship between genetic variation and disease susceptibility. However, a single SNP analysis usually provides limited information. Factors affecting the disease may exist within or near the haplotypes, so a better approach may be to analyze the haplotypes derived from SNPs [30–33].

The results of this study provide important information for *GRP78* gene and the susceptibility of

asthenozoospermia. rs3216733 may alter transcriptional activity and affect the differential expression of serum GRP78 protein resulting in an increased risk of asthenozoospermia. However, the limitations of this study should be pointed out. Firstly, this study only explored the polymorphism of *GRP78* promoter region. Secondly, our study was limited to the subjects of Chinese population, and it was unclear whether the results were consistent with those in other populations. Therefore, further functional studies are necessary to clarify the exact role of *GRP78* gene in asthenozoospermia pathogenesis.

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Compliance with ethical standards

This study was approved by the ethics review board of the Affiliated Hospital of Youjiang Medical University for Nationalities, consistent with provisions of the Declaration of Helsinki. Voluntary written informed consent was obtained.

Conflict of interest The authors declare that they have no competing interests.

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