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DEFB1 gene polymorphisms modify vitiligo extent and response to NB-UVB phototherapy

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Abstract

Human beta defensin-1(hBD-1); an antimicrobial peptide, has immune regulatory effects which may be involved in autoimmunity. The aims were to evaluate the association between defensin beta 1 (DEFB1) (-44 C/G) and (-20 G/A) gene polymorphisms with the risk of vitiligo development, the extent of the disease and the response to NB-UVB treatment in a sample of Egyptian population. 178 active non-segmental vitiligo patients and 182 control subjects were included in this prospective case control study. Vitiligo extent was evaluated using vitiligo area scoring index (VASI). Gene polymorphisms in all participants were studied by RFLP PCR technique. Patients were treated by three narrowband UVB (NB-UVB) treatment sessions per week. After 12 weeks, the patients were reevaluated clinically to assess the extent of the disease using VASI scoring again and to evaluate the type of repigmentation, if any. AA genotype of DEFB1 (-20G/A) has a protective role against vitiligo development, while (DEFB1 -44 C/G) GG genotype and G allele increase the risk of vitiligo development about two folds. Patients carrying polymorphism in DEFB1 (-20G/A) only showed the lowest VASI scores (14.23 ± 2.77) and the highest percentage of improvement (66.12 ± 18.01%), while patients carrying polymorphism in DEFB1(-44 C/G) only showed the highest baseline VASI scores (38.87 ± 6.7) and the lowest therapeutic response (23.79 ± 19.42%) among all patients groups. Different DEFB1 gene polymorphisms may modify the risk of vitiligo development, the disease extent and the response to NB-UVB phototherapy.

KEYWORDS
gene polymorphism, hBD-1, phototherapy, vitiligo

1 | INTRODUCTION

Vitiligo is a chronic systemic acquired disease characterized by the appearance of macules and achromic or hypochromic patches on the skin and mucous membranes. The cause of vitiligo is not fully understood yet, however, there are multiple hypotheses to explain the pathogenesis of vitiligo which include the genetic, neural, autoimmune, biochemical, and melanocytorrhagy theories.[1]

Defensins represent a large antimicrobial peptides family which can be classified according to the variations in the structure into α-, β-, and θ-defensins. β-defensins include about 17 members which are expressed in mucosal and epithelial cells.[2] In the skin, hBDs are localized to the outer epidermal layers supporting its vital role in cutaneous innate immunity.[3]

Human β-defensin (hBD)-1 is an antimicrobial peptide that is encoded by the DEFB1 gene. It is initially secreted in a primary form composed of 68 amino acids. Later on, this pro-peptide

becomes processed to produce the mature version of hBD-1 that is composed of 36 amino acids only. This human β-defensin member; hBD-1, is the only defensin that is expressed in the tissues in a constant manner.[4]

Besides its main function as an active antimicrobial peptide, hBD-1 is also an immunomodulator that is upregulated in different inflammatory conditions suggesting its immunoregulatory effects.[5] It is involved in the chemoattraction and maturation of dendritic cells, facilitating their antigen uptake, processing and presentation actions which are essential in the pathogenesis of various allergic and autoimmune diseases.[6]

Although, the association between several HBD gene polymorphisms and the development of different inflammatory and autoimmune diseases has been studied,[7,8] there is a scarcity in literature discussing the hBD gene polymorphisms' role in vitiligo.

The current study aimed to evaluate the association between defensin beta 1 (DEFB1) (-44 C/G) and (-20 G/A) gene polymorphisms with the risk of vitiligo development, the extent of the disease and the response to NB-UVB treatment in a sample of Egyptian population.

2 | SUBJECTS AND METHODS

This prospective case-control study included 360 participants; 178 patients suffering from active non segmental vitiligo, in addition to 182 apparently healthy control subjects of matched age and sex. All subjects were recruited from the outpatient clinic and the phototherapy treatment unit of the Dermatology Department of Benha University Hospitals.

The study was approved by the local ethics committee on research involving human subjects of the Faculty of Medicine, Benha University. A written informed consent was obtained from each participant before samples collection.

Subjects with other autoimmune or inflammatory skin or systemic diseases as well as those with chronic infectious diseases or chronic systemic disorders (eg, hepatic, renal, cardiac... etc.) or malignancies were excluded from the study.

At the baseline visit, all patients were subjected to detailed history taking and clinical cutaneous examination to evaluate the extent of vitiligo using vitiligo area scoring index (VASI) score.[9] Gene polymorphisms in all participants were studied by RFLP PCR technique using the DNA extracted from the blood.

Included patients were treated in the phototherapy treatment unit in the Dermatology Department, Benha University. Patients received three narrowband UVB (NB-UVB) treatment sessions per week. Totonchy and Chiu protocol[10] was followed in adjusting the initial and the subsequent incremental UVB doses.

After 12 weeks of regular continuous NB-UVB treatment sessions, the patients were reevaluated clinically to assess the extent of the disease using VASI scoring again and to evaluate the type of repigmentation, if any.

2.1 | DNA extraction and genetic analysis

Two milliliter of venous blood were withdrawn in EDTA containing vacutainer under complete aseptic conditions from all cases and controls. DNA extraction was performed from peripheral blood leucocytes using the DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions then the extracted DNA samples were stored at −20°C.

The DEFB1 gene polymorphisms at positions -20 and -44 in the 5 untranslated region were determined by polymerase chain reaction-restriction fragment length polymorphism.

(PCR-RFLP). The DNA was amplified by PCR using a sense primer (5′-GTGGCACCTCCCTTCAGTTCCG-3′) and an antisense primer (5′CAGCCCTGGGGATGGGAAACTC-3′). The amplicon produced by PCR is 260 bp. PCR reaction was performed using total volume 60 μl using 100 ng of genomic DNA, 200 μM dNTPs, 75 μM of each primer and 0.5 U Taq polymerase (Invitrogen) in ×10 PCR buffer (Invitrogen) with 1.5 mM MgCl2.

The PCR program was an initial denaturing step at 95°C for 15 min followed by 34 cycles of denaturation at 95°C for 15 s, annealing for 30 s at 67°C and extension at 72°C for 30 s with a final extension at 72°C for 5 min.

After PCR amplification, the PCR product 260 amplicon was subjected to restriction digestion overnight at 37°C with 2 U Scr FI restriction endonuclease (New England BioLabs, Inc.) and 4 U Hga I restriction endonuclease (New England BioLabs, Inc.) to detect variations at positions -20 and -44, respectively.

Electrophoresis analysis of the digested PCR products was performed using 3% agarose gel stained with ethidium bromide. The digested RFLP product was used to identify the study subjects with the DEFB1 gene polymorphism at positions -20 and -44.

Regarding -20 G/A, the presence of two fragments of 254 and 6 bp indicated the presence of homozygous AA genotype while three fragments of 136, 118, and 6 bp indicated the presence of homozygous GG. The presence of four fragments of 254, 136, 118, and 6 bp product size confirmed the presence of for heterozygous AG genotype.

Regarding -44C/G genotype, the homozygous GG genotype should be represented by three fragments of 71, 30, and 159 bp while homozygous CC genotype should br represented by two fragments of 71 and 189 bp. The heterozygous CG genotype was represented by four fragments of 189,159, 30, and 71 bp.

To ensure validity of the test, the gel was assigned by two independent blind investigators, in addition the samples were run using internal positive and negative controls and 20% of samples was repeated randomly.

2.2 | Statistical analysis

The collected data were revised, coded, tabulated and statistically analyzed using the Statistical package for Social Science (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0.

Armonk, NY: IBM Corp). Data were presented and suitable analysis was done according to the type of data obtained for each parameter.

Shapiro-Wilk test was done to test the normality of data distribution. Significant data was considered to be nonparametric.

I. Descriptive statistics:

- Mean, SD (±SD) for numerical data.
- Frequency and percentage of nonnumerical data.

II. Analytical statistics:

- Student *T* Test was used to assess the statistical significance of the difference between two study group means.
- For the comparison of the three groups' means, one way analysis of variance (ANOVA) was used.
- Chi-square test was used to examine the relationship between two qualitative variables
- Fisher's exact test: was used to examine the relationship between two qualitative variables when the expected count is <5 in more than 20% of cells.
- Regression analysis: Logistic and linear regression analyses were used for prediction of risk factors. Odds ratio and 95% confidence interval were calculated. Odds ratio and 95% confidence interval were calculated. Odds ratios are used to determine whether a particular exposure is a risk factor for a particular outcome, OR = 1 Exposure does not affect the outcome, OR > 1 Exposure associated with higher risk of outcome; OR < 1 Exposure associated with lower risk of outcome.
- Deviations from Hardy-Weinberg equilibrium expectations were determined using the chi-squared test.

III. The power was calculated using CATS power calculator software program, developed by (Skol A.D. (2006), for case-control studies. We conducted power calculations under disease allele frequency of 0.33, disease prevalence of 2%, case-to-control ratio of 1:1, odds ratio of 1.768, level of significance of 0.05, sample size of cases of 178, and that of control of 182, revealed power of 89% (CaTS Power Calculator. Retrieved March 31, 2017, from http://csg.sph.umich.edu//abecasis/CaTS/).

IV. *P* value: *P* > .05: Nonsignificant.

3 | RESULTS

The current study included 178 active nonsegmental vitiligo patients, as well as 182 apparently healthy control subjects. There was nonsignificant difference between patients and control subjects regarding age (*P* = .27), gender (*P* = .76) and BMI (*P* = .104; Table 1).

The mean disease duration in the current sample was 12.6 ± 3.95 years. Regarding the clinical presentation of the disease, 126 patients (70.78%) had vitiligo vulgaris, while 52 patients only (29.21%) presented with acrofacial vitiligo. The most common Fitzpatrick skin type in the present patients were type III; 74 patients

TABLE 1 Sociodemographic criteria of the studied groups

	Patients 178	Control 182	Test	*P*
Age	32.43 ± 4.21	31.86 ± 5.65	*t* = −1.08	.27
Male	97 (54.49%)	103 (56.59%)	χ^2 = 0.16	.76
Female	81 (45.5%)	79 (43.40%)		
BMI	23.59 ± 6.23	22.41 ± 7.46	*t* = −1.62	.104

Note: χ^2, Chi-square test; *T*, Students test.

(41.75%) and type IV; 69 patients (38.76%). Types II and V represented 7.86% and 11.79% only of our sample.

Both control and patients' groups were in Hardy-Weinberg equilibrium regarding both SNPs. There was significant difference in the frequencies of the studied genotypes and alleles between vitiligo patients and control subjects groups. AA genotype of DEFB1 (-20G/A) was significantly less frequent in patients than in controls (*P* = <.0001) suggesting a protective role of this genotype against vitiligo development. Regarding DEFB1 (-44 C/G) SNP, the GG genotype and G allele were significantly predominant in vitiligo patients (*P* < .0001 and .009, respectively) increasing the risk of vitiligo development about two folds (Table 2). There was insignificant difference in the genotypes distribution of both studied SNPs between acrofacial vitiligo and vitiligo vulgaris (Table 3;Figure 1).

The percentage of improvement of patients with acrofacial vitiligo (22.6 ± 15.2) was significantly lower than that in the patients with vitiligo vulgaris (51.8 ± 19.7; ANOVA = 91.7; *P* = <.0001). However, DEFB1 (-20G/A) gene polymorphism was associated with significantly better therapeutic response in both vitiligo types, while DEFB1 (-44 C/G) gene polymorphism was associated with poorer response in both types (Table 4).

For more clarification of the different effects of these gene polymorphisms on the disease extent and the therapeutic response to NB-UVB phototherapy, patients were further classified into four groups according to their genotypes;

- Group I: The wild group which included 42 patients (23.6%) carrying the wild genotypes of both SNPs; DEFB1 (-20G/A) GG genotype and DEFB1 (-44 C/G) CC genotype.
- Group II: This group included 33 patients (18.5%) having polymorphism in DEFB1 (-20G/A) only; GA and GG genotypes and DEFB1 (-44 C/G) CC genotype.
- Group III: This group included 31 patients (17.4%) having polymorphism in DEFB1 (-44 C/G) only; CA and CG genotypes and DEFB1 (-20G/A) GG genotype.
- Group IV: This group included 72 patients (40.44%) who carry polymorphisms in both SNPS.

Patients carrying polymorphism in DEFB1 (-20G/A) only (Group II) showed significantly lower VASI scores (14.23 ± 2.77) and significantly higher percentage of improvement (66.12 ± 18.01%) after treatment when compared to other groups.

TABLE 2   DEFB1 -20G/A and DEFB1 -44 C/G genotypes and alleles frequencies in the studied groups

		Control N = 182		Cases N = 178				
		N	%	N	%	P	OR	95% CI
DEFB1 -20G/A	GG	52	28.6	73	41.0	–	1	(reference)
	GA	49	26.9	70	39.3	<.001	0.309	0.210-0.456
	AA	81	44.5	35	19.7	<.001	0.466	0.347-0.625
	GA + AA	130	71.4	105	59.0	.013	0.708	0.538-0.930
	G	153	42.0	216	60.7	–	1	(reference)
	A	211	58.0	140	39.3	<.001	0.624	0.518-0.751
DEFB1 -44 C/G	CC	114	62.6	75	42.1	–	1	(reference)
	CG	16	8.8	10	5.6	.016	1.926	1.131-3.279
	GG	52	28.6	93	52.2	.039	1.738	1.028-2.938
	CG + GG	68	37.4	103	57.9	.039	1.738	1.028–2.938
	C	244	67.0	160	44.9	–	1	(reference)
	G	120	33.0	196	55.1	<.001	1.768	1.466-2.132

Note: CI, confidence interval; OR, odds ratio.

TABLE 3   Genotypes distribution of both studied SNPs in acrofacial vitiligo and vitiligo vulgaris

The SNP	Genotypes	Acrofacial N = 52	Vulgaris N = 126			
		N (%)	N (%)	P	OR	95% CI
DEFB1 -20G/A	GG	24 (46.15)	49 (38.8)	–	1	(reference)
	GA + AA	28 (53.84)	77 (61.1)	.371	1.197	0.807-1.774
	P	0.36	0.37	–		
DEFB1 -44 C/G	CC	22 (42.3)	53 (42.1)	–	1	(reference)
	CG + GG	30 (57.69)	73 (57.9)	.976	1.006	0.679-1.492
	P	.97	.8	–		
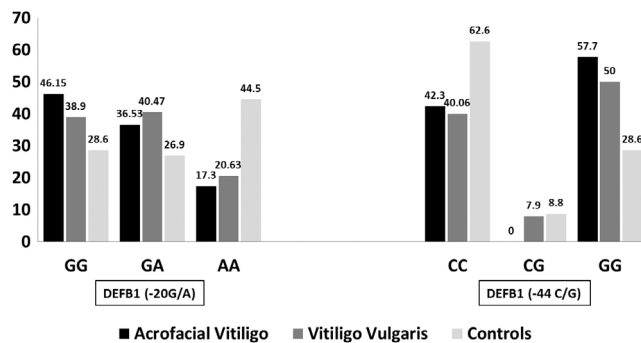
FIGURE 1   Genotypes distribution percentage in acrofacial vitiligo, vitiligo vulgaris and control subjects

While patients carrying polymorphism in DEFB1(-44 C/G) only (Group III) showed the highest baseline VASI scores (38.87 ± 6.7) and the lowest therapeutic response (23.79 ± 19.42%) among all patients groups. There was insignificant difference between the wild group (Group I) and the group with gene polymorphisms in both SNPs (Group IV) regarding the percentage of improvement in VASI scores following NB-UVB phototherapy (Table 5). In fact, it seems that the A allele of DEFB1 (-20G/A) is associated with significantly higher improvement percentage (48.4 ± 22.) when compared to the G allele (40.9 ± 23.5; *P* = .01), while the polymorphic allele of DEFB1(-44 C/G); the G allele is associated with significantly lower improvement percentage (34.9 ± 19.9) when compared to the wild allele (42.25 ± 22.9; *P* = .008). There was insignificant difference in the percentage of improvement in patients with different skin types; 40.87 ± 25.5 in skin type II, 42.8 ± 24.1 in type III, 43.02 ± 21.1 fin type IV and 47.6 ± 22.24.

In type V (*f* = 0.3, *P* = .8).

Marginal repigmentation was the most common type of repigmentation among Group II patients, while the diffuse repigmentation was the most common form in the other groups (Table 6).

4 | DISCUSSION

The studied SNPs are located within defensin beta 1 (DEFB1) gene at the short arm of chromosome 8 (8p23). DEFB1 gene is a constitutive gene which encodes human beta defensin-1; the "restless

TABLE 4 Percentage of improvement of both studied vitiligo types according to different genotypes

	DEFB1	Genotypes	% improvement	P	OR	95% CI
Acrofacial N = 52	-20G/A	GG	15.81 ± 14.9	–	1	(reference)
		GA + AA	28.46 ± 13.03	.023	1.425	1.050-1.933
	-44 C/G	CC	30.7 ± 18.8	–	1	(reference)
		CG + GG	16.6 ± 8.08	<.001	0.420	0.339-0.521
Vulgaris N = 126	-20G/A	GG	45.9 ± 17.4	–	1	(reference)
		GA + AA	55.6 ± 20.25	.021	1.210	1.030-1.422
	-44 C/G	CC	64.8 ± 13.06	–	1	(reference)
		CG + GG	42.5 ± 18.4	<.001	0.656	0.567–.759

TABLE 5 VASI scores before and after treatment in all patients

The patients groups	N (%)	VASI score	VASI after	% of improvement	P	OR	95% CI
Group I; Wild group	42 (23.6)	23.81 ± 4.37	13.14 ± 5.31	45.12 ± 19.16	–	1	(reference)
Group II; polymorphism in DEFB1 (-20 G/A) only	33 (18.5)	14.23 ± 2.77	4.52 ± 2. 17	66.12 ± 18.01	<.001	0.465	0.370-0.583
Group III; polymorphism in DEFB1 (-44 C/G) only	31 (17.4)	38.87 ± 6.7	28.04 ± 3.89	23.79 ± 19.42	.009	1.777	1.644-1.938
Group IV; polymorphism in both SNPs	72 (40.44)	28.15 ± 9.56	16.09 ± 5.482	39.8 ± 18.25	.017	1.310	1.049-1.637
ANOVA	–	66.78	141.46	30.28			
P value	–	.00001	<.00001	<.00001			

TABLE 6 Type of repigmentation in the different patients groups

		Type of repigmentation				
The patients groups	N (%)	Marginal	Diffuse	Perifollicular	Chi square	P value
Group I; Wild group	42 (23.6)	1 3(30.9)	25 (59.5)	4 (9.5)	13.15	.04
Group II; polymorphism in DEFB1 (-20G/A) only	33 (18.5)	17 (51.5)	9 (27.3)	7 (21.21)		
Group III; polymorphism in DEFB1 (-44 C/G) only	31 (17.4)	8 (25.8)	14 (45.16)	9 (29)		
Group IV; polymorphism in both SNPs	72 (40.44)	19 (26.38)	36 (50)	17 (23.61)		

warrior" which is an active soldier in the continuous battle of the body against microbes and malignant changes. It can be upregulated in various inflammatory and infectious conditions. Single-nucleotide polymorphisms (SNPs) of this gene can occur at different sites of the first exon's 5′ noncoding region. DEFB1 gene polymorphisms at this region regulate the levels of the gene expression and can also affect the activity of its product; hBD-1. Among the described SNPs, rs1800972 (-44C/G), and rs11362 (-20A/G) have been studied in different infectious and autoimmune diseases.[4,11,12]

DEFB1 (-20G/A) (rs11362) is located at 6877877 base and DEFB1(-44 C/G) (rs1800972) is located at 6877901 base, with <1 kb apart.[13] DEFB1 (-20G/A) has A and G alleles. The wild allele is the G allele while the polymorphic allele is the A allele. The A allele decreases the levels of hBD-1 expression at the tissue level.[8]

The current study revealed that DEFB1 (-20G/A) AA genotype showed significantly lower frequency in vitiligo patients when compared to the control subjects. In addition, DEFB1 (-20G/A) polymorphism was significantly associated with less extensive disease

according to VASI scores when compared to the other patients suggesting the protective effect of this genotype against vitiligo development. Ochoa-Ramírez et al[5] also suggested the protective effect of DEFB1 (-20G/A) AA genotype against vitiligo, however, its relation to the diseases extent was not studied. Similar protective effect of the same genotype was reported against other autoimmune diseases, for example, SLE,[7] Crohn disease,[8] and type-II diabetes mellitus.[14]

Regarding the DEFB1 (-44C/G) SNP (rs1800972), the G allele -the polymorphic allele- increases the production of HBD-1 via enhancing DEFB1 mRNA transcription rates.[15] In the current study, DEFB1(-44 C/G) GG genotype as well as the G allele showed significantly higher frequency in vitiligo patients when compared to the control subjects and also were significantly associated with the highest VASI scores among all patients. Ochoa-Ramirez et al[5] also found that the G allele and GG genotype at position -44 were associated with an increased risk of developing vitiligo.

Multiple clinical,[16] laboratory and genetic factors[17] have been previously suggested to affect the response of vitiligo to NB-UVB phototherapy. Our vitiligo patients were classified as mentioned

before into four groups to study the effect of the currently studied gene polymorphisms on the response to NB-UVB phototherapy. After 12 weeks of phototherapy, patients with polymorphism in HBD (-20G/A) only (group II) showed the highest percentage of improvement, while those with HBD (-44C/G) polymorphism only showed the lowest percentage of improvement.

The protective or the provocative effects of different polymorphisms in the same gene and the association with different therapeutic responses can be attributed to the variable impact of different polymorphisms on the hBD-1 expression levels—as discussed before—with the subsequent changes in its active participation in the process of autoimmunity. DEFB1 expression is constitutive, however, it can be upregulated in infectious or inflammatory conditions.[5] Upregulation of HBD-1 can play a role in the local inflammatory reactions via chemoattracting different inflammatory cells either directly through chemokine receptor (CCR)6 signaling or indirectly by inducing hBD-3 upregulation. The chemoattracted inflammatory cells include neutrophils, T cells and immature dendritic cells.[18] Beside attracting the immature dendritic cells; the key cells in many autoimmune reactions, hBD-1 enhances the maturation of these cells and activates them by upregulating the CD91 scavenger receptor expression on their surfaces.[19] These effects would potentiate the main functions of dendritic cells; antigen uptake, processing and presentation, with subsequent induction of antigen-specific immune responses.[20] The recruitment and activation of dendritic and memory T cells together with the environment full of "danger" signals (eg, oxidative stress and elevated interleukins 6 and 8 as well as heat shock protein 70) may be associated with the autoantigen specific reaction against melanocytes.[5] Moreover, the disturbed levels and tissue expression of hBD-1 associated with its gene polymorphism may affect the function of melanocrtin receptors. Melanocortin receptors are among the regulators of melanogenesis, and hBD-1 has been suggested as one of the intrinsic ligands of these receptors.[21]

It was surprising to find out that the extent of the disease in group IV patients was midway between that of groups II and III patients. Moreover, there was nonsignificant difference between the percentage of improvement in patients with wild genotypes (group I) and those with both polymorphisms (group IV). It seems like the protective effect of DEFB1 (-20G/A) counteracts the provocative effect of DEFB1 (-44C/G) polymorphism.

There are three clinical patterns of repigmentation reported in vitiliginous patches undergoing treatment; marginal, perifollicular and diffuse. In the current sample, the most common repigmentation pattern in group II was the marginal pattern, while the diffuse pattern predominated in the three other groups. This suggests a better prognosis in patients with HBD (-20G/A) polymorphism as the marginal pattern is the most stable pattern as reported previously by Parsad et al.[22]

Several treatment options are now available for management of vitiligo, however, the therapeutic response is highly variable between patients. Moreover, each treatment option may have its own limitations and unwanted side effects. That's why finding new options is necessary. The new options would never emerge unless we try to understand the pathogenesis of the disease better and to discover new players in its complicated process. Targeting new players may provide a new hope for the patients of this devastating disfiguring disorder.

5 | CONCLUSION

Polymorphisms at different SNPs of this gene can modify the susceptibility to vitiligo development, the extent of the disease and its response to NB-UVB phototherapy differently according to the effect of this polymorphism on the gene expression. These findings may help better understanding of the complex pathogenesis of vitiligo and suggest a promising new therapeutic target for managing this disfiguring disorder.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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