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Heterogeneous spectrum of *EXT* gene mutations in Chinese patients with hereditary multiple osteochondromas

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Abstract

Hereditary multiple osteochondroma (HMO) is one of the most common genetic skeletal disorders. It is caused by mutations in either *EXT1* or *EXT2* resulting in abnormal skeletal growth and morphogenesis. However, the spectrum and frequency of *EXT1* and *EXT2* mutations in Chinese patients with HMO was not previously investigated.

Mutations were identified by performing Sanger sequencing analysis of the complete coding regions and flanking intronic sequences of *EXT1* and *EXT2*, followed by multiplex ligation-dependent probe amplification (MLPA) analysis to detect gene deletions or duplications that could not be identified by the Sanger sequencing method.

The present study identified pathogenic mutations in 93% (68/73) of unrelated HMO probands from 73 pedigrees. Mutations in *EXT1* and *EXT2* were identified in 53% (39/73) and 40% (29/73) of families. We identified 58 distinct mutations in *EXT1* and *EXT2*, including 20 frameshift mutations, 16 nonsense mutations, 7 missense mutations, 9 splice site mutations, 5 large deletions, and 1 in-frame deletion mutation. Twenty-six of these mutations were novel and 32 were previously reported. Most of the mutations in *EXT1* were base deletions or insertions (21/33), whereas the majority of those in *EXT2* were single base substitution (18/25).

Complete sequencing of both the *EXT1* and *EXT2* followed by MLPA analysis is recommended for genetic analysis of Chinese patients with HMO. This study provides a comprehensive characterization of the genetic aberrations found in Chinese patients with HMO and highlights the diagnostic value of molecular genetic analysis in this particular disease.

Abbreviations: HGMD = Human Gene Mutation Database, HMEs = hereditary multiple exostoses, HMO = hereditary multiple osteochondroma, HS = heparan sulfate, MLPA = multiplex ligation-dependent probe amplification, OMIM = Online Mendelian Inheritance in Man, SCMC = Shanghai Children's Medical Center.

Keywords: Chinese, *EXT1*, *EXT2*, gene mutation, hereditary multiple osteochondromas

1. Introduction

Hereditary multiple osteochondroma (HMO), previously known as hereditary multiple exostoses (HMEs), is characterized by the growth of multiple osteochondromas (benign, cartilage-capped bone tumors) mainly in the metaphyses and diaphyses of long bones.[1,2] Patients with HMO may present a variety of orthopedic deformities such as malformations of the forearm, inequality in limb length, varus or valgus angulation of the knee, deformity of the ankle, and disproportionate short stature.[3] The risk of malignant degeneration to osteochondrosarcoma increases with age, although the lifetime risk of malignant degeneration is low (~1%).[1,2] The prevalence of HMO is estimated to be at least 1 in 50,000 in the general population[4] and seems to be higher in males (male to female ratio 1.5:1).[3]

The HMO is inherited in an autosomal dominant manner.[1] Two genes, *EXT1* (OMIM: 608177) and *EXT2* (OMIM: 608210), which are located respectively at 8q24.11 and 11p11.2, have been identified to cause HMO.[5–7] These genes encode homologous, Golgi-associated glycosyltransferases that are involved in the chain elongation step of heparan sulfate (HS) biosynthesis.[8,9] Studies have indicated that HS influences various important processes in skeletogenesis, skeletal growth, and morphogenesis.[8,9] Loss-of-function mutations in *EXT1* and *EXT2* lead to HS deficiency.[8,9] Hundreds of mutations have been reported in the Human Gene Mutation Database (HGMD,

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JW and JT contributed equally to this work and should be considered as co-first authors.

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Informed consent was obtained from all individual participants included in the study.

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http://www.hgmd.cf.ac.uk/ac/search.html) and Multiple Osteochondromas Mutation Database (MOdb, http://medgen.ua.ac.be/LOVD). Although most reported mutations involve one or several base changes in patients with HMO, the Sanger method of DNA sequencing is unable to detect all types of mutations.

In the present study, Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA) were employed to determine the spectrum of mutations in *EXT1* and *EXT2* of 73 Chinese patients diagnosed with HMO.

2. Patients and methods

2.1. Patients

The study was approved by the ethics committee of Shanghai Children's Medical Center (SCMC), and written informed consent was obtained from the patients' parents or guardians. Patients were initially diagnosed on the basis of radiology, and genetic analysis of the patients was performed subsequently. A personal medical history and physical examination were conducted. The diagnosis of HMO was confirmed by clinical and radiographic findings of multiple exostoses (arising from the area of the growth plate in the juxtaphyseal region of long bones or from the surface of flat bones), with the majority of cases have a positive family history.[1–4]

2.2. Sanger method of DNA sequencing

Genomic DNA was extracted from peripheral blood samples of the probands and family members using a QIAamp Blood DNA mini kit (Qiagen GMBH, Hilden, Germany). Primers used in the amplification of *EXT1* and *EXT2* (GenBank accession numbers NM_000127.2 and NM_207122.1, respectively) were designed using the Primer3 online software (primer sequences are listed in Table 1). All exons and exon–intron boundaries of each gene were amplified by polymerase chain reaction (Takara Bio, Dalian, China). The amplified products were purified from an agarose gel using a QIAquick Gel extraction kit (Qiagen GMBH) and sequenced via an ABI3730XL sequencer (Applied Biosystems, Foster City, CA). An additional group of 105 Chinese patients without skeletal deformities were recruited as ethnicity-matched controls to examine the allele frequencies of various *EXT1* and *EXT2* sequence variants.

2.3. Copy number variation analysis

The MLPA analysis was performed according to the manufacturer's protocol using the "SALSA MLPA probemix P215-B2 EXT" kit (MRC Holland, Amsterdam, the Netherland). The probe mix included in this kit contains 41 different probes with amplification products between 130 and 453 bp, including 13 specific probes for *EXT1*, 16 specific probes for *EXT2*, and 12 reference probes for the control. Data analysis and interpretation were performed using GeneMarker software (Softgenetics, State College, PA). This program identifies peaks as "normal" when the ratio of sample peak height or area to that of control samples falls in the range from 0.75 to 1.33; as "deleted" when the ratio is <0.75, and as "duplicated" when the ratio is >1.33.

2.4. In silico prediction of the novel missense mutations

In silico prediction of the identified variant was performed using prediction tools. Alamut Visual 2.7.1 (http://www.interactive-biosoftware.com/alamut-visual/, which included tools such as

Table 1

Sanger sequencing primers for *EXT1* and *EXT2* genes.

Exon	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product size, bp
EXT1 gene (NM_000127.2)			
1_1	GGAAAGGCATCCAGAGAAGGT	GCACATACTGAGGTGACAACTGG	662
1_2	ATCGAGGGCTCCAGGTTCTAC	ATTTGCTCAGTTCCAGGCTCA	690
2	ACTGGGCAAACCAAATTGTTG	TAGGCCAAGCTGGCAATTAGA	640
3	CAAGGCCAGTCGTCTCTATGG	GCTCCCATTCTTTACCTGCAA	636
4	GGTTGTTCATGTGCAAGGTCA	CCATGGCAAAGCAGGTAAAAG	610
5	TTTTGGAATGAGCATGGACTC	TGCAATGCTCTGCTCTGTTTT	605
6	GGCAAAGGATGTCAAAGCAAG	AACGAGGCAGGATGAATGAAA	519
7	CCGGACACAGTTGGTTTTGTT	TCAAGACCCAGATTTCCCTGA	546
8	GTTGCTCCATCCTGTGGTCTC	GCAAGGTGCTAACAGGAATCG	606
9	TTATGGGGCAAAATGTCAAGC	TGCCAAGAGGTTTCACTGGTT	510
10	CCTGCCTTGTAGGCTCCTTATG	TGGGTGGAACAGCTAGAGGAA	506
11	CTTGGTCCCAAGTGCAAAGAG	CACAATCTGGCTCTGCTGATG	627
EXT2 gene (NM_207122.1)			
1 (noncoding)			
2	TTCAAGTGTCATTTGCCATCC	CCCTTCCCTTTAGTTCCCTGA	696
3	GCAGGTCTGTATGGGACAAGC	GCACAATCCAGAGTGGGAAAA	554
4	GGGAGGTAGCAGAGAGGCTGT	CTCAGTGCCTCAAGGACCCTA	537
5	CATGCGCTCTCAGCTTAGCAT	TTCGCTGGGCTCAATTTTAAC	566
6	TTTCAGAAGGCCAACAGTGGT	GCCTTGGTTTGTGAACTGCTC	561
7	TGGAGGCAGGGTGAAAGATTA	CATTCAGCTCCTGTCCCTCTG	425
8	CACCCCCATCCCTACAACTTT	AAGTCACCGGGATGTCTTTGA	553
9	GCAAATTTTGAGGAGGGGAAG	GAGAAAAATGGAGGCATGCTG	480
10	AGAGCCGTGGATACAAGCTGA	GCACAGTTGCCATTTTGGAAT	488
11	GGAACATCTCCAGAATCCCATT	GCAAGCTGGAAATAGCACCTG	557
12	GGTCACTTGACCAAAAGCATTC	CAATGTGACCGCATCAATCAT	459
13	TCGCCCTTATGGCTACAAGAA	TGCACATGGAGGTGACTATGG	547
14	AGAACCTGGGAGCAGACTGTG	CTTCCACTTGGCATTTTCGAG	554

Table 2

Spectrum of *EXT1* and *EXT2* mutations in Chinese patients with hereditary multiple osteochondromas.

Gene	Patient ID	Genotype	Novel	Gene	Patient ID	Genotype	Novel
EXT1	19	c.194_195delinsAA; p.Phe65*	Yes	*EXT2*	33	c.67C>T; p.Arg23*	
	41,84	c.247dupC; p.Arg83Profs*106			85	c.382C>T; p.Arg128Trp	
	15	c.335delA; p.Asn112Thrfs*24			7	c.398T>G; p.Leu133Arg	
	93	c.354dupA; p.Tyr119Ilefs*70	Yes		37	c.426C>G; p.Tyr142*	Yes
	17	c.361C>T; p.Gln121*			12	c.544C>T; p. Arg182*	
	67	c.635delG; p.Gly212Alafs*40	Yes		38	c.594_621del; p.Asp198Glufs*63	Yes
	3	c.651_664delinsTTT; p.Lys218Leufs*3			29	c.626+2_626+5delTAGG	Yes
	90	c.659G>A; p.Ser220Asn			55	c.627-2_630delinsT	Yes
	6	c.680delG; p.Arg227Hisfs*25			71	c.666C>G; p.Tyr222*	
	42	c.838_839delAG; p.Arg280Glufs*8	Yes		34	c.678C>A; p.Tyr226*	Yes
	27	c.942_943delAG; p.Asp315Glnfs*5	Yes		23	c.743+1G>T	
	56	c.1019G>A; p.Arg340His			10	c.751C>T; p.Gln251*	
	13	c.1108G>T; p.Glu370*			43	c.800_801delAG; p.Glu267Glyfs*18	Yes
	59	c.1165-1G>T	Yes		78,87	c.910C>T; p.Q304*	Yes
	11	c.1182delG; p.Arg394Serfs*9			45	c.924C>A; p.Tyr308*	Yes
	36	c.1431dupC; p.Ser478Leufs*43			62	c.925_928dupCCAC; p.Gln310Profs*46	Yes
	32,60	c.1469delT; p.Leu490Argfs*9			89	c.940-2A>G	Yes
	22	c.1469dupT; p.Val491Glyfs*30	Yes		4,5,9,61	c.1016G>A; p.Cys339Tyr	
	54	c.1551G>A; p.Trp517*			25	c.1072T>C; p.Trp358Arg	Yes
	1,26	c.1567delC; p.Leu523Tyrfs*24			24	c.1075A>T; p.Lys359*	Yes
	53	c.1705delG; p.Val569Cysfs*52	Yes		73	c.1181G>A; p.Trp394*	
	2	c.1722+2T>G			57	c.1182delG; p.Trp394Cysfs*42	Yes
	70	c.1723-2A>G	Yes		44	c.1188G>A; p.Trp396*	
	28	c.1784_1785delGC; p.Arg595Glnfs*6			65	c.1758G>A; p.Trp586*	Yes
	18	c.1879_1881delCAC; p.His627del			16	All Exon deletion	
	77	c.1883+1G>A					
	86	c.1884-1G>C					
	94	c.1911C>A; p.Tyr637*	Yes				
	58	c.1930A>T; p.Asn644Tyr	Yes				
	81	Exon1 deletion					
	48,83	Exon2-11 deletion					
	80	Exon4 deletion	Yes				
	30,74,79	All Exon deletion					

SIFT, PolyPhen-2, and MutationTaster) was used for the identification of novel missense mutations.

3. Results

3.1. Mutations identified

Between August 2008 and December 2015, 73 patients from different families with HMO were recruited at Shanghai Children's Medical Center (SCMC) China, including 50 boys and 23 girls; the mean age was 7.38 years (range 4.8–15.1 years). Sanger sequencing and MLPA were used for genetic analysis and revealed mutations in 93% of these probands (68/73; Table 2). We detected 33 different *EXT1* mutations in 39 families and 25 different *EXT2* mutations in 29 families. Among the 58 *EXT1/EXT2* mutations, 26 were novel, whereas 32 had been previously reported. The mutation types included 20 frameshift (16 in *EXT1* and 4 in *EXT2*), 16 nonsense (3 in *EXT1* and 13 in *EXT2*), 7 missense (3 in *EXT1* and 4 in *EXT2*), 9 splice site (5 in *EXT1* and 4 in *EXT2*), 5 large deletions (4 in *EXT1* and 1 in *EXT2*), and 1 small in-frame deletion (*EXT1*) (Fig. 1).

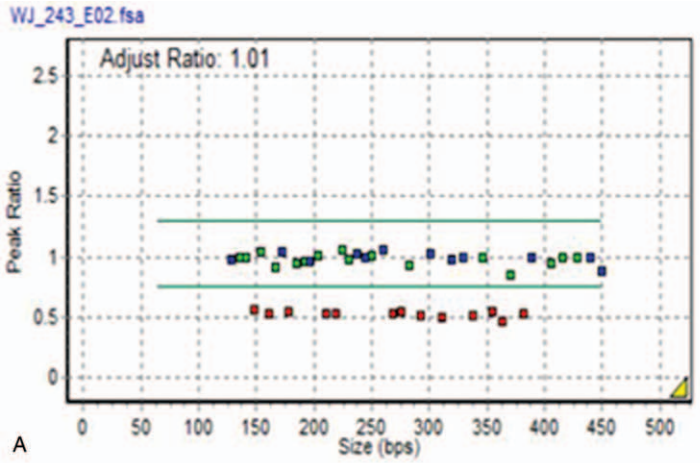
3.2. Pathologic prediction of novel missense mutations

Of the 26 novel mutations, 24 affected the length of the EXT protein by introducing a premature stop codon or changing the conserved splice site positions and thus were considered pathogenic. These mutations were not detected in 307 unrelated control individuals, nor were they reported in the Exome Aggregation Consortium (ExAC) database (http://exac.broadinstitute.org/).

In the case of the 2 novel amino acid substitutions (*EXT1*: c.1930A > T; p.Asn644Tyr; *EXT2*: c.1072T > C; p.Trp358Arg), the possibility that the changes represented nonpathologic polymorphisms was examined. The effect of amino acid changes on protein stability was examined using Alamut software. The prediction results are listed in Table 3. Both variants were predicted to be harmful to protein function with different in silico tools. In addition, the 2 patients' parents were wild type indicating the de novo status of the 2 variants and offering strong pathogenic evidence for their causal relationship to the disease.

4. Discussion

The HMO is an autosomal, dominant, benign tumor that is characterized by abnormal growth of long bone cartilaginous caps (osteochondroma). It is one of the most common skeletal dysplasias in adolescents. About 80% of patients with HMO develop clinical symptoms before 10 years of age and it can cause various complications, leading to skeletal deformities and short stature.[1,10,11]



B

	Probe Name	Bin Size	WJ_243_E02
1	Ctrl_05q31	128.1	1.027
2	Ctrl_06p12	196.7	0.996
3	Ctrl_12p13	319.8	0.999
4	Ctrl_13q12	299.6	1.052
5	Ctrl_16q23	171.8	1.099
6	Ctrl_18q11	237.5	1.028
7	Ctrl_19q12	243.7	1.043
8	Ctrl_1p22	329.0	1.018
9	Ctrl_21q11	438.7	0.989
10	Ctrl_22q11	449.8	0.991
11	Ctrl_2q13	259.3	1.060
12	Ctrl_5p13	388.9	1.000

C

13	EXT1_A_E1	291.8	0.524
14	EXT1_B_E1	336.8	0.573
15	EXT1_C_E2	275.4	0.546
16	EXT1_D_E3	310.4	0.540
17	EXT1_E_E4	381.4	0.518
18	EXT1_F_E5	210.4	0.588
19	EXT1_G_E6	147.5	0.581
20	EXT1_H_E7	160.4	0.563
21	EXT1_I_E8	177.5	0.584
22	EXT1_J_E9	218.7	0.544
23	EXT1_K_E10	363.2	0.497
24	EXT1_L_E11	268.4	0.573
25	EXT1_M_E11	354.5	0.566

D

26	EXT2_A_E1	154.0	1.036
27	EXT2_B_E2	224.6	1.088
28	EXT2_C_I2	282.7	0.978
29	EXT2_D_E3	250.2	1.064
30	EXT2_E_E4	184.4	1.010
31	EXT2_F_E5	346.4	1.014
32	EXT2_G_E6	406.1	1.001
33	EXT2_H_E7	203.2	1.051
34	EXT2_I_E8	191.1	1.036
35	EXT2_J_E10	166.1	1.000
36	EXT2_K_E11	415.1	0.987
37	EXT2_L_E12	370.4	0.910
38	EXT2_M_E13	135.8	1.039
39	EXT2_N_E14	141.0	1.014
40	EXT2_O_E15	230.2	1.007
41	EXT2_P_E16	427.7	0.991

Figure 1. Example of MLPA detection results in *EXT1/EXT2*. Heterozygous deletion of the entire *EXT1* in patient 30. (A) Distribution of the peak ratio of all probes. (B) The peak ratio values of control probes. (C) The peak ratio values of *EXT1* probes. (D) The peak ratio values of *EXT2* probes.

Table 3

In silico evaluation of novel missense mutations in the *EXT1* and *EXT2* genes.

Mutation type	Alamut software describe	Pathogenicity clues splicing predict
EXT1: c.1930A>T; p.Asn644Tyr	Transversion from A to T in exon 10. Missense substitution. Asn at position 644 is changed to Tyr	• Highly conserved nucleotide (phyloP: 0.99 [−5.2;1.1]) • Moderately conserved amino acid (considering 15 species) • Large physicochemical difference between Asn and Tyr (Grantham dist.: 143 [0–215]) • This variant is in protein domains: *EXTL2*, α-1,4-*N*-acetylhexosaminyltransferase Nucleotide-diphospho-sugar transferases • Align GVGD: C0 (GV: 353.86 - GD: 0.00) • SIFT: Deleterious (score: 0.03, median: 4.32) • MutationTaster: disease causing (*P*-value: .977) • PolyPhen-2: Possibly damaging (sensitivity: 0.82, specificity:0.81)
EXT2: c.1072T > C; p.Trp358Arg	Transition from T to C in exon 6. Missense substitution. Trp at position 358 is changed to Arg	• Highly conserved nucleotide (phyloP: 0.99 [−5.2;1.1]) • Highly conserved amino acid, up to Fruitfly (considering 11 species) • Moderate physicochemical difference between Trp and Arg (Grantham dist.: 101 [0–215]) • This variant is in protein domain: Exostosin-like • Align GVGD: C0 (GV: 268.54 - GD: 82.54) • SIFT: Deleterious (score: 0, median: 3.63) • MutationTaster: disease causing (*P*-value: 1) • PolyPhen-2: Probably damaging (sensitivity: 0.00, specificity: 1.00)

The EXT family of genes has been implicated in the pathogenesis of HMO. At least 5 members have been cloned and defined. *EXT1* (NM_000127.2) consists of 11 exons that encode a 746-amino-acid protein, whereas *EXT2* (NM_207122) comprises 14 exons that encode a 718-amino-acid protein. *EXT1* and *EXT2* are widely expressed genes and are highly homologous, particularly in terms of the carboxyl terminal sequences.[12] HS, which is modified by EXT1 and EXT2 proteins, is generally distributed on the cell surface and the extracellular matrix. As a co-enzyme, HS is involved in cell adhesion, blood coagulation, and angiogenesis, as well as in the regulation of cell growth factors and various other biologic processes. In addition, based on its sulfate-modifying function, HS is involved in the distribution of various signal proteins in target cells, including a bone formation protein family (bone morphogenetic proteins), that are expressed in the bone growth plate. Research evidence suggests that mutations in either *EXT1* or *EXT2* may trigger the abnormal synthesis of the HS chain, which in turn leads to the development of HMO.[8,13–16] Moreover, 3 EXT-like genes (*EXTL1*, *EXTL2*, and *EXTL3*) are also involved in HS synthesis and are believed to be correlated with HMO, although no mutations of these have been detected in patients with HMO.[17,18]

Previous reports indicated that mutations in either *EXT1* or *EXT2* are responsible for nearly 90% of HMO cases, of which the majority involve *EXT1* (60–70%). *EXT1* mutations are more often found in the first 6 exons, whereas those of *EXT2* involve the first 8 exons. Mutations in *EXT1* are the most frequent cause of HMO in Europe and North America.[19,20] The reported mutation frequencies of *EXT1* and *EXT2* are apparently similar (39/68 vs 29/68), yet differ from those observed in 36 Chinese families (14% in *EXT1* and 33% in *EXT2*).[21] These findings suggest that regional differences in *EXT1* and *EXT2* mutation frequencies occur in China. Moreover, the use of MLPA has dramatically increased the detection of mutations: it can identify large fragment deletions and duplications based on hybridization and ligation, followed by amplification of the ligation products.

To date, 463 mutations in *EXT1* have been reported, including 154 missense/nonsense mutations, 223 small insertion/deletion mutations, 50 splice site mutations, and 36 other types, whereas mutations in *EXT2* consist of 71 missense/nonsense mutations, 94 small insertion/deletion mutations, 24 splice site mutations, and 27 other types (data from HGMD). The percentage of small insertion/deletion mutations is higher than others in both *EXT1* and *EXT2*. In the present study, most of the detected *EXT1* mutations were base deletions or insertions (21/33). In contrast, the major mutation type in *EXT2* involved single-base substitution (18/25). These findings add new insights into the spectrum of *EXT* mutations in Chinese patients with HMO. Furthermore, we detected 26 novel mutations in *EXT1* and *EXT2*, which may be utilized in the clinical diagnosis of HMO.

Similar to most other single-gene inherited diseases, most of the mutations in *EXT1* and *EXT2* in HMO are caused by changes in a single or several bases, including missense/nonsense, splice site, and small insertion or deletion mutations.[11] These small mutations are identified by using the Sanger sequencing technology; however, a few large gene fragments often go undetected and thus may result in an incorrect clinical diagnosis. Here, we introduced the MLPA assay to improve our detection specificity, as it can identify large fragment deletions and duplications (copy number aberrations) based on hybridization and ligation, followed by the amplification of the ligation products.[22–24] Eight novel large deletions in *EXT1* and *EXT2* were detected by Sanger sequencing and verified by using the MLPA assay, with 93% of patients with HMO receiving verified diagnostic results. The limitation of this study is that no *EXT1* or *EXT2* mutations were detected in 5 patients with HMO, indicating that other genes that yet have to be identified may be responsible for this particular disorder. Next generation sequencing may be the best approach in identifying the candidate genes.[25] In addition, this study only includes 73 samples that might result in the statistics bias of the variation spectrum. Moreover, functional studies of the missense mutations need be performed in the future studies, which can better understand the pathogenicity of these mutations.

In conclusion, we have successfully detected pathogenic mutations in 93% (68/73) of unrelated probands from 73 pedigrees. A total of 58 mutations were identified, which included 26 novel mutations. The range of *EXT* mutations slightly varied from those previously reported in Chinese and other ethnic groups around the world. Molecular genetic analysis is useful for the clinical diagnosis and genetic counseling of patients with HMO.

Author contributions

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