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Genetic Analysis of Korean Adult Patients with Nontuberculous Mycobacteria Suspected of Primary Ciliary Dyskinesia Using Whole Exome Sequencing

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Purpose: Nontuberculous mycobacteria (NTM) is ubiquitous in the environment, but NTM lung disease (NTM-LD) is uncommon. Since exposure to NTM is inevitable, patients who develop NTM-LD are likely to have specific susceptibility factors, such as primary ciliary dyskinesia (PCD). PCD is a genetically heterogeneous disorder of motile cilia and is characterized by chronic respiratory tract infection, organ laterality defect, and infertility. In this study, we performed whole exome sequencing (WES) and investigated the genetic characteristics of adult NTM patients with suspected PCD.

Materials and Methods: WES was performed in 13 NTM-LD patients who were suspected of having PCD by clinical symptoms and/or ultrastructural ciliary defect observed by transmission electron microscopy. A total of 45 PCD-causing genes, 23 PCD-candidate genes, and 990 ciliome genes were analyzed.

Results: Four patients were found to have biallelic loss-of-function (LoF) variants in the following PCD-causing genes: *CCDC114*, *DNAH5*, *HYDIN*, and *NME5*. In four other patients, only one LoF variant was identified, while the remaining five patients did not have any LoF variants.

Conclusion: At least 30.8% of NTM-LD patients who were suspected of having PCD had biallelic LoF variants, and an additional 30.8% of patients had one LoF variant. Therefore, PCD should be considered in patients with NTM-LD with symptoms or signs suspicious of PCD.

Key Words: Primary ciliary dyskinesia, nontuberculous mycobacteria, whole exome sequencing

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INTRODUCTION

Nontuberculous mycobacteria (NTM) are ubiquitous organisms commonly isolated from environmental sources.[1] Since exposure to these organisms is inevitable, otherwise healthy individuals who develop NTM lung disease (NTM-LD) are likely to have specific susceptibility factors that make them vulnerable to these infections. Of particular note, NTM-LD is associated with disorders of mucociliary clearance, such as primary ciliary dyskinesia (PCD).[2]

PCD is a genetically heterogeneous disorder of motile cilia that is characterized by chronic respiratory tract infection, organ laterality defect, and infertility.[3] The 9+2 motile cilia are located on the apical surface of epithelial cells in the upper and lower respiratory tracts, paranasal sinuses, middle ear, ventricles of the central nervous system, fallopian tubes, and sperm flagella.[4] Therefore, defects in 9+2 motile cilia lead to recurrent respiratory infections, chronic sinusitis, chronic otitis media, hydrocephalus, and infertility. Recurrent respiratory infections, chronic sinusitis, and chronic otitis media lead to bronchiectasis, anosmia, and hearing loss, respectively. On the other hand, 9+0 motile cilia are located in the nodal cilia, which are responsible for left-right asymmetry during embryogenesis.[5,6] Therefore, defects of 9+0 motile cilia lead to laterality defect.

PCD patients are vulnerable to respiratory infections due to impaired mucociliary clearance. Approximately 18% of PCD patients have been reported to have at least one positive sputum culture for NTM.[7] In addition, ciliary abnormalities, such as reduced nasal nitric oxide production and low ciliary beat frequency, were observed in NTM-LD patients.[8] Therefore, PCD is considered a genetic susceptibility factor for NTM-LD.[9,10]

In general, neonatal respiratory distress occurs in about 80% of PCD patients within the first 24 hours of life.[11] Therefore, PCD should be suspected in full-term infants who have unexplained neonatal respiratory distress, especially when accompanied by an organ laterality defect. The median age at PCD diagnosis was 5.3 years in pediatric patients.[12] However, diagnosis can be delayed by the lack of knowledge of PCD and a low level of suspicion. Moreover, the mean age at PCD diagnosis was delayed to 17.2 years when adult patients were included.[13] In addition, the prevalence of PCD in adult bronchiectasis patients ranged from 1% to 13%,[14-22] which was higher than the estimated prevalence of PCD, 1:10000–20000 live births.[12] Therefore, there might be several undiagnosed adult PCD patients, and PCD should be considered not only in children, but also in adults.

In this study, we performed whole exome sequencing (WES) for adult NTM-LD patients suspected of having PCD. We analyzed the known PCD-causing genes and investigated the prevalence of PCD in patients with NTM-LD. Furthermore, we aimed to discover novel relationships between PCD and PCD-candidate/ciliome genes in humans.

MATERIALS AND METHODS

Study population

From April 2015 to April 2019, 15 adult NTM-LD patients with suspected PCD were included in this study. All patients met the criteria for NTM-LD according to the American Thoracic Society guidelines.[23] Clinical suspicion of PCD was based on the clinical criteria for PCD diagnosis of the PCD Foundation: daily wet cough/bronchiectasis, daily nasal congestion/pansinusitis, and laterality defect.[24] Patients with two or more symptoms/signs were suspected to have PCD. Two patients who had only one symptom/sign were excluded. Finally, a total of 13 patients were included in this study, and the clinical characteristics and transmission electron microscopy (TEM) findings were obtained. The informed consent was obtained from all participants. This study was approved by the Samsung Medical Center Institutional Review Board (approval number: 2013-10-019).

Whole exome sequencing

Genomic DNA was extracted from peripheral blood leukocytes using a Wizard Genomic DNA Purification kit according to the manufacturer's instructions (Promega, Madison, WI, USA). DNA quality was assessed by PicoGreen® dsDNA Assay (Invitrogen, Life Technologies, Waltham, MA, USA) and 1% agarose gel electrophoresis. Sequencing libraries were prepared according to the Agilent SureSelect Target Enrichment Kit preparation guide using SureSelect Human All Exon V4 or V6 (Agilent Technologies, Santa Clara, CA, USA). The libraries were sequenced with the Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA). Reads were aligned to the human reference genome GRCh37/hg19 using Burrows-Wheeler Aligner 0.7.10 or 0.7.12. Duplicate marking was conducted using Picard-tools 1.118 or 1.130. Indel realignment, base recalibration, variant calling, and filtering were conducted by GATK v.3.4.0. Annotation was performed using in-house custom-made script.

Data analysis and variant interpretation

A total of 45 PCD-causing genes, 23 PCD-candidate genes, and 990 ciliome genes were selected for analysis (Supplementary Table 1, only online). PCD-causing genes were defined as genes previously reported as causative of PCD in humans. PCD-candidate genes were defined as genes demonstrated to have been associated with PCD in animal studies, but not confirmed in humans. The list of ciliome genes was adapted from the study by Li, et al.[25] Taking into account the prevalence of PCD (1/15000), we filtered out the variants with an allele frequency greater than 0.008 in a population database (Genome Aggregation Database, 1000 Genome Project, and Exome Variant Server). Then, loss-of-function (LoF) variants, such as nonsense, frameshift, and canonical splice site variants, were selected. When only one LoF variant was identified in one of the analyzed genes, non-synonymous variants identified in the gene were further

considered. Since all of these patients were suspected of having PCD, based on the clinical criteria for PCD diagnosis of the PCD Foundation,[24] if two variants were found in one gene, they were assumed to be biallelic variants even if family study was not performed. The selected variants were interpreted according to the 2015 American College of Medical Genetics and Genomics–Association for Molecular Pathology (ACMG-AMP) guidelines.[26] Since a gene-disease relationship has not been clearly identified in ciliome genes, the ACMG-AMP guidelines were not applied to variants identified in ciliome genes.

RESULTS

Patient characteristics

Among 13 patients, 4 (30.8%) were male and 9 (69.2%) were female. The median age was 38 years (range 16–59 years). The proportion of patients with bronchiectasis, sinusitis, and laterality defect was 100% (13/13), 76.9% (10/13), and 30.8% (4/13), respectively. Ten patients underwent TEM analysis of the respiratory epithelium, and three patients did not. Among the 10 patients with TEM analysis, seven had abnormal ciliary structure, and three had normal ciliary structure. Abnormal ciliary structure included outer dynein arm (ODA) defect, inner dynein arm defect, radial spoke (RS)/central pair (CP) defect, and microtubular disorientation (Fig. 1). The baseline characteristics of the 13 patients are summarized in Table 1.

Diagnostic yield

Among 13 patients, four had biallelic LoF variants and were compatible with PCD, resulting in a 30.8% diagnostic yield. These four patients had biallelic LoF variants in PCD-causing genes (*CCDC114*, *DNAH5*, *HYDIN*, and *NME5*), which included one patient with a novel *NME5* variant that had been reported in our previous work.[27] The variants identified in *DNAH5* were known,[28-30] and the other variants were novel. According to the 2015 ACMG-AMP guidelines, the variants identified in *DNAH5* were interpreted as pathogenic variants, and the others were interpreted as likely pathogenic variants (LPVs). Detailed evidence of variant interpretation is summarized in Table 1.

A total of 30.8% (4/13) of patients had only one LoF variant. Among them, Patient 11 had c.2749_2753delinsTAACT (p.Gln917*) in *CROCC*. When viewed on the Integrative Genomics Viewer 2.4.19 (Broad Institute of MIT and Harvard, Cambridge, MA, USA), the c.2753G>T variant was in cis with the

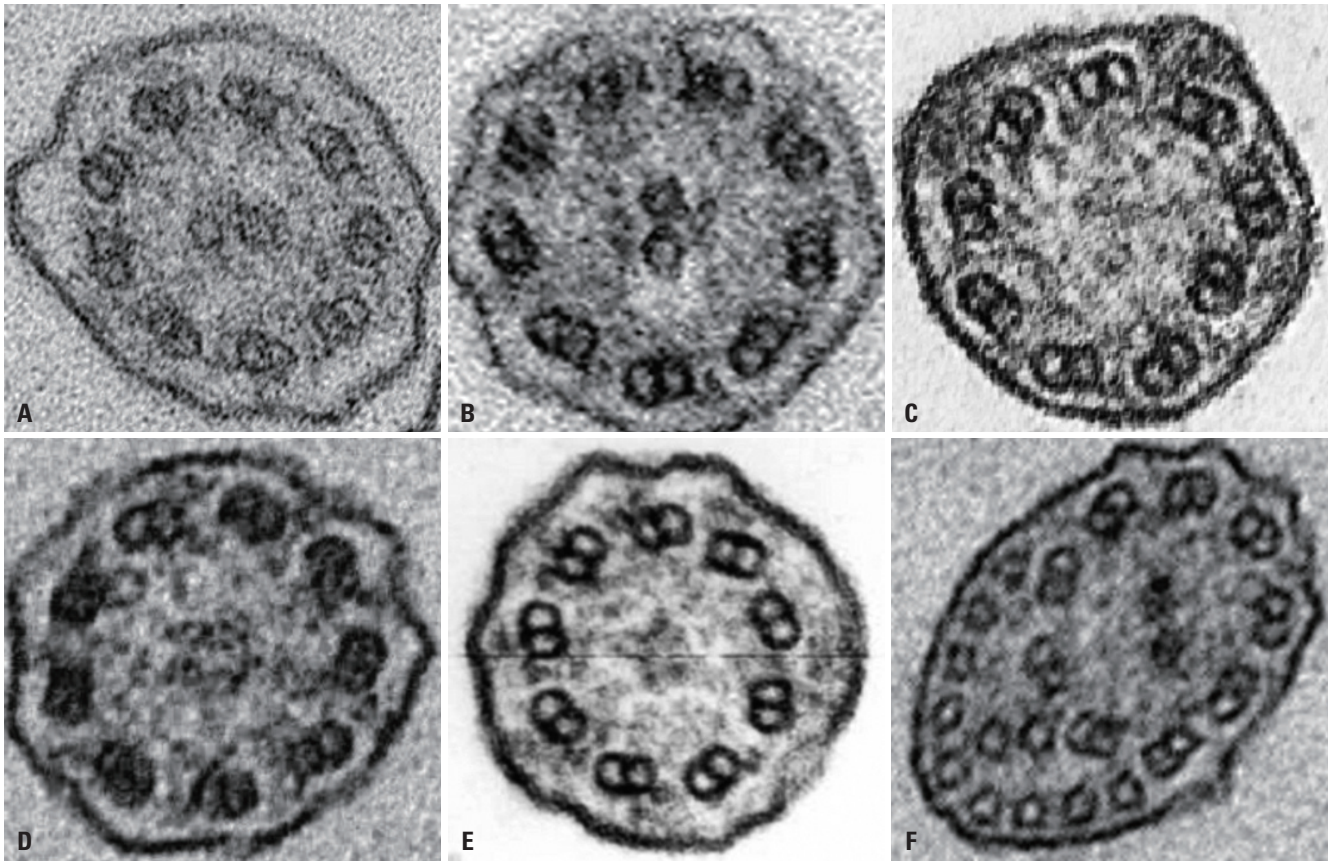

Fig. 1. Abnormal transmission electron micrographs of respiratory cilia. (A) IDA defects observed in Patient 6. (B) ODA and IDA defects observed in Patient 12. (C and D) RS/CP defect observed in Patient 4 and Patient 9. (E) ODA, IDA, and RS/CP defects observed in Patient 1. (F) RS/CP defect with microtubular disorganization observed in Patient 8. IDA, inner dynein arm; ODA, outer dynein arm; RS, radial spoke; CP, central pair.

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Table 1. Clinical and Genetic Characteristics of 13 NTM-LD Patients with Suspected PCD

Case	Sex	Age at suspicion of PCD* (yr)	Situs inversus	Bronchiectasis	Sinusitis	TEM finding	Filtered variants						
							Gene	Refseq	Nucleotide change	Protein change	Zygosity	Reference	ACMG-AMP class
1	F	20	N	Y	Y	ODA defect, IDA defect, RS/CP defect	N/D	N/A	N/A	N/A	N/A	N/A	N/A
2	M	43	N	Y	Y	N/A	N/D	N/A	N/A	N/A	N/A	N/A	N/A
3	F	49	Y	Y	N	ODA defect, IDA defect, RS/CP defect, MTD	*DNAH11* *FBF1*	NM_001277115.1 NM_001319193.1	c.2709del c.31+1G>A	p.(Trp904Glyfs*5) p.(?)	Het Het	Novel Novel	LPV (PVS1, PM2) N/A†
4	F	24	N	Y	Y	RS/CP defect	*NME5*	NM_003551.3	c.572G>A	p.(Trp191*)	Hom	Novel (27)	LPV (PVS1_strong, PM1, PM2)
5	F	49	Y	Y	Y	N/A	*CCDC114* *CCDC114*	NM_144577.3 NM_144577.3	c.-41-2A>C c.702_705dup	p.(?) p.(Pro236Alafs*11)	Het Het	Novel Novel	LPV (PVS1, PM2) LPV (PVS1, PM2)
6	M	38	N	Y	Y	IDA defect	N/D	N/A	N/A	N/A	N/A	N/A	N/A
7	F	52	Y	Y	N	N/A	N/D	N/A	N/A	N/A	N/A	N/A	N/A
8	F	28	N	Y	Y	RS/CP defect, MTD	*DNAH5* *DNAH5* *PKD2L1*	NM_001369.2 NM_001369.2 NM_016112.2	c.5367del c.13458dup c.349+2T>G	p.(Asn1790Ilefs*14) p.(Asn4487*) p.(?)	Het Het Het	28 29, 30 Novel	PV (PVS1, PM2, PP5) PV (PVS1, PM2, PP5) N/A†
9	F	30	N	Y	Y	RS/CP defect	*HYDIN* *HYDIN* *DNAH6* *DNAH6*	NM_001270974.1 NM_001270974.1 NM_001370.1 NM_001370.1	c.10558-1G>C c.15333del c.7899del c.709A>G	p.(?) p.(Lys5111Asnfs*6) p.(Lys2633Asnfs*4) p.(Ser237Gly)	Het Het Het Het	Novel Novel Novel Novel	LPV (PVS1, PM2) LPV (PVS1_moderate, PM2, PM3) LPV (PVS1, PM2) VUS (PM2, PM3)
10	F	57	N	Y	Y	Normal	N/D	N/A	N/A	N/A	N/A	N/A	N/A
11	M	59	N	Y	Y	Normal	*CROCC*	NM_014675.4	c.2749_2753delinsTAACT	p.(Gln917*)	Het	Novel	N/A†
12	M	24	N	Y	Y	ODA defect, IDA defect	*RAD54L* *PDE11A*	NM_001142548.1 NM_016953.3	c.2133dup c.221dup	p.(Lys712*) p.(Gly75Trpfs*65)	Het Het	Novel Novel	N/A† N/A†
13	F	16	Y	Y	N	Normal	*SLC49A3* *SLC49A3*	NM_001294341.1 NM_001294341.1	c.1003C>T c.604C>T	p.(Gln335*) p.(Pro202Ser)	Het Het	Novel Novel	N/A† N/A†

NTM-LD, nontuberculous mycobacterial lung disease; PCD, primary ciliary dyskinesia; TEM, transmission electron microscopy; ACMG-AMP, American College of Medical Genetics and Genomics–Association for Molecular Pathology; F, female; N, no; Y, yes; ODA, outer dynein arm; IDA, inner dynein arm; RS, radial spoke; CP, central pair; N/D, not detected; N/A, not applicable; M, male; MTD, microtubular disorganization; Het, heterozygous; LPV, likely pathogenic variant; Hom, homozygous; PV, pathogenic variant; VUS, variant of uncertain significance.

*The age at TEM study was described in all patients except Patients 2, 5, and 7. Since Patients 2, 5, and 7 did not undergo TEM study, the age at study enrollment was described, †For variants identified in ciliome genes, the ACMG-AMP guidelines were not applied since a gene-disease relationship has not been clearly identified in ciliome genes.

c.2749C>T variant, which could be described as c.2749_2753delinsTAACT. Patient 13 had another missense variant in addition to a LoF variant in *SLC49A3*. The missense variants identified in *SLC49A3* showed different prediction results according to the in-silico algorithms (C0 in Align GVGD, tolerated in SIFT, possibly damaging in PolyPhen-2). In 38.5% (5/13) of the patients, no LoF variants were identified. The variants identified in the 13 patients are summarized in Table 1.

Genotype-phenotype correlation

Patient 4 was homozygous for c.572G>A (p.Trp191*) in *NME5*, and a parent study revealed that her parents were heterozygous.[27] The patient had no situs inversus, and CP was absent in a TEM study. Since *NME5* encodes RS neck component,[31] these findings were compatible with the phenotypes expected from a defect of *NME5*.

Patient 5 was compound heterozygous for c.-41-2A>C and c.702_705dup (p.Pro236Alafs*11) in *CCDC114*. Since *CCDC114* encodes ODA docking complex, its defect causes the absence of ODA, and affected patients have a 50% chance of laterality defect.[32] The patient's situs inversus was confirmed, but the absence of ODA could not be evaluated, as TEM study was not conducted.

Patient 8 was compound heterozygous for c.5367del (p.Asn1790Ilefs*14) and c.13458dup (p.Asn4487*) in *DNAH5*. Since DNAH5 encodes ODA, its defect causes absence of ODA.[33] However, contrary to the expected phenotype, TEM study showed some cilia exhibiting microtubular disorganization with loss of the CP.

Patient 9 was compound heterozygous for c.10558-1G>C and c.15333delA (p.Lys5111Asnfs*6) in *HYDIN*. *HYDIN* encodes projection of the CP, and the changes in projection of the CP are too subtle to be identified by TEM.[34] Therefore, most of the patients with *HYDIN* defect showed normal ciliary structure in TEM studies. However, contrary to our expectation, TEM study showed some cilia exhibiting outer singlet microtubules with loss of the CP.

DISCUSSION

PCD is a genetically heterogeneous disorder of motile cilia and is considered a genetic susceptibility factor of NTM-LD.[9,10] In this study, we performed WES in 13 NTM-LD patients with suspected PCD, and demonstrated that 30.8% of patients had biallelic LoF variants and an additional 30.8% of patients had one LoF variant. Although patients with suspected PCD were enrolled in the present study, the diagnostic yield was lower than the 67.6% diagnostic yield reported in a previous study,[35] which might be due to the patient demographics in our study. In general, PCD patients are diagnosed at pediatric age, as they have experienced respiratory symptoms that begin in the neonatal period. According to the previous study by Kuehni, et al.,[12] the median age at PCD diagnosis in children was 5.3 years. On the other hand, the median age of the patients in our study was 38 years, which was higher than the median age reported in a previous study. The delayed diagnosis in our patients might be due to the unavailability of diagnostic tests for PCD diagnosis, such as nasal nitric oxide measurement and high speed videomicroscopy, in Korea.

Among the 15 LoF variants identified in this study, three variants (c.572G>A in *NME5*, c.15333del in *HYDIN*, and c.2133dup in *RAD54L*) were located in the last exon and would escape nonsense-mediated decay; therefore, we applied the refining PVS1 criterion addressed by the ClinGen Sequence Variant Interpretation Working Group.[36] In the case of *RAD54L*, ACMG-AMP guidelines were not applied, since the gene-disease relationship has not been clearly identified. In *NME5*, the last exon encodes the DPY-30 domain, which is important for the assembly of RS.[37] Finally, c.572G>A in *NME5* was interpreted as LPV (PVS1_strong, PM1, PM2). The c.15333del in *HYDIN* disrupt the last 11 amino acid residues, which do not encode a specific domain. Therefore, the effect of this variant was not clear. As another *HYDIN* variant identified in the same patient was interpreted as LPV, c.15333del was interpreted as LPV (PVS1_moderate, PM2, PM3).

In the present study, four of the nine patients who did not have biallelic LoF variants had only one LoF variant. If copy number analysis had been performed for the patients with only one LoF variant, the diagnostic yield might have been increased further. In addition, there is a possibility that digenic interaction can contribute to PCD, as shown in a previous study by Li, et al.[25] Patients 3 and 12 had one LoF variant in each of two different genes, *DNAH11/FBF1* and *RAD54L/PDE11A*, respectively. Assessment of digenic interaction between these genes can help improve diagnostic yield.

Among four patients with biallelic LoF variants, except one patient who did not undergo TEM analysis, only one showed the expected TEM results in the gene with biallelic LoF variants. The genotype-phenotype correlation was not evident in the other two patients, perhaps because a single TEM study was conducted in the patients in this study. A single TEM study can lead to a false-positive result, as it is difficult to distinguish between primary and secondary changes.[38] Since the patients in this study suffered from prolonged NTM-LD, secondary changes from NTM infections can cause nonspecific TEM findings, which might lead to poor genotype-phenotype correlation and even cause false-positives in PCD diagnoses.

This study had several limitations. First, family studies were not conducted, except for one patient who had a homozygous *NME5* variant. Therefore, the assumed biallelic variants might not actually be biallelic, and the diagnostic yield could be changed according to the result of the family study. Second, we did not confirm whether or not the variants identified in *HYDIN* were in *HYDIN2*, the highly homologous pseudogene. Third, we did not focus on variants other than LoF variants.

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The pathogenicity of variants other than LoF variants was difficult to assess without functional and/or family studies. Fourth, we did not enroll all suspected PCD patients. Instead, we focused on suspected PCD patients among NTM-LD patients. The difference in diagnostic yield from previous studies might be due to this characteristic of our enrolled patients.

In conclusion, at least 30.8% of patients with NTM-LD who were suspected of having PCD had biallelic LoF variants, and an additional 30.8% of patients had one LoF variant. Therefore, PCD should be considered in NTM-LD patients with PCD-associated symptoms. Early suspicion makes early PCD diagnosis possible, which leads to improved patient prognosis.

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AUTHOR CONTRIBUTIONS

Conceptualization: Chang-Seok Ki and Hee Jae Huh. Data curation: Eun Hye Cho. Formal analysis: Eun Hye Cho and Hee Jae Huh. Funding acquisition: Hee Jae Huh. Investigation: Eun Hye Cho and Hee Jae Huh. Methodology: Chang-Seok Ki and Hee Jae Huh. Project administration: Chang-Seok Ki, Hee Jae Huh, and Nam Yong Lee. Resources: Sun Ae Yun, Su-Young Kim, Byung Woo Jhun, and Won-Jung Koh. Supervision: Hee Jae Huh and Nam Yong Lee. Writing—original draft: Eun Hye Cho and Chang-Seok Ki. Writing—review & editing: Hee Jae Huh and Nam Yong Lee. Approval of final manuscript: all authors.

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