Pilot study of expanded newborn screening for 573 genes related to severe diseases in China: results from 1173 newborns

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Abstract

Background: Current newborn screening (NBS) in China is mainly aimed at detecting biochemical levels of metabolites in blood, which may generate false positive/negative results. **Methods:** We designed a next-generation sequencing (NGS) panel of 573 genes related to severe diseases and performed NBS for 1173 individuals, who had been screened for tandem mass spectrometry (MS/MS), phenylalanine (Phe), thyroid-stimulating hormone (TSH), 17- α -hydroxyprogesterone (17-OHP) and glucose-6-phosphate dehydrogenase (G6PD) as conducted in traditional biochemical NBS in September 2016. We compared their biochemical results with genetic variants and investigated the carrier frequencies of 77 genes related to disorders of MS/MS in these newborns. **Results:** Biochemical results showed that four newborns were positive for G6PD enzymatic assay (all males), the other biochemical values including MS/MS, Phe, TSH and 17-OHP were negative. Genetic analysis results showed that all the four newborns with positive G6PD values carried G6PD hemizygous mutations. Besides, NGS results revealed an individual (ID 84123) carrying two common SLC22A5 mutations (c.760C>T/p.R254* and c.1400C>G/p.S467C) in Chinese population which were verified in trans later, was biochemical negative in 2016. MS/MS results in June 2019 showed free carnitine deficiency, which was consistence with genetic analysis results. Carrier frequencies data suggested the top five genes with the highest carrier frequencies in these newborns were PAH (2.04%), ETFDH (1.24%), MMACHC (1.15%), SLC25A13 (0.98%), and GCDH (0.80%). **Conclusion:** Our study provides data that combine biochemical results with genetic variants in 1173 newborns. This is the first study that reveals carrier frequencies of 77 genes causing inherited metabolic diseases (IMDs) in China.

Background

Newborn screening (NBS) is a successful public health program that prevents morbidity and mortality through early detection and management of specific conditions, it can improve lives of children by allowing timely therapeutic interventions. It is a routine component of neonatal care to screen newborns for inherited metabolic diseases (IMDs) and other common genetic disorders in many countries now. tandem mass spectrometry (MS/MS) is widely used to detect metabolites related to amino acid metabolism, organic acid metabolism and fatty acid oxidation in dried blood spots (DBS). Currently MS/MS is the principal method used in NBS centers to detect biochemical metabolites (1). In China, apart from MS/MS test, traditional biochemical NBS also includes tests for phenylalanine (Phe), thyroid-stimulating hormone (TSH), 17- α -hydroxyprogesterone (17-OHP) and glucose-6-phosphate dehydrogenase (G6PD) enzymatic analysis.

The advent of next-generation sequencing (NGS) technologies has enabled the simultaneous and high-throughput detection of genes to identify variants. The rapid development of NGS has reduced the cost of sequencing and genetic analysis to the extent that NGS could be used in NBS. Genome-wide sequencing in the US has been discussed and applied in 159 newborns (2, 3), and the results suggested that newborn genomic sequencing can effectively detect the risk and carrier status for a wide range of disorders that are not detectable by current NBS assays (4). Genomic sequencing for newborns can avoid diagnostic

odyssey for ill newborns, allow optimal early treatment strategies for affected newborns (5, 6), and identify carrier status that could help in future reproductive planning. Despite the anticipated benefits, the major challenges hindering its application include data interpretation and appropriate reporting of information. In our previous research, we designed a panel of 77 genes related to over 40 IMDs and demonstrated that genomic DNA extracted from DBS could generate reliable sequencing data, enabling NGS to function as a second-tier diagnostic test for NBS (7).

Considering the benefits and challenges mentioned, we launched a project to screen newborns for genes related to severe diseases. A total of 573 genes related to birth defects were selected based on penetrance, age of onset, and the severity of causal diseases. In total, 1173 newborns that were born in September 2016 were randomly enrolled in the NBS center of Xinhua Hospital in Shanghai. The newborns had all been screened for MS/MS, Phe, TSH, 17-OHP, and G6PD as in traditional biochemical NBS. The biochemical results and the genetic variants obtained using NGS of the 1173 newborns were compared.

Previous studies showed that the frequency of different Mendelian disorders in China varies with diverse ethnic backgrounds and areas (8). Majority of the newborns recruited in this study were born in Shanghai (1097, 93.52%), and most of them were of the Han ethnicity, which is the largest ethnic group in China. IMDs, a group of rare diseases with an overall incidence of 1/500 in all ethnic cohorts, are caused by enzyme or transporter abnormalities and alterations in biochemical pathways for metabolism (9). IMDs are genetically heterogeneous disorders and are frequently transmitted in an autosomal recessive inheritance pattern; patients with IMDs may present severe symptoms. In the present study, we investigated the carrier frequencies of IMDs related to amino acid metabolism, organic acid metabolism, and fatty acid oxidation in these random selected newborns.

Methods

Subjects

A total of 1173 residual DBS specimens from newborns (600 males and 573 females) at Xinhua Hospital in Shanghai were included in the research. All the 1173 newborns were randomly selected in September 2016 with consecutive number. Among these newborns, 93.52% (total 1097, 557 males and 540 females) were born in Shanghai and 6.48% (total 76, 43 males and 33 females) were born in Macao. All the newborns had undergone traditional biochemical NBS, including MS/MS, Phe, TSH, 17-OHP, and G6PD analysis. Written informed consent was obtained from parents/legal guardians. This study reused the NBS samples and was approved by Xinhua Hospital Ethics Committee Affiliated to Shanghai Jiao tong University School of Medicine (XHEC-C-2017-021-2),

Target diseases and genes

Causative genes for severe diseases including hematological, cardiovascular, rheumatic immunological, gastrointestinal, endocrinological, skeletal, neurological, otolaryngological, muscular, dermatological,

urinary, respiratory, and IMDs were included. A total of 573 genes related to more than 200 birth defects diseases were selected based on penetrance, age of onset, pathogenicity and mode of inheritance (Additional file 1). Most diseases were inherited recessively, and rare diseases with extremely low incidence rates were excluded.

NGS and data analysis

Target-specific primers were generated based on the hg19/GRCh37 human reference genome. The primers were designed to have similar length, GC content, and similar amplicon fragment size to maximize the amplification efficiency. The amplicon fragments included the exons and flanking intronic sequences. DNA extracted from a single DBS was generally enough for target capture and library preparation. Amplicons were sequenced on the NovaSeq 6000 instrument (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Paired-end reads were aligned to the NCBI reference sequence (hg19/GRCh37) using BWA and variant calls were made using GATK. Primary analyses of NGS data included sequence alignment, post-processing, and variant calling.

Variants with allele counts of more than 3 were removed. Allele frequencies lower than 0.005 in ExAC database, gnomAD exome database, and 1000Genomes of East Asian database were further evaluated for pathogenicity according to the ACMG Standards and Guidelines (10). Variants that were selected met either of the following criteria: (1) variants classified as disease-causing mutations in the Human Gene Mutation Database (HGMD); or (2) frameshift, stop-gained, stop-lost, and splice donor/acceptor variants that were supposed to have a high impact on individuals. Traditional biochemical data were compared with the information on genetic variants.

Results

NGS Assay

Among the 1173 DBS specimens, genomic DNA extracted from 1127 samples (551 females and 576 males) was subjected to quality control and sequencing, while the other 46 DBS failed to meet quality control standards. The average coverage of the target region was 99.97%, and 99.2% of reads were properly paired and mapped to the reference genome. The mean coverage of sequencing was 305×, and 99.4% of bases were sequenced to at least 20× coverage.

Traditional biochemical screening compared with genetic variants

Among these 1173 newborns, there were four newborns (all males, ID:83162, 83841, 83847, 84010) showed low levels of G6PD enzymatic activity (Table 1). MS/MS, Phe and TSH values at birth were negative for all of them. Newborns with G6PD deficiency may suffer from neonatal jaundice and acute

hemolytic anemia, which is triggered by exogenous primaquine or fava beans (11). *G6PD* hemizygous variants were detected in all four individuals and the variants were classified as disease-causing mutations according to NGS results. The biochemical values of G6PD were consistent with the information from genetic variants, which indicated the reliable results for G6PD tests.

Besides, there were three newborns with abnormal high levels of 17-OHP at birth and the results turned to be negative when they were called back soon in October 2016 and tested again. This result indicated the false-positives of 17-OHP enzymatic screening.

There were 1173 samples in total subjected to NGS analysis and 1127 samples returned qualified data. Apart from the 4 samples with *G6PD* hemizygous variants mentioned above, there were 2 individuals with negative biochemical results carrying genetic variants (heterozygous variants for recessively inherited genes were excluded), including one individual with two *DUOX2* variants (ID:84066) and one individual with two *SLC22A5* variants (ID:84123). In addition, there was one more individual (ID:84193) with two *DUOX2* mutations, since integrative genomics viewer showed that the two *DUOX2* variants (c.1588A>T/p.K530* and c.1606C>T/p.R536*) were in one allele and were thus not compound heterozygous variants, this case was excluded in Table 1. In April 2019, we contacted the parents of the two children (ID:84066 and 84123) to verify whether the variants were in trans.

We failed to contact the parents of ID 84066. As for ID 84123, *SLC22A5* compound heterozygous mutations were verified, including c.760C>T(p.R254*) inherited from his father and c.1400C>G(p.S467C) inherited from his mother (Fig. 1). The two variants are common mutations in Chinese population. MS/MS was performed for ID 84123 again in May 2019, the results showed free carnitine deficiency, which was consistence with genetic analysis results, indicating false-negative MS/MS result at birth.

Carrier frequencies for IMDs

The screening population included 1173 newborns with an average age of 3.7 days; 1097 (93.52%) of them were born in Shanghai and the other 76 (6.48%) of them were born in Macao. Among the 1173 DBS specimens, genomic DNA extracted from 1127 (576 males and 551 females) samples were qualified and sequenced. Carrier frequencies of 77 OMIM genes (Additional file 2) related to amino acid metabolism, organic acid metabolism, and fatty acid oxidation were calculated in this study. Variants with allele counts of more than 3 or those with allele frequency higher than 0.005 in ExAC database, gnomAD exome database, and 1000Genomes of East Asian database were excluded. Only variants that were either (1) classified as a disease-causing mutation in HGMD or (2) frameshift, stop-gained, stop-lost, and splice site variants were used to calculate carrier frequencies (Additional file 3). Based on our criteria, 51 genes listed in Table S2 were identified in newborns (Table 2).

Discussion

The development of NGS revolutionized genetic research and has been proven to be effective for detecting genetic disorders (12). Our previous study has demonstrated that DNA extracted from DBS could be used for NGS; we designed a gene panel incorporating 77 genes related to amino acid metabolism, organic acid metabolism, and fatty acid oxidation, and compared the specificity and sensitivity of the sequencing data (7). This cohort included 1173 DBS, among which 1127 (96.1%) qualified for NGS. There may be several factors affecting the yield, including the individual differences in white blood cells and DNA degradation rates because the DBS samples had been stored for over a year before being retrieved for NGS. Subjects in our study were born in September 2016, and their NBS samples were continuously numbered from ID 83038 to 84210.

Comparison of biochemical screening with genetic results

There were four newborns with low levels of G6PD enzyme and all of them were males that carried hemizygous variants classified as DM in the HGMD database. The biochemical results for G6PD were all consistent with the genetic variants, indicating the high sensitivity and specificity of G6PD. G6PD deficiency is an X-linked, genetic defect due to a mutation in *G6PD* and is the most common human enzyme defect. The most frequent clinical manifestations of G6PD deficiency are neonatal jaundice and acute hemolytic anemia, which is usually triggered by an exogenous agent. Heterozygous females generally have less severe clinical manifestations than G6PD-deficient males (11). The highest frequencies of G6PD deficiency were detected in tropical Africa, tropical/subtropical Asia, the Mediterranean region, and the Middle East. The global distribution of G6PD deficiency is similar to the epidemic areas of malaria, which provides evidence that G6PD deficiency confers resistance against malaria (13, 14). In this study, four male individuals showed low levels of G6PD based on enzymatic assay results, including two males born in Shanghai (ID 83162, 84010) and two males born in Macao (ID 83841, 83847). In this cohort, 557 males were born in Shanghai, and two of them were identified with G6PD deficiency. While 43 males were born in Macao, which is in the subtropical area of Asia, there were also two males identified with G6PD deficiency. This also indicated the higher prevalence of G6PD deficiency in Macao than in Shanghai.

There were three infants with high levels of 17-OHP at birth, the results turned out to be normal when they were called back soon and tested again in October 2016. The false-positives of 17-OHP enzymatic screening at birth could be time consuming for further reexamination. Thus, a second-tier test may be needed to improve the efficacy of the screening and reduce the number of false-positives.

Apart from the cases mentioned above, there are two children with negative biochemical results carrying *DUOX2*, and *SLC22A5* variants, respectively.

DUOX2 is an autosomal recessive gene related to thyroid hormone synthesis dysfunction (15). There was one case (ID 84066) identified with two *DUOX2* variants, c.3391G>T(p.A1131S) and c.2202G>A(p.W734*). Whether the two *DUOX2* variants were in the same allele could not be distinguished by integrative genomics viewer, and samples from parents were needed to verify. Our study had an obvious limitation.

The proband-only sequencing approach makes it hard to distinguish whether the two variants are bi-allelic or located at one allele of the recessive gene, as we did not have the parents' blood samples, unless the two variants were not far and could be distinguished via integrative genomics viewer.

Homozygous or compound heterozygous mutations in *SLC22A5* can cause systemic primary carnitine deficiency (SPCD) and lead to impaired fatty acid oxidation in muscles (16). There was one case (ID 84123) with *SLC22A5* compound heterozygous variants. His C0 (free carnitine) value was 11.6umol/L (reference: 10–60umol/L) at birth in September 2016. When he was called back in April 2019 due to *SLC22A5* mutations, the C0 value was 4.3umol/L, which was lower than normal range and could be classified into SPCD. SPCD encompasses a broad clinical spectrum including metabolic decompensation in infancy; childhood myopathy involving heart and skeletal muscle; pregnancy-related decreased stamina or exacerbation of cardiac arrhythmia; fatigability in adulthood or absence of symptoms (17). Genomic screening can avoid diagnostic odyssey and allow optimal early treatment strategies for affected newborns. The child is near 3 years old now, he has not shown symptoms yet. It is important for this child to have physical examination regularly and possible treatment to maintain plasma carnitine and prevent primary manifestations.

Carrier frequencies

Our team focused on IMDs, and we investigated the carrier frequencies of 77 genes related to disorders of MS/MS including amino acid metabolism, organic acid metabolism and fatty acid oxidation, which are listed in Table S2. A total of 51 genes were identified in 1127 subjects, and the other 26 genes with LP/P variants were not detected according to our criteria. The top five genes with the highest carrier frequencies in these newborns were *PAH* (2.04%), *ETFDH* (1.24%), *MMACHC* (1.15%), *SLC25A13* (0.98%), and *GCDH* (0.80%). This is the first study that reveals the carrier frequencies of this many genes in China.

In 2018, a pilot study of expanded carrier screening in China investigated 11 recessive diseases, including *PAH* deficiency, and revealed that the carrier frequency for *PAH* in all ethnicities in China was 3.59%. The results varied among different ethnicities and the carrier frequency for *PAH* in the Han ethnicity was 3.30% (8), which was higher than what was calculated in our study (2.04%). The subjects in our study were mainly born in Shanghai and the population was mostly of the Han ethnicity. One of the reasons for the varying carrier frequencies obtained in the two studies may be the different criteria employed for determining variants.

Another study in 2016 performed a molecular approach for NBS for four genetic diseases in Guizhou Province of south China, including beta-thalassemia, G6PD deficiency, PKU, and non-syndromic hearing loss. They included 515 newborns and selected 10 common mutations of *PAH* in the Chinese population, and the carrier frequency for *PAH* they calculated in their study was 0.78% (18). The frequency they calculated was much lower than that in our study (2.04%). Our data indicated 23 newborns carrying variants with mutations in 18 different positions, and all 18 variants were classified as disease-causing

mutations. This comparison also suggests that *PAH* is better screened as whole exons instead of as variants based on mutations in several positions.

Conclusions

Our study provide data that combine biochemical results with genetic variants of newborns, which could be helpful for the evaluation of other expanded NBS strategies. We identified an individual (ID 84123) carrying two common *SLC22A5* mutations (c.760C>T/p.R254* and c.1400C>G/p.S467C) in Chinese population which were verified in trans later, was biochemical negative in 2016. MS/MS results in June 2019 showed free carnitine deficiency, which was consistence with genetic analysis results. This is also the first study that reveals carrier frequencies of 77 genes causing IMDs related to amino acid metabolism, organic acid metabolism, and fatty acid oxidation in China.

Abbreviations

NGS: next-generation sequencing; NBS: newborn screening; MS/MS: tandem mass spectrometry; Phe: phenylalanine, TSH: thyroid-stimulating hormone; 17-OHP: 17- α -hydroxyprogesterone; G6PD: glucose-6-phosphate dehydrogenase; IMDs: inherited metabolic diseases; DBS: dried blood spots; SPCD: systemic primary carnitine deficiency.

Declarations

Ethics approval and consent to participate

This study reused the NBS samples and was approved by Xinhua Hospital Ethics Committee Affiliated to Shanghai Jiao tong University School of Medicine (XHEC-C-2017-021-2). Written informed consent was obtained from parents/legal guardians.

Consent for publication

Not applicable

Availability of data and materials

The data generated or analyzed during this study are included in this article and its supplementary files.

Competing interests

The authors declare that they have no conflict of interest.

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Author contributions

XG and YY conceived and designed the study. FX, JG and JY provided biochemical results of all the DBS samples. LL and ZL performed the NGS experiments. YS interpreted the NGS data. XL analyzed the sequencing data and drafted the manuscript. All authors reviewed and approved the final manuscript.

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Tables

Table 1 Biochemical and genetic results of the newborns

ID	Sex	Biochemical results [#]					Genetic Variants*	Zygosity
		MS	Phe	TSH	17-OHP	G6PD		
83162	M	N	N	N	N	P	<i>G6PD</i> c.1114C>T(p.L372F)	Hemizygous
83841	M	N	N	N	N	P	<i>G6PD</i> c.1478G>A(p.R493H)	Hemizygous
83847	M	N	N	N	N	P	<i>G6PD</i> c.1478G>A(p.R493H)	Hemizygous
84010	M	N	N	N	N	P	<i>G6PD</i> c.496C>T(p.R166C)	Hemizygous
84066 ⁺	F	N	N	N	N	N	<i>DUOX2</i> c.3391G>T(p.A1131S), c.2202G>A(p.W734*)	unknown
84123 ⁺⁺	M	P	N	N	N	N	<i>SLC22A5</i> c.760C>T(p.R254*), c.1400C>G(p.S467C)	Compound heterozygous

Abbreviations: MS=MS/MS, M=Male, F=Female, N=Negative, P=Positive,

Transcripts: *G6PD*(NM_000402.4), *DUOX2*(NM_014080.4), *SLC22A5*(NM_003060.3)

[#]There were three more newborns with abnormal levels of 17-OHP at birth which are not listed in the table because the 17-OHP results turned to be negative when they were called back soon and tested again.

*There was one more newborn (ID 84193) with two *DUOX2* variants, integrative genomics viewer showed that the two variants were in one allele and were thus excluded in the table.

⁺Parents of this individual could not be contacted.

⁺⁺The MS/MS test for ID 84123 at birth was negative, but the plasma free carnitine value turned to be positive when he was called back in April 2019 to confirm the data.

Table 2 Carrier frequencies of 77 genes causing IMDs in the cohort of 1127 newborns

Disease	Gene	N	%	1 in _
Hyperphenylalaninemia	<i>PAH</i>	23	2.04	49
	<i>PTS</i>	6	0.53	188
	<i>GCH1</i>	2	0.18	564
Sepiapterin reductase deficiency	<i>SPR</i>	1	0.09	1127
Maple syrup urine disease	<i>BCKDHB</i>	2	0.18	564
Carbamoyl phosphate synthetase I deficiency	<i>CPS1</i>	4	0.35	282
Ornithine transcarbamylase deficiency	<i>OTC</i>	1	0.09	1127
Citrullinemia	<i>ASS1</i>	4	0.35	282
	<i>SLC25A13</i>	11	0.98	102
Argininosuccinic aciduria	<i>ASL</i>	2	0.18	564
Argininemia	<i>ARG1</i>	5	0.44	225
Hyperornithinemia-hyperammonemia-homocitrullinuria syndrome	<i>SLC25A15</i>	1	0.09	1127
Ornithine aminotransferase deficiency	<i>OAT</i>	1	0.09	1127
Nonketotic hyperglycinemia	<i>GLDC</i>	3	0.27	376
	<i>AMT</i>	3	0.27	376
Homocystinuria	<i>CBS</i>	3	0.27	376
Methylenetetrahydrofolate reductase deficiency	<i>MTHFR</i>	3	0.27	376
Methionine synthase deficiency	<i>MTR</i>	1	0.09	1127
Methionine synthase reductase deficiency	<i>MTRR</i>	1	0.09	1127
Hypermethioninemia	<i>MAT1A</i>	2	0.18	564
S-adenosylhomocysteine hydrolase deficiency	<i>AHCY</i>	2	0.18	564
Methylmalonic aciduria	<i>MUT</i>	8	0.71	141
	<i>MMAA</i>	2	0.18	564
	<i>MMAB</i>	1	0.09	1127
	<i>MMACHC</i>	13	1.15	87
Propionic acidemia	<i>PCCA</i>	2	0.18	564
	<i>PCCB</i>	1	0.09	1127
Isovaleric acidemia	<i>IVD</i>	1	0.09	1127
Glutaric aciduria	<i>GCDH</i>	9	0.80	125
	<i>SUGCT</i>	2	0.18	564
3-Methylcrotonyl-CoA carboxylase deficiency	<i>MCCC1</i>	2	0.18	564
	<i>MCCC2</i>	3	0.27	376
Holocarboxylase synthetase deficiency	<i>HLCS</i>	3	0.27	376
Biotinidase deficiency	<i>BTB</i>	8	0.71	141
HMG-CoA lyase deficiency	<i>HMGCL</i>	2	0.18	564
Alpha-methylacetoacetic aciduria	<i>ACAT1</i>	1	0.09	1127
Malonyl-CoA decarboxylase deficiency	<i>MLYCD</i>	1	0.09	1127
2-Methylbutyrylglycinuria	<i>ACADSB</i>	4	0.35	282
Isobutyryl-CoA dehydrogenase deficiency	<i>ACAD8</i>	6	0.53	188
Succinic semialdehyde dehydrogenase deficiency	<i>ALDH5A1</i>	4	0.35	282
Mevalonic aciduria	<i>MVK</i>	2	0.18	564
HMG-CoA synthase-2 deficiency	<i>HMGCS2</i>	2	0.18	564
Primary carnitine deficiency	<i>SLC22A5</i>	8	0.71	141
Carnitine palmitoyltransferase deficiency	<i>CPT1A</i>	1	0.09	1127
	<i>CPT2</i>	2	0.18	564

Short-chain acyl-CoA dehydrogenase deficiency	<i>ACADS</i>	7	0.62	161
Medium-chain acyl-CoA dehydrogenase deficiency	<i>ACADM</i>	7	0.62	161
Very long-chain acyl-CoA dehydrogenase deficiency	<i>ACADVL</i>	7	0.62	161
3-Hydroxyacyl-CoA dehydrogenase deficiency	<i>HADH</i>	1	0.09	1127
Trifunctional protein deficiency	<i>HADHB</i>	2	0.18	564
Other acyl-CoA dehydrogenase deficiency diseases	<i>ETFDH</i>	14	1.24	81

The other 26 genes were not detected in the cohorts: *QDPR*, *PCBD1*, *BCKDHA*, *DBT*, *DLD*, *FAH*, *TAT*, *HPD*, *GCSH*, *HGD*, *MMADHC*, *MCEE*, *AUH*, *TAZ*, *OPA3*, *DNAJC19*, *ASPA*, *ETHE1*, *L2HGDH*, *D2HGDH*, *IDH2*, *SLC25A20*, *ETFA*, *ETFB*, *DECR1*, *HADHA*.

Figures

Figure 1

Sequencing analysis of the SLC22A5 gene mutations. The arrows show the site of the c.760 and c.1400 of the family.

Supplementary Files

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- [supplement1.xlsx](#)
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