Expression profile analysis of long non-coding RNA in acute myeloid leukemia by microarray and bioinformatics

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INTRODUCTION

Acute myeloid leukemia (AML) is the most common form of hematological malignant tumor that threatens human health. In the last decade, the rapid evolution in the detection of molecular abnormalities has brought more and more precise prediction of prognosis and diagnosis, which can efficiently guide post-remission therapy and personal treatment.1 The development of high-throughput screening technologies (HTS), such as next-generation sequencing and cDNA microarray, has identified several new mutations (including DNMT3A, IDH1, IDH2, and TET2) in AML.2,3 However, the vast majority of transcripts that were detected by HTS do not appear to be protein-coding genes. This phenomenon is notable in non-coding RNAs, which have been vividly described as the “dark matter” of the genome.4 In some diseases there are no point mutations in the protein-coding genes. The pathogenic mechanisms may be multifactorial and likely to involve genetic elements additional to small non-coding RNAs, as seen in 13q14.3 of solid tumors and hematopoietic malignancies. Long non-coding RNAs (lncRNAs) are probably the chief culprits.5
In contrast to small non-coding RNAs, what we know about lncRNAs is confined to mRNA-like transcripts lacking significant ORFs and over 200 nt in length. Recent studies indicate that lncRNAs may play a key role in cancer pathways. In AML, lncRNAs are important fractions of the biomarkers detected by microarray. Its expression may discriminate acute leukemia molecular subtypes, which may provide a more precise tool to categorize leukemia and stratify patients. Various lncRNAs were reported to be implicated in AML. For example, lncRNA CCAT1 can regulate microRNA-155 that can target for c-Myc, which in turn can activate CCAT1. The study was just a glance of the complex world constructed by mRNA, microRNA, and lncRNA. Furthermore, Garrow et al built a prognostic lncRNA score system for older patients (≥60 years) with cytogenetically normal AML. It is anticipated that lncRNAs will be used in clinical diagnosis and treatment after large-scale clinical trials and functional studies are completed in the near future.

This study analyzed the expression profiles of lncRNAs and mRNAs in AML vs iron deficiency anemia (IDA) controls, with a focus on lncRNAs playing a big part in AML in the modularization process. In particular, using public databases, we identified the clinical significance of lncRNAs, and validated their expression in AML patients by quantitative real-time PCR (qRT-PCR).

2 MATERIALS AND METHODS

2.1 Patients and samples

Bone marrow specimens were obtained from AML patients at the Department of Hematology, Second Affiliated Hospital of Xi’an Jiaotong University, and Xi’an Jiaotong University (both Xi’an, China) in 2016. This study was approved by the Medical Ethics Committee of the Second Affiliated Hospital of Xi’an Jiaotong University (#2015182), and written informed consent was obtained from all parents or guardians. Diagnosis of AML was made in accordance with the revised French-American-British classification. One hundred and fifty-one patients included in The Cancer Genome Atlas (TCGA), investigated using RNA sequencing technology, were analyzed to evaluate prognostic values of lncRNAs. The ID numbers of the patients are shown in Table S1. To weaken the variation between samples as much as possible, bone marrow samples from IDA were used as controls, for the deficiency of bone marrow samples from normal donors in clinic. Bone marrow mononuclear cells were isolated using lymphocyte separation liquid within 8 hours after bone marrow samples were harvested and subjected for isolation of total cellular RNA, then stored at −80°C. Detailed information of all cases in the study is summarized in Table S2.

2.2 Profiling of lncRNA expression

Arraystar Human lncRNA Array version 4.0 was used to profile the expression of lncRNAs, which was performed by KangChen Bio-tech (Shanghai, China). Briefly, RNA samples from bone marrow mononuclear cells were purified to remove rRNA and were amplified and transcribed into fluorescent cRNAs along the entire length of the transcripts without 3’ bias. Then, each cRNA was hybridized to the Arraystar Human lncRNA Array. The array was designed for the global expression profiling of human lncRNA and protein-coding mRNA transcripts, which can detect a total of 40 173 lncRNAs in two tiered compilations: gold standard lncRNAs for 7506 well-annotated functionally studied and experimentally supported full-length lncRNAs, and reliable lncRNAs for 32 667 high confidence lncRNAs as the comprehensive collection. The lncRNAs were carefully constructed using the most highly respected public transcriptome databases (e.g. RefSeq, UCSC known genes, and Ensembl), as well as landmark publications. The array also includes an entire collection of 20 730 protein-coding mRNAs further supported by the UniProt catalog. Data were deposited in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo, accession no. GSE103828).

2.3 Mapping and identification of differentially expressed genes

We applied Agilent GeneSpring GX version 12.1 to screen out the differentially expressed genes using the following criteria: (i) fold change >2 for upregulation or downregulation; (ii) P-value <.05; and (iii) false discovery rate (FDR) <0.05.

2.4 Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses

Gene Ontology (GO) analysis was undertaken to facilitate the understanding of the unique biological significance of the genes in the distinctive or representative profiles of the differentially expressed genes. Pathway analysis of differentially expressed genes was carried out to find out the important pathways, based on the latest Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The significant GO terms and pathways were identified by Fisher’s exact test, and FDR was used to correct the P-values.

2.5 Correlation and coexpression analysis

The coexpression analysis was based on weighted correlation network analysis (WGCNA), a systems biology method for constructing relationship patterns. Compared to general methods, such as Pearson’s correlation coefficient, WGCNA uses the soft threshold, which can provide more extensive and exact correlation between transcripts. Differentially expressed lncRNAs and mRNAs with fold change ≥4, P < .05, and FDR <0.05 were analyzed. The value of parameter soft threshold =0.98 and P-value <.05 was recommended for the coexpression analysis. k-Core scoring was used to determine core transcripts of coexpression networks. A higher k-core score means a more central location of a transcript within a network. The soft threshold was adjusted to 0.8 to obtain the lncRNA coexpressed mRNA cluster for further functional analysis of lncRNAs.
TABLE 1  Real-time quantitative PCR primer sequences used in this study

| Primer name | Forward (5'-3') | Reverse (5'-3') |
|-------------|----------------|----------------|
| β-actin (H) | GTGCGCGAGGACTTTGAT | CCTGTAACAACGATCTCAAT |
| RP1122k16.2 | CTAAACTTTTGAGTCGCT | CCCATTCGGACATCTCAT |
| AC092580.4 | GACCAAGAAGAAGAGAAAGC | CAAGAGAGACAGATCGTCCAG |
| linc00944 | CCCGAACACATCATCATT | GAGTTACAGGACCAGAGCA |
| linc00899 | CCCAAAGGAAGGTCTGGT | TCAGTGCTGAGTACATCT |
| RP1-109B7.5 | CACTGAGAGAGACAGAAGG | CAAGCTTAACCTCCTCAG |
| RP1-305O6.3 | TGCTTAACCCTCCTCAGT | GTGAGGAACAGAGGAAGT |

FIGURE 1  Volcano plots and heat map showing expression profiles of long non-coding RNAs (lncRNAs) (A) and mRNAs (B) in acute myeloid leukemia. Left panels, plots are based on the expression values of all lncRNAs and mRNAs detected by microarray. Middle and right panels, maps showing significantly changed lncRNAs and mRNAs with fold change ≥2.0 and ≥10.0 respectively (P < .05; false discovery rate <0.05)
2.6 | Gene Ontology annotations of IncRNA-correlated mRNAs

The IncRNA coexpressed mRNAs, calculated by WGCNA, were analyzed by DAVID tools for GO analysis. Fisher’s exact test was applied to identify the significant GO terms, and FDR was utilized to correct the P-values.

2.7 | Validation of IncRNAs by qRT-PCR

Expression of six IncRNAs was validated by qRT-PCR. The cDNA was synthesized by reverse transcription using a PrimeScript RT reagent kit with random primers according to the manufacturer’s protocols (TaKaRa). Then, qRT-PCR was carried out using SYBR Premix Ex Taq II (Tli RNaseH Plus; TaKaRa). Primers for RP11-222k16.2, AC092580.4, LINC00944, LINC00899, RP1-109B7.5, RP11-305O.6, and β-actin were synthesized by Invitrogen (Shanghai, China). All qRT-PCR primer sequences are shown in Table 1.

2.8 | Statistical analysis

Statistically significant differences between groups were estimated by the Mann-Whitney U-test for the expression of IncRNAs using SPSS 23.0 (IBM, SPSS, Chicago, IL, USA); P < .05 was considered statistically significant. Correlation of transcripts were evaluated using Pearson’s correlation. The Kaplan-Meier survival curves were used to show the differences in patients’ overall survival (OS) between the high expression group and low expression group, and the statistical significance
chromosome Y are excluded to eliminate gender
somes covering chromosome X and Y. The transcripts located in
(Figure 1).

of lncRNAs and mRNAs in AML differ from those in IDA controls
and mRNAs among samples. The data suggested that the expression
analysis showed systematic variations in the expression of lncRNAs
was obtained using the two-sided log-rank test. Cox regression was
used to analyze the significance of IncRNAs for OS more deeply.

3 | RESULTS

3.1 | Differentially expressed IncRNAs and mRNAs
in AML

Volcano plots were used for assessing gene expression variation
between AML and IDA patient groups. In total, 3564 IncRNAs dis-
played differential expression in AML, including 1872 upregulated
IncRNAs and 1692 downregulated IncRNAs. Of 3106 mRNAs that
showed differential expression, 1084 were upregulated and 2022
were downregulated. Among them, 37 IncRNAs and 42 mRNAs were
significantly upregulated, and 112 IncRNAs and 317 mRNAs were
significantly downregulated >10-fold in AML. Hierarchical clustering
analysis showed systematic variations in the expression of IncRNAs
and mRNAs among samples. The data suggested that the expression
of IncRNAs and mRNAs in AML differ from those in IDA controls
(Figure 1).

These IncRNAs and mRNAs are widely distributed in all chromo-
somes covering chromosome X and Y. The transcripts located in
chromosome Y are excluded to eliminate gender’s effect (Figure 2A).
The well-annotated IncRNAs (totally 1216) were classified into six
categories: 14.1% were intronic antisense, 5.8% were intron sense-
overlapping, 5.5% were bidirectional, and 1.1% were exon sense-
overlapping. There are overlaps between these four categories
(Figure 2B). Intergenic and natural antisense IncRNAs constitute the
largest number in all differentially expressed IncRNAs, and comprised
54.5% and 19.0%, respectively, in this study. We also noted that,
among the 1498 and 231 pair relationships, 57.9% of intergenic
IncRNAs and 76.6% of natural antisense were positively correlated
with their neighboring genes (Figure 2C).

3.2 | Functional analysis of differentially expressed
genes

Until now, the functions of most IncRNAs have not been well anno-
tated. Therefore, by analyzing differentially expressed mRNAs, we
can forecast the role that IncRNAs play in AML. The GO and KEGG
pathway analyses of differentially expressed mRNAs could provide a
cue about the AML disease process. We utilized all differentially
expressed mRNAs for the GO analysis and found that the most
enriched GO targeted by upregulated and downregulated transcripts
were involved in anterior/posterior pattern specification, immune
system processes, and immune response (Figure 3A). In the KEGG

pathway analysis, the down- and upregulated mRNAs were found to
be mostly enriched in hematopoietic cell lineage and glycerophos-
pholipid metabolism, respectively (Figure 3B). Many genes involved
in hematopoietic cell differentiation were dysregulated (Figure 3C). A
pathway network was constructed using 20 of the most significantly
enriched pathways to illustrate the critical pathways in the process
of AML. The hematopoietic cell lineage pathway and cell adhesion
molecules pathway were considered to be the most central functions
in the net because the exchanges with other pathways strongly
depended on their existence (Figure 3D).

To further investigate the function of genes at the protein level,
and to reveal the core mRNAs in the cellular process of AML, a bio-
 logical database, Search Tool for the Retrieval of Interacting Genes/
Proteins (STRING) was used to filter functional genes, thus providing an
intuitive network for annotating structural and functional properties
of proteins.15 The highest confidence score (0.9) was adopted to eval-
uate the protein interactions for the differentially expressed genes.
The network contains 1000 nodes and 636 edges (Figure 4A). The k-
score was used to assess the importance of genes, and the 30 highest
k-score genes constitute two important subnetworks (Figure 4B). The
networks are enriched in the G-protein-coupled receptor signaling
pathway and T-cell receptor signaling pathway (Figure 4C).

3.3 | Long non-coding RNA/mRNA coexpression
network in AML

Transcriptome regulation involves a huge network, among which
many transcripts form a complex web to function. Coexpression net-
works facilitate the intricate network based on gene screening meth-
ods that can be used to identify candidate biomarkers or therapeutic
targets. The coexpression network comprised 676 network nodes
and 1283 connections, and several prominent subnetworks were
formed. The most crucial subnetwork was constructed by the tran-
scripts with a high k-score, which would be the core regulatory mod-
ules of the entire coexpression network (Figure 5). What is more,
the transcripts in this subnetwork are widely distributed in all chro-
mosomes, indicating the widely interconnected regulation network
between IncRNAs and mRNAs (Figure 2A). This subnetwork includes
four IncRNAs, RP11-222K16.2, G005087, G044640, and
ANKRD36BP2, constituting probably the core of the network.

3.4 | Coexpression and GO annotations to predict
the probable functions of IncRNAs

It is well known that IncRNAs could regulate the expression of
neighboring coding genes by cis-pattern, and affect distant genes
by trans-pattern, even in different chromosomes.\textsuperscript{16,17} We con-
structed mRNA functional modules to clarify the biological role of
differently expressed lncRNAs. The mRNAs coexpressed with
lncRNA were annotated by GO terms. The enriched GO terms
($P < 0.05$) could reflect the function of lncRNAs. The analysis flow
chart of RP11-222K16.2 is shown as an example (Figure 6A,B). To

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6}
\caption{G-protein-coupled receptor signaling pathway}
\end{figure}
confirm the correlation, the multi-experiment matrix was applied to obtain more correlated mRNAs, if the lncRNAs were included in the database. Then, by their connections with mRNAs, lncRNAs were functionally annotated. We analyzed all the lncRNAs with a fold change >10 and constructed three separate networks to show the connections of lncRNAs with GO annotations. The core biological process GO terms of each separate system were: GO: 0002250, adaptive immune response; GO: 0009952, anterior/posterior pattern formation; and GO: 0015671, oxygen transport. Referring to the results of GO annotations for differentially expressed mRNAs, the core GO terms for lncRNAs correctly reflect the biological process of AML.

3.5 | Survival analysis using TCGA for lncRNAs

After the systemic analysis of lncRNA and mRNA expression profiling, we obtained the core lncRNAs from the coexpression network and the GO annotations enrichment map. Through analysis of the TCGA dataset, which provides extensive genetic studies of human gene expression and specific disease associations, we found several...
In lncRNAs that are involved the prognosis of AML patients. Of 151 patients in the AML dataset, 130 had full transcript sequencing and survival time data. Hundred and forty seven lncRNAs in our microarray had fold change $>10$ or belonged to the core of the coexpression network, and 44 of these 147 lncRNAs had the expression profiling in the TCGA dataset. Based on the median value of lncRNA expression, patients were divided into high expression and low expression groups for each lncRNA. The Kaplan-Meier survival curves of 130 patients with AML showed that expression of eight lncRNAs was correlated with OS (Figure 7A). Among the eight lncRNAs, lncRNA RP11-222k16.2 is the core of the coexpression network, and lncRNA HOXB-AS3 has been reported to be upregulated in NPM1-mutated AML. In order to find independent factors for patients’ OS, a multivariate regression analysis was further carried out on the expression levels of eight lncRNAs with OS, and other individual clinical features were also considered. The results showed RP11-222k16.2, LINC00899 and RP11-305O.6 were significant to independently predict patients’ OS ($P < .05$). The significant factors are summarized in Table 2. All 130 patients were assigned to a high-risk group or a low-risk group using the median risk score as the cut-off point. The Kaplan-Meier analysis showed that there are significant differences in patients’ OS between high-risk and low-risk
groups ($P < .001$, log-rank test; Figure 7B). Patients in the high-risk group had significantly shorter mean OS (569.7 days) than those in the low-risk group (1448.5 days). In conclusion, the three lncRNAs not only expressed abnormally in AML, but also had important clinical significance. Further functional studies for these lncRNAs would be valuable.

### 3.6 Validation of dysregulated lncRNAs in AML vs IDA controls

To validate the microarray data, we used qRT-PCR to detect lncRNA expression. RP11-222k16.2 and AC092580.4 that belonged to the core of the coexpression network and had the highest GO

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**FIGURE 7** Expression of eight long non-coding RNAs (from The Cancer Genome Atlas [TCGA]) correlates with survival in acute myeloid leukemia. A, Kaplan-Meier survival curves of 130 patients with acute myeloid leukemia. The median value of expression was set as the cut-off point. B, Kaplan-Meier analysis for overall survival of patients with high-risk or low-risk scores in the TCGA dataset. C, Risk score distribution and survival status of the risk score model in 130 patients of TCGA dataset.
annotation enrichment (Figures 5 and 6C) were confirmed in bone marrow from 82 AML patients (51 newly diagnosed, 8 relapses, and 23 complete response) and 17 IDA patients. LINCO0944, LINCO0899, RP6-109B7.5, and RP11-305O.6 were detected in 22 newly diagnosed AML and 10 IDA cases. Through our confirmation, lncRNA RP11-222K16.2, AC092580.4, and RP11-305O.6 had different expressions in AML and IDA, but LINC00944 lost significance. Furthermore, RP11-222K16.2 and AC092580.4 had validated difference in newly diagnosed and recurrent AML between complete response patients (Figure 8).

3.7 | Long non-coding RNA RP11-222K16.2 involved in the immune system through cis-regulated Eomes

As mentioned, RP11-222K16.2 was the core lncRNA in the coexpression network. The GO annotations showed that it was involved in the immune system. What is more, its expression level was correlated with AML patients’ OS. To understand how lncRNA RP11-222K16.2 functions in AML, we had the deeper insight into the AML transcripts’ sequencing data from the TCGA. We calculated the Spearman’s correlation of lncRNA RP11-222K16.2 with approximately 37 100 transcripts in 151 AML patients. Eomes is the mRNA with the highest correlation with lncRNA RP11-222K16.2. Eomes was significantly downregulated in the AML group. The top-scoring gene in the immune system development category was Eomes. This gene was differentially expressed in our microarray data, and the analysis by string software showed that it was the core mRNA in AML (Figure 4B). Eomes is a master regulator of CD8 T-cell function, and a transcription factor that is critical for terminal natural killer (NK) cell differentiation.23,24 A recent study noted the NK cells from leukemic mice and humans with AML showed lower level of Eomes, which at least partly led to the blocking of NK cell differentiation, and then it enabled AML to evade mature NK cell surveillance.25 Based on these results, we predicted that, through the lncRNA-mediated dysregulation of Eomes, blocking of NK cell differentiation occurs, promoting the immunized evasion of AML. Additional studies are underway to probe into this additional mechanism of immune escape in cancer.

### DISCUSSION

It is well known that the mutation of genes and chromosomes contribute to the pathogenesis of leukemia.25 However, lncRNAs, the rising stars in biology, have just begun to be understood, and the majority of them have not yet been researched. To provide some insights into the biological functions of lncRNAs in the pathogenesis of AML, we undertook a comprehensive analysis of lncRNA and mRNA profiling data from AML and IDA patients, together with data from a public database. We identified the core lncRNAs and their functional annotations, and validated their expression by qRT-PCR. Overall, our work uncovered an interlaced transcripts network that is involved in AML development, in which lncRNAs play an indispensable role.

We explored the expression patterns of transcripts between AML patients and IDA controls’ bone marrow. Microarray data identified vast lncRNAs and mRNAs, supporting an extensive involvement of lncRNAs in AML. There were two important concepts that ran throughout our studies to handle the mass data. First, we simplified the complex transcript network by modularization. The GO and KEGG pathway analyses divided mRNAs into several functional modules, which are related to immunity, hematopoiesis, and cancer, indicating the validity of the microarray. STRING and WGCNA were used to construct coexpressed networks from the public and our microarray data, respectively, and then the networks were facilitated into several subnetworks through the k-score method. In the same way, lncRNAs were attributed their correlated functional mRNA modules through the coexpressed network and GO annotations. Overall, modularization contributes to simplifying the intricate network into modules, which were like “big genes”. Second, the AML dataset from

| Variables in the equation | Hazard ratio | Coefficient | Wald | P-value |
|---------------------------|-------------|-------------|------|---------|
| RP11-222K16.2             | 0.562       | -0.577      | 3.915| .048    |
| LINC00899                 | 1.811       | 0.594       | 4.973| .026    |
| RP11-305O.6               | 0.464       | -0.767      | 7.487| .006    |
| Age                       | 1.027       | 0.027       | 10.230| .001    |
| Cytogenetics risk category| 1.632       | 0.490       | 5.620| .018    |
| White blood cell          | 1.007       | 0.007       | 4.164| .041    |

The backward step (likelihood ratio) method was used. Variables are summarized. —, not available.

TABLE 2 Multivariable Cox regression analysis of eight long non-coding RNAs and other individual clinical features
TCGA was applied to analyze the clinical significance of lncRNAs from the modules. Eight lncRNAs might have prognostic application in AML. Among them, RP11-222K16.2 gained our attention as it belonged to the most important subnetwork, and its expression is associated with patients’ OS. Further analysis of public data showed that the lncRNA may regulate Eomes to block NK cell differentiation, leading to the immunized evasion of AML. Finally, three lncRNAs, RP11-222K16.2, AC092580.4, and RP11-305O.6, were confirmed as significantly differentially expressed in AML patients and IDA controls by qRT-PCR.

Our work clearly indicated an important role for lncRNAs in AML. However, many lncRNAs were excluded as they failed to be allocated to functional modules and have not been included in public data. It was difficult to originally understand the functions and targets of these lncRNAs, which may also play a key role in AML. In addition, our analysis showed that lncRNA RP11-222K16.2 was highly correlated with Eomes, its neighboring gene. The lncRNA may be directly or indirectly correlated with Eomes and there may be additional transcripts involved in the lncRNA-associated biological process. The lncRNA’s biological functions need to be validated further.

The last decades witnessed the discovery of biological functions for non-coding RNA, which triggered the recognition that RNA is not only a simple hinge of the central dogma but also directly takes part in the regulation of biological networks. With the development of next-generation sequencing, especially in terms of depth and scale, significant data has been accumulated. We have to recognize the system is so complex that it is beyond the initial recognition. Fortunately, the progress of methodology simplifies the networks. Through modularization, thousands of transcripts can be facilitated into several “big genes,” and then the core lncRNAs of each module can be researched in details. Moreover, the active application of accumulated public data will help us to make the functions of lncRNAs more clear. Hopefully, this study can provide a reference for the broad analysis of HTS data.

**FIGURE 8** Expression of RP11-222K16.2, AC092580.4, RP11-305O.6, and LINC00944 in samples from patients with newly diagnosed acute myeloid leukemia (AML), disease relapse (R), or complete response (CR), and iron deficiency anemia (IDA) controls. RP11-222K16.2 and AC092580.4 were significantly downregulated in newly diagnosed and relapsed AML. RP11-305O.6 was significantly upregulated in newly diagnosed AML. LINC00944 was downregulated in newly diagnosed AML, but lost its significance.
FIGURE 9  Functional analysis of long non-coding RNA (lncRNA) RP11-222K16.2 in acute myeloid leukemia (AML). A, Schematic representation of the composition of the Eomes gene and the RP11-222K16.2 loci on human chromosome 3p24.1. B, Pearson's correlation of the lncRNA RP11-222K16.2 with Eomes in the AML dataset from The Cancer Genome Atlas (TCGA). C, Pearson's correlation of the lncRNA RP11-222K16.2 with Eomes in 10 cancers from TCGA. Data were acquired from the CHIP-BASE database. The size of the points represents the number of patients included in the dataset. D, E, Gene Set Enrichment Analysis (D) and map (E) for all protein-coding genes with Spearman correlations >0.4 with lncRNA RP11-222K16.2
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CONFLICT OF INTEREST

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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