Prognostic significance of long non coding maternally expressed gene 3 in pediatric acute myeloid leukemia

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Abstract

The purpose of this study was to evaluate the correlation of long non-coding RNA maternally expressed gene 3 (Lnc-MEG3) with disease features, treatment response, and survival in pediatric acute myeloid leukemia (AML) patients.

Among 92 de novo pediatric AML patients (before treatment and after 1 course of induction) and 40 controls, bone marrow mononuclear cells were obtained. Then, Lnc-MEG3 expression was determined by reverse transcription quantitative polymerase chain reaction. After 1 course of standard induction therapy of pediatric AML patients, complete remission (CR) was assessed. Furthermore, event-free survival (EFS) and overall survival (OS) were determined according to follow-up data.

Lnc-MEG3 was reduced in pediatric AML patients compared with controls. In pediatric AML patients, Lnc-MEG3 was correlated with French-American-British subtypes and lower Chinese Medical Association risk stratification, while it was not associated with cytogenetic features, FLT3-ITD mutation, CEBPA mutation, NPM1 mutation, WT1 mutation, or National Comprehensive Cancer Network risk stratification. After 1 course of treatment, Lnc-MEG3 exhibited an up-regulation trend. Furthermore, Lnc-MEG3 was of no difference before treatment between patients with and without CR, while elevated Lnc-MEG3 and change of Lnc-MEG3 after 1 course of treatment were associated with increased CR rate. Additionally, increased Lnc-MEG3 expression before treatment was associated with longer EFS but not OS, while enhanced Lnc-MEG3 expression after 1 course of treatment was correlated with both prolonged EFS and OS.

Lnc-MEG3 may have clinical significance as a biomarker for assisting with disease management, treatment optimization, and prognosis improvement in pediatric AML patients.

Abbreviations: AML = acute myeloid leukemia, CR = complete remission, EFS = event-free survival, Lnc-MEG3 = long non-coding RNA maternally expressed gene 3, OS = overall survival, WBC = white blood cell.

Keywords: complete remission, long non-coding RNA maternally expressed gene 3, pediatric acute myeloid leukemia, risk stratification, survival

1. Introduction

Pediatric acute myeloid leukemia (AML) is a hematopoietic malignancy which is characterized by accumulated chromosomal alterations and clonal expansion of aberrantly differentiated myeloblasts, and pediatric AML accounts for approximately 25% of all reported pediatric acute leukemia.[1,2] The usual treatment regimen includes intensive chemotherapy (mainly based on cytarabine and an anthracycline) and the judicious use of hematopoietic stem cell transplantation.[3] Although the long-term survival rate reaches as high as 60% in pediatric AML patients with current treatment paradigms, high refractory, relapse, and/or mortality rates remain as prominent concerns owning to heterogeneity of disease, drug resistance of AML cells, and treatment-related toxicities (including infections and cardiac failure).[4] The concerns mentioned above emphasize the need for the identification of biomarkers enabling risk stratification, therapy optimization, and prognosis improvement in pediatric AML patients.

Long non-coding RNA maternally expressed gene 3 (Lnc-MEG3), an imprinted gene, is located on human chromosome 14q32.3 within DLK1-MEG3 locus, which is extensively expressed in multiple tissues such as adrenal gland and liver.[5] Abnormal expression of Lnc-MEG3 functions as a tumor suppressor via interacting with its target microRNAs to regulate the downstream cancer-related signaling pathways (such as p53-dependent pathway and Wnt/β-catenin pathway) in hematologi-
catal malignancies including chronic myeloid leukemia and AML. In chronic myeloid leukemia, Lnc-MEG3 suppresses cell proliferation but promotes cell apoptosis and/or cell sensitivity to chemotherapy via sponging microRNA-184, microRNA-21, or microRNA-147. As to AML, earlier experimental studies uncover the involvement of Lnc-MEG3 in the inhibition of leukemogenesis and the reduction of drug resistance via the regulation of ALG9 and in a p53-dependent manner. In clinical aspects, only one previous study shows resistance via the regulation of ALG9 and in a p53-dependent experimental studies uncovers the involvement of Lnc-MEG3 in microRNA-21, or microRNA-147. As to AML, earlier sensitivity to chemotherapy via sponging microRNA-184, cell proliferation but promotes cell apoptosis and/or cell

2. Methods

2.1. Patients and specimens

Between January 2016 and May 2020, a total of 92 de novo pediatric AML patients were screened for analysis. All analyzed patients satisfied the following criteria: diagnosed as AML in accordance with the World Health Organization (WHO) classification of AML; age at diagnosis <16 years old; had cryopreserved bone marrow (BM) specimens, which were collected before treatment and after 1 course of induction treatment; had remission evaluation data after 1 course of induction treatment. Patients were excluded for analysis if they had diagnoses of acute promyelocytic leukemia (M3), bone marrow failure syndromes, juvenile myelomonocytic leukemia, or Down syndrome, or had a history of cancers (including hematological malignancies). For each patient, the BM specimen before treatment and the corresponding BM specimen after 1 course of induction treatment were collected for analysis. Meanwhile, 40 BM specimens from untreated pediatric patients with non-malignant disease were collected as controls in the current study. Those pediatric patients with nonmalignant disease were idiopathic thrombocytopenic purpura patients or hemolytic anemia patients, with age at diagnosis <16 years, without other histories of bone marrow disease or malignancies. Institutional Review Board approved the present study. For patients with age at diagnosis <10 years, their guardians signed the informed consents; as for patients with age at diagnosis >10 years, they and their guardians all signed the informed consents.

2.2. Clinical features collection

The medical charts of pediatric AML patients were checked and the following data were collected: age, sex, height, weight, French-American-British (FAB) classification, cytogenetic features, molecular abnormalities, white blood cell (WBC) level, percentage of bone marrow (BM) blasts, and risk stratifications. The risk stratification included the National Comprehensive Cancer Network (NCCN) risk stratification (based on cytogenetics and molecular abnormalities) and Chinese Medical Association risk stratification (based on risk factors including morphology and cytogenetics features, age at diagnosis, WBC ≥100 × 10^9/L at diagnosis, karyotype-7, myelodysplastic syndrome (MDS)-AML, and no remission after 1 course of standard induction therapy. The details of the NCCN risk stratifications were as follows: favorable risk, including inv (16), t (16;16), t (8;21), or t (15;17) for cytogenetics or normal cytogenetics with NPM1 mutation in the absence of FLT3-ITD or isolated biallelic CEBPA mutation for molecular abnormalities; intermediate risk, including normal cytogenetics, +8 alone, t (9;11), other cytogenetic abnormalities not classified as favorable or poor-risk or t (8;21), inv (16) or t (16;16) with c-KIT5 mutation for molecular abnormalities; poor risk, including complex (3 clonal chromosomal abnormalities), monosomal karyotype, -5, 5q-, -7q-, 11q23 - non t (9;11), inv (3), t (3;3), t (6;9), or t (9;22) for cytogenetics or normal cytogenetics with FLT3-ITD mutation for molecular abnormalities. The details of the Chinese Medical Association risk stratifications were as follows: low risk, including M3, M2b or M4 EO for FAB classification or inv (16) for molecular abnormalities; intermediate risk, including not meeting the standards for low risk, and not meeting any of the following conditions of poor prognosis for pediatric AML patients: age <1 years old; WBC ≥100 × 10^9/L at diagnosis; karyotype of -7; MDS-AML; no remission after 1 course of standard induction therapy; high risk, including any of the following conditions of poor prognosis for pediatric AML patients: age ≥1 years old; WBC <100 × 10^9/L at diagnosis; karyotype of -7; MDS-AML; no remission after 1 course of standard induction therapy.

2.3. Lnc-MEG3 determination

After collection of cryopreserved BM specimens, Ficoll density gradient centrifugation was performed for separation of BM mononuclear cells (BMMCs). Then the BMMCs were used for lnc-MEG3 determination by Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) assay. Total RNA was isolated from BMMCs using TRIzol Reagent (Invitrogen, Carlsbad, CA). Subsequently, the cDNA synthesis was conducted using Scribe cDNA Synthesis Kit (Bio-Rad, Hercules, CA). After that, the cDNA was amplified using SYBR Premix DimerEraser (Takara, Dalian, Liaoning, China). Lastly, the Lnc-MEG3 relative expression was calculated by the 2^-ΔΔCt method, with GAPDH as the internal reference. The primers applied in the qPCR procedures were listed as below: lncRNA MEG3, forward primer (5'-3') TACACTCTACAGGGGACTA, reverse primer (5'-3') CAGGGCTTAATGCCCAATGC; GAPDH, forward primer (5'-3') TACACCTCACGAGGGCACTA, reverse primer (5'-3') GCCTGCTTCACCACCTTCTTGA.

2.4. Treatment and remission evaluation

The pediatric AML patients received standard induction therapy with DA/E or IA regimen for 1 course. The DA/E regimen included daunorubicin (DNR) 40 mg/m^2 d, day 1–3, intravenous drip for 30 minutes; cytarabine (Ara-c) 200 mg/m^2 d, day 1–7, every 12 hours (q12h), hypodermic injection; etoposide (E) 100 mg/m^2 d, day 5–7, intravenous drip for 3 to 4 hours. The IA regimen included idarubicin (IDA) 10 mg/m^2 d, day 1–3, intravenous drip for 30 minutes; Ara-c 200 mg/m^2 d, day 1–7, q12h, hypodermic injection. After 1 course of induction therapy, remission status of pediatric AML patients was assessed in terms of remission assessment criteria recommended by the international expert panel, in which complete remission (CR) was defined as (all of the following conditions should be satisfied): BM blasts <5%; absence of blasts with Auer rods; no extramedullary disease; absolute neutrophil count >1.0 × 10^9/L; platelet count <100 × 10^9/L at diagnosis, karyotype-7, myelodysplastic syn-
>80 \times 10^9/L; independence of red cell transfusions. After 1 course of induction therapy, CR patients repeated the original regimen for consolidation chemotherapy, followed by further chemotherapy or hematopoietic stem cell transplantation (HSCT); non-CR patients received the original regimen for the next course, followed by consolidation chemotherapy, HSCT, or salvage chemotherapy.

### 2.5. Follow-up and survival assessment

Management and surveillance of pediatric AML patients were performed as recommended by guidelines.\(^{[15,17]}\) Disease status during follow-up surveillance was recorded for survival evaluation. The event-free survival (EFS) was defined as the time duration from initiation of induction therapy to failure to achieve CR or PR, or relapse from CR, or death. If patients did not suffer from any of the above events, they were censored on the date that they were last examined. The overall survival (OS) was defined as time duration from initiation of induction therapy to death.

### 2.6. Statistical analysis

Statistical processing and analysis were carried out with the use of SPSS 24.0 (IBM, Armonk, NY). Statistical graphs were plotted using GraphPad Prism 7.01 (GraphPad Software Inc., San Diego, CA). Quantitative data were described as mean with standard deviation (SD) or median with interquartile range, based on normality by Kolmogorov-Smirnov test. Qualitative data were described as the number with a percentage. The difference of Lnc-MEG3 expression before and after treatment, or Kruskal-Wallis H rank-sum test. In the survival analysis, according to the median value of Lnc-MEG3 expression, pediatric AML patients were categorized into Lnc-MEG3 high expression group and low expression group. The correlation of Lnc-MEG3 expression with risk stratification was determined by Spearman rank correlation test. Change of Lnc-MEG3 expression was calculated using the Lnc-MEG3 expression before 1 course of treatment minus the Lnc-MEG3 expression after 1 course of treatment. The correlation of Lnc-MEG3 expression with molecular genetics, no. (%) was determined by Spearman rank correlation test. In the survival analysis, according to the median value of Lnc-MEG3 expression, pediatric AML patients were categorized into Lnc-MEG3 high expression group and low expression group. The correlation of Lnc-MEG3 expression with risk stratification was determined by Spearman rank correlation test. Change of Lnc-MEG3 expression was calculated using the Lnc-MEG3 expression before 1 course of treatment minus the Lnc-MEG3 expression after 1 course of treatment. The correlation of Lnc-MEG3 expression with molecular genetics, no. (%) was determined by Spearman rank correlation test. In the survival analysis, according to the median value of Lnc-MEG3 expression, pediatric AML patients were categorized into Lnc-MEG3 high expression group and low expression group. The correlation of Lnc-MEG3 expression with risk stratification was determined by Spearman rank correlation test. Change of Lnc-MEG3 expression was calculated using the Lnc-MEG3 expression before 1 course of treatment minus the Lnc-MEG3 expression after 1 course of treatment.

### 3. Results

#### 3.1. Baseline characteristics of pediatric AML patients

In pediatric AML patients, the mean age was 6.9 ± 2.9 years, and there were 46 (50.0%) men/46 (50.0%) women (Table 1). As to FAB classification, 5 (5.4%), 38 (41.3%), 17 (18.5%), and 32 (34.8%) patients presented with M1, M2, M4, and M5 AML, respectively. Regarding molecular genetics, 25 (27.2%), 14 (15.2%), 21 (22.8%), and 12 (13.0%) patients exhibited FLT3-ITD mutation, CEBPA mutation, NPM1 mutation, and WT1 mutation, respectively. In terms of risk stratification, 30 (32.6%), 28 (30.4%), and 34 (37.0%) patients displayed favorable, intermediate, and poor risk based on NCCN risk stratification, respectively; meanwhile, 14 (15.2%), 41 (44.6%), and 37 (40.2%) patients had low, intermediate, and high risk based on Chinese Medical Association risk stratification, respectively. Other detailed information about height, weight, cytogenetics, WBC, and BM blasts of pediatric AML patients was exhibited in Table 1.

#### 3.2. Lnc-MEG3 expression

Lnc-MEG3 expression was reduced in pediatric AML patients compared with controls (median [interquartile range]: 0.262 [0.180–0.444] vs 1.004 [0.621–1.488]) (P < .001) (Fig. 1).

#### 3.3. Association of Lnc-MEG3 with FAB classification, cytogenetics, and molecular genetics

Lnc-MEG3 was highest in pediatric AML patients with M2 type, followed by pediatric AML patients with M4 type and pediatric AML patients with M5 type, then lowest in pediatric AML patients with M1 type (P = .007) (Table 2). As for the association of Lnc-MEG3 with cytogenetics and molecular genetics, no

### Table 1

| Pediatric AML patients' characteristics. | Pediatric AML patients (N = 92) |
|-----------------------------------------|---------------------------------|
| Age, yr, mean±SD | 6.9±2.9 |
| Gender, no. (%) | Male 46 (50.0) Female 46 (50.0) |
| Height, cm, mean±SD | 119.2±19.0 |
| Weight, kg, mean±SD | 24.3±9.2 |
| FAB classification, no. (%) | M1 5 (5.4) M2 38 (41.3) M4 17 (18.5) M5 32 (34.8) |
| Molecular genetics, no. (%) | FLRT3-ITD mutation 25 (27.2) CEBPA mutation 14 (15.2) NPM1 mutation 21 (22.8) WT1 mutation 12 (13.0) |
| NCCN risk stratification, no. (%) | Better 30 (32.6) Intermediate 28 (30.4) Poor 34 (37.0) |
| Chinese Medical Association risk stratification, no. (%) | Low 14 (15.2) Mediate 41 (44.6) High 37 (40.2) |

AML = acute myeloid leukaemia, CK = complex karyotype, FAB classification = French-American-British classification, NK = normal karyotype, SD = standard deviation.

* Others, not included in better or poor risk according to NCCN guidelines; FLRT3-ITD, internal tandem duplications in the FMS-like tyrosine kinase 3; CEBPA, CCAAT/enhancer-binding protein a; NPM1, nucleophosmin 1; WT1, Wilm tumor gene 1; WBC, white blood cell; IQR, interquartile range; BM, bone marrow; NCCN, national comprehensive cancer network.
Association was revealed of Lnc-MEG3 with cytogenetics ($P = .381$), FLT3-ITD mutation ($P = .252$), CEBPA mutation ($P = .086$), NPM1 mutation ($P = .878$), or WT1 mutation ($P = .266$) in pediatric AML patients.

### 3.4. Association of Lnc-MEG3 with risk stratification

In pediatric AML patients, no association was found of Lnc-MEG3 with cytogenetics, FLT3-ITD mutation, CEBPA mutation, NPM1 mutation, or WT1 mutation in pediatric AML patients.

### 3.5. Association of Lnc-MEG3 with CR after 1 course of induction treatment

Lnc-MEG3 displayed an increasing trend after 1 course of treatment in pediatric AML patients ($P < .001$) (Fig. 3). After 1 course of treatment, 61 (66.3%) pediatric AML patients achieved CR (grouped as CR patients), while the remaining 31 (33.7%) pediatric AML patients did not achieve CR (grouped as non-CR patients) (Fig. 4A). Furthermore, Lnc-MEG3 before treatment ($P = .114$) (Fig. 4B) was of no difference between CR patients and non-CR patients, while Lnc-MEG3 after 1 course of treatment ($P < .001$) (Fig. 4C) and change of Lnc-MEG3 ($P < .001$) (Fig. 4D) were increased in CR patients compared with non-CR patients.

### 3.6. Association of Lnc-MEG3 with survival

Lnc-MEG3 high expression before treatment was associated with prolonged EFS ($P = .034$) (Fig. 5A), but not OS ($P = .092$) (Fig. 5B) in pediatric AML patients. Regarding Lnc-MEG3 high expression after 1 course of treatment, it was associated with both longer EFS ($P = .003$) (Fig. 6A) and OS ($P = .016$) (Fig. 6B) in pediatric AML patients.

### 3.7. Association of chemotherapy regimen with achievement of CR after 1 course of induction treatment and Lnc-MEG3

In pediatric AML patients, the chemotherapy regimen was not associated with achievement of CR after 1 course of induction treatment ($P = .185$) (Figure S1A, Supplemental Digital Content, http://links.lww.com/MD2/A324) or change of Lnc-MEG3 ($P = .577$) (Figure S1B, Supplemental Digital Content, http://links.lww.com/MD2/A324).

### 3.8. Prognostic value of age in pediatric AML patients

According to the Cox proportional regression analysis, age ≤ 1 year had no influence on the overall survival ($P = .576$) (Table S1, Supplemental Digital Content, http://links.lww.com/MD2/A325).

### 4. Discussion

Preceding evidence illuminates that Lnc-MEG3 modulates malignant cell activities and chemosensitivity in chronic myeloid leukemia and AML.[6–12] In chronic myeloid leukemia, the overexpression of Lnc-MEG3 inhibits cell proliferation while downregulating the expression of multidrug transporters and enhancing cell apoptosis, which results in increased cell sensitivity to imatinib in chronic myeloid leukemia.[11] In AML, based on prior experimental studies, Lnc-MEG3 represses cell proliferation but facilitates G0/G1 cell cycle arrest and cell apoptosis through p53-dependent signaling pathway and retinoblastoma/mouse double minute 2 homolog signaling pathway; meanwhile,
Lnc-MEG3 might act as a competing endogenous RNA to sponge microRNA-155 and further relieve the absorption effect on microRNA-155 to ALG9, and the latter promotes proliferation, induces drug sensitivity and sphere formation in AML cells.[8,10] Clinically, only one study reveals that in adult AML patients, Lnc-MEG3 is linked with favorable-risk stratification, CR, and prolonged survival benefits.[13] However, the clinical significance of Lnc-MEG3 in pediatric AML patients has not been evaluated.

Preceding data display that Lnc-MEG3 is linked with favorable risk stratification (by NCCN risk stratification) in adult AML patients, while the association of Lnc-MEG3 with clinicopathological features in pediatric AML patients remains to be elucidated.[13] In the present study, it was found that Lnc-MEG3 was highest in M2 type pediatric AML patients, followed by M4 type pediatric AML patients and M5 type pediatric AML patients, then lowest in M1 type pediatric AML patients; furthermore, Lnc-MEG3 was associated with lower Chinese Medical Association risk stratification but not NCCN risk stratification in pediatric AML patients. The possible explanations were listed as below: Lnc-MEG3 probably suppressed the proliferative capacity and induced cell cycle arrest at G0/G1 phase progression of myeloblasts, which resulted in varied differentiation state of myeloblasts and different FAB subtypes in pediatric AML patients.[8,10] Chinese Medical Association risk stratification was based on 2 key risk factors: morphology features and remission status after 1 course of induction therapy, while NCCN risk stratification was mainly based on cytogenetics and molecular abnormalities; besides, the present study also found that Lnc-MEG3 was linked with FAB subtypes (based on the morphology of leukemia cells) and CR in pediatric AML patients, therefore Lnc-MEG3 was correlated with lower Chinese Medical Association risk stratification but not NCCN risk stratification in pediatric AML patients.[13]

From previous evidence, Lnc-MEG3 high expression is correlated with higher treatment response, longer OS, and EFS in adult AML.[13] While in pediatric AML patients, the prognostic value of Lnc-MEG3 is still unknown. In the present study, Lnc-MEG3 was detected in pediatric AML patients and controls, then the data revealed that Lnc-MEG3 was decreased in pediatric AML patients compared with controls. One potential explanation might be that: according to the preceding evidence, Lnc-MEG3 probably repressed AML cell proliferation while mediated G0/G1 cell cycle arrest and facilitated AML cell apoptosis, which inhibited leukemogenesis, hence, Lnc-MEG3 was expressed at reduced levels in pediatric AML patients.[8,10]

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study, it was displayed that in pediatric AML patients, the increment of Lnc-MEG3 after induction therapy was linked with CR; furthermore, Lnc-MEG3 before treatment was only associated with improved EFS, while Lnc-MEG3 after induction therapy was associated with both better EFS and OS. The potential reasons were listed as below: firstly, Lnc-MEG3 might downregulate the expressions of multidrug-resistant drug transporters (such as MRP1 and ABCG2) and then increases AML cell sensitivity to drug treatment, therefore, the increment of Lnc-MEG3 was linked with CR in pediatric AML patients.[11] Furthermore, Lnc-MEG3 might hinder AML cell proliferation while facilitating G0/G1 cell cycle arrest and AML cell apoptosis through its downstream pathways (including the p53-dependent manner and RB-DNMT3A signaling pathway), which repressed

![Figure 4. Lnc-MEG3 correlated with CR in pediatric AML patients. Response rate after 1 course of treatment in pediatric AML patients (A). Comparison of Lnc-MEG3 expression before treatment between CR and non-CR pediatric AML patients (B). Comparison of Lnc-MEG3 expression after 1 course of treatment between CR and non-CR pediatric AML patients (C). Comparison of change of Lnc-MEG3 expression between CR and non-CR pediatric AML patients (D). AML= acute myeloid leukemia, CR= complete remission, Lnc-MEG3= long non-coding RNA maternally expressed gene 3, non-CR= non-complete remission.](image)

![Figure 5. Lnc-MEG3 before treatment correlated with EFS but not OS in pediatric AML patients. Comparisons of EFS (A) and OS (B) between pediatric AML patients with Lnc-MEG3 high (before treatment) and pediatric AML patients with Lnc-MEG3 low (before treatment). AML= acute myeloid leukemia, EFS= event-free survival, Lnc-MEG3= long non-coding RNA maternally expressed gene 3, OS= overall survival.](image)
tumor growth, mitigated leukemogenesis and disease severity, resulting in prolonged survival in pediatric AML patients.[8]

Additionally, according to our findings, Lnc-MEG3 was associated with lower Chinese Medical Association risk stratification and higher CR, which contributed to longer survival in pediatric AML patients.

Although the present study was the first to investigate the clinical value of Lnc-MEG3 in pediatric AML patients, some limitations should be addressed. Firstly, as it was hard to enrol untreated pediatric patients with non-malignant disease as controls, the sample size of the controls was relatively small, which probably decreased the statistical power of the analysis. Secondly, the effect of Lnc-MEG3 on regulating malignant cell activities in AML was not investigated, further in vivo and in vitro experimental studies were needed. Thirdly, since only de novo pediatric AML patients were enrolled and included for the study analyses, our findings might not be applicable to relapsed pediatric AML patients. Fourthly, nowadays the WHO classification is widely used in clinical practice, while at the time this study analyses, our newly diagnosed pediatric AML patients were enrolled and included for the study analyses, our findings might not be applicable to relapsed pediatric AML patients. Lastly, Lnc-MEG3 was only assessed before treatment and after 1 course of treatment, while the longitudinal change of Lnc-MEG3 during the whole disease course was not evaluated, hence, further studies would be desirable.

To sum up, pretreatment Lnc-MEG3 correlates with different FAB subtypes and lower risk stratification by Chinese Medical Association; furthermore, the increment of Lnc-MEG3 after 1 course of induction therapy is linked with higher CR and prolonged survival in pediatric AML patients.

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