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Expression Profiling of MicroRNAs in Hippocampus of Rats Following Traumatic Brain Injury*

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Summary: The changes of microRNA expression in rat hippocampus after traumatic brain injury (TBI) were explored. Adult SD rats received a single controlled cortical impact injury, and the ipsilateral hippocampus was harvested for the subsequent microarray assay at three time points after TBI: 1st day, 3rd day and 5th day, respectively. We characterized the microRNA expression profile in rat hippocampus using the microRNA microarray analysis, and further verified microarray results of miR-142-3p and miR-221 using quantitative real-time PCR. Totally 205 microRNAs were identified and up-/down-regulated more than 1.5 times. There were significant changes in 17 microRNAs at all three time points post-TBI. The quantitative real-time PCR results of miR-142-3p and miR-221 indicated good consistency with the results of the microarray method. MicroRNAs altered at different time points post-TBI. MiR-142-3p and miR-221 may be used as potentially biological markers for TBI assessment in forensic practice.
Key words: forensic clinical medicine; traumatic brain injury; microRNA; microarray technique; hippocampus

Traumatic brain injury (TBI) is a common trauma in industrial accident, traffic accident and fight. Around 10 million people worldwide suffer TBI event each year[1]. Identification of TBI becomes a major responsibility in forensic clinical medicine. Currently, medical technologies for identification of TBI are limited, and it is one of the hot-topics and difficult problems that offer relatively objective and accurate identification for ascertaining the pathophysiological changes after TBI. Many survivors of TBI remain some functional disorders to some extent, especially enduring cognitive deficits, including impaired learning and memory[2]. The hippocampus is crucial for learning and memory, but it is quite vulnerable to TBI, displaying more essential and earlier pathological changes than other brain regions[3, 4]. However, the underlying mechanism of the alterations in gene expression in the hippocampus neuron following TBI is still elusive.

MicroRNA (miRNA) is a novel class of small non-coding RNAs, which consist of about 22 nucleotides that function as regulators of posttranscriptional gene expression, by binding to the 3′-untranslated region of target mRNA and thus leading to the translational inhibition or degradation of the target mRNA[5–7]. Recent studies suggested that a number of miRNAs are highly expressed in the mammalian central nervous system as the key modulator of cell differentiation, proliferation, apoptosis, neuronal development, neuroprotection and synaptic plasticity[8, 9]. The present study was undertaken to reveal the changes in the expression of miRNAs in rat hippocampus at day 1, 3 and 5 post-TBI by microarray analysis and qRT-PCR, and further investigate the applied value of miRNAs in the forensic medicine.

1 MATERIALS AND METHODS

1.1 Animals and Surgical Procedures

Adult Sprague-Dawley (SD) rats of either gender weighing 220–250 g were purchased from Laboratory Animals Center of Tongji Medical College of Huazhong University of Science and Technology (China) and housed in a controlled temperature environment under a 12 h light/12 h dark cycle, with access to food and water *ad libitum*. Twenty SD rats were randomly divided into four groups (n=5 each): one sham-operated group and three TBI groups according to the time points of experiments: 1st day, 3rd day and 5th day post-TBI. Animal use was in accordance with “Control Ordinance of Laboratory Animals of Hubei Province, China”. Surgical procedures were approved by the Institutional Animal Care and Use Committee. An intraperitoneal injection of 10% chloral hydrate (3 mL/kg) was performed to anesthetize the rats before controlled cortical impact (CCI) injury. The surgery commenced once pedal foot reflexes disappeared. Rats were placed on a stereotaxic device. Body temperature was monitored and maintained using a heated pad. Surgical areas of the rat brain were shaved and treated with iodine. After exposure of the left parietal, a 4 mm×4 mm unilateral craniectomy was performed to expose the dura with the frontal edge 1 mm posterior to bregma and with the medial edge 2 mm lateral to the

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midline. Each rat received a single contusion using an electromagnetic piston at a velocity of 3.5 m/s, duration of 200 ms and depth of 2 mm. For sham rats, the incision was made and craniotomy was performed as in the CCI rats, but without CCI injury. Then the incision was sutured, and antibiotic ointment was applied. The animals were monitored during recovery from anesthesia, and then were placed back to their home cages. At 1st day, 3rd day and 5th day post-TBI, animals were killed by decapitation. The ipsilateral hippocampus was dissected immediately in the ice-cold artificial cerebrospinal fluid, and instantly placed into cryopreservation tubes and stored in liquid nitrogen cans for the subsequent tests.

1.2 Total RNA Quality

Total RNA was harvested using TRIzol (Invitrogen, USA) and miRNeasy mini kit (Qiagen, USA) according to manufacturer's instructions. RNA quality and quantity were measured using NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, USA). The ratio of the readings at 260 nm and 280 nm (260/280) was calculated to estimate the purity of RNA with respect to contaminants that absorb in the UV, such as protein. The ratio of absorbance (A) at 260 and 280 nm (A_{260}/A_{280}) should be in a range from 1.8 to 2.1, which is the acceptable RNA purity. It was indicated that there was no detectable protein contamination of the RNA sample. And A_{260}/A_{230} ratio greater than 1.8 was usually considered an acceptable indicator of RNA purity.

1.3 MicroRNA Microarray Assay and Data Analysis

After having passed RNA quality measurement, the samples were labeled using the miRCURY™ Hy3™/Hy5™ Power labeling kit (Exiqon, Vedbaek, Denmark). Then, the Hy3™-labeled samples were hybridized on the miRCURY™ LNA Array (v.18.0) (Exiqon, Vedbaek, Denmark) according to the array manual. Following hybridization, the slides were achieved, washed several times using Wash buffer kit (Exiqon, Vedbaek, Denmark), and finally dried by centrifugation for 5 min at 400 r/min. Then the slides were scanned using the Axon GenePix 4000B microarray scanner (Axon Instruments, USA) and imported into GenePix Pro 6.0 software (Axon Instruments, USA) for grid alignment and data extraction. Data were analyzed by subtracting the background and then normalizing the signals using a LOWESS (Locally-Weighted Regression) filter. Target miRNAs differentially expressed in hippocampus were screened out through microarray analysis.

1.4 Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

We further confirmed target miRNAs expression obtained from microarray analysis using qRT-PCR. MiRNAs were transcribed into cDNAs using Gene Amp PCR System 9700 (Applied Biosystems, USA). Real-time PCR was performed with the ABI PRISM 7900 system (Applied Biosystems, USA) using sequence-specific primer and fluorescently labeled probe. U6 was also quantified as an endogenous reference to normalize input amount. A no-template control was used as a negative control. Data analysis was performed by using $2^{-\Delta\Delta CT}$ method.

2 RESULTS

2.1 Total RNA Quality

Total RNA was extracted from each sample of rat hippocampus, separately. For spectrophotometer, the results (table 1) showed that A_{260}/A_{280} and A_{260}/A_{230} were more than 2.0.

Table 1 Quality control data of RNA purity and concentration

Groups	A_{260}/A_{280} ratio	A_{260}/A_{230} ratio	Concentration (ng/μL)
Sham	2.001±0.013	2.089±0.161	871.102±207.462
TBI			
Day 1	2.012±0.018	2.143±0.158	943.257±147.909
Day 3	2.019±0.007	2.156±0.128	1044.476±106.243
Day 5	2.009±0.015	2.087±0.139	757.423±103.662

Table 2 List of significantly changed microRNAs with fold change at all three time points post-TBI

Gene name	Time points following TBI		
	Day 1	Day 3	Day 5
rno-miR-146b	1.540	1.753	1.626
rno-miR-142-3p	0.480	0.241	2.464
rno-miR-221	2.855	5.139	4.589
rno-miR-376b-3p	0.641	0.662	0.546
rno-miR-186	1.719	2.321	3.174
rno-miR-190	0.528	7.744	9.685
rno-miR-7a	0.144	0.631	0.564
rno-miR-153	1.699	2.136	3.600
rno-miR-490	3.093	5.247	1.873
rno-miR-1193-3p	2.871	3.702	1.588
rno-let-7d*	2.531	4.369	3.845
rno-miR-758*	2.548	3.347	1.858
rno-miR-138-2*	2.618	4.118	2.623
rno-miR-3573-3p	9.179	8.629	6.280
rno-miR-3572	2.416	2.024	1.586
rno-miR-466d	20.328	41.463	23.999
rno-miR-551b	0.539	0.585	0.578

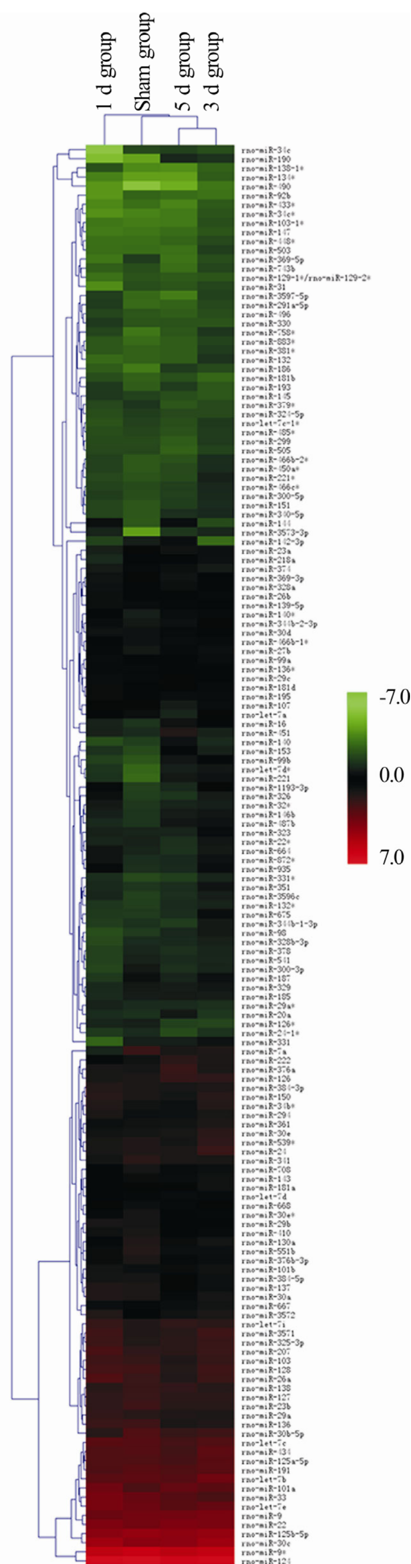

Fig. 1 Hierarchical cluster analysis of microRNAs in hippocampus following TBI

Each column represents a group, and each row represents a single microRNA. Colors indicate relative signal intensities: green for negative values (down-regulation), black for zero (no change), and red for positive values (up-regulation) 1 d group, 3 d group, 5 d group: 1st day, 3rd day, 5th day post-TBI group, respectively.

2.2 Microarray Analysis

A total of 205 miRNAs were identified. At 1st day post-injury, 41 miRNAs were more than 1.5 times up-regulated, and 31 miRNAs were more than 1.5 times down-regulated; at 3rd day post-injury, 81 miRNAs were more than 1.5 times up-regulated, and 11 miRNAs were more than 1.5 times down-regulated; at 5th day post-injury, 52 miRNAs were more than 1.5 times up-regulated, and 41 miRNAs were more than 1.5 times down-regulated. A dendrogram of a hierarchical clustering analysis of the 4 sets of array data in sham group and TBI groups at three time points post-injury is shown in fig. 1. The top 10 up-regulated or down-regulated miRNAs and their fold changes at 1st, 3rd or 5th day after TBI are shown in fig. 2. Table 2 shows the 17 miRNAs 1.5 times up-/down-regulated in ipsilateral hippocampus after TBI in all three experimental groups and their fold changes.

2.3 qRT-PCR Validation

We used quantitative real-time PCR method to validate the microarray results of miR-142-3p and miR-221. The stem-loop RT primers for use in reverse transcriptase reactions and specific RT primers for amplification of U6, miR-142-3p and miR-221 are shown in table 3. Fig. 3 displays a comparison of the results of miR-142-3p and miR-221 from quantitative RT-PCR and microarray. A strong correlation between our microarray profiling and quantitative RT-PCR data was found, suggesting that the microarray data were reliable for further study.

3 DISCUSSION

In the present study, microarray analysis revealed that 205 rat miRNAs showed significant expression changes in the hippocampus after CCI injury. It represented that brain injury dramatically altered a large number of mature miRNA transcripts, revealing the miRNA's extremely sensitive reactions to TBI and the wide extent of influence. It is in concordance with the theory of multi-level pathological responses caused by brain injury[10]. Some studies have demonstrated that each miRNA can regulate hundreds of mRNA targets and each mRNA can be regulated by many miRNAs[11, 12]. The expression changes of many miRNAs involved in this study indicated that the effect of feedback and regulation on the injury and its interventions are through complicated biological network, rather than single path.

Up-/down-regulation of the differentially-expressed miRNAs in the injured hippocampus was detected at different time points post-TBI. Hierarchical cluster analysis showed that each period post-injury had a unique miRNA expression profile. The identities of differentially-expressed miRNAs were different at the same time point, and the expression level of the same miRNA was significantly changed at the different time points after TBI. Most miRNAs were selectively up/down-regulated at one or two time points, however, 17 miRNAs were 1.5 times up-/down-regulated in all three experimental groups, as follows: miR-146b, -142-3p, -221, -376b-3p, -186, -190, -7a, -153, -490, -1193-3p, -758*, -138-2*, -3573-3p, -3572, -466d, -551b and let-7d*. In consideration of different time-serials property of different

miRNAs, as well as the impact on lots of neuronal genes caused by the alteration of miRNAs and their key role in the apoptosis, oxidation, transduction and specific signaling pathways in nerve cells[13–15], it was suggested that the mechanisms of the alteration in miRNA expression at various time points post-TBI varied. We speculate that differentially-expressed miRNAs in hippocampus post-injury, as a form of mediation, affect synaptic plasticity by regulating apoptosis and regeneration, and then change the brain's electrical activity and metabolism, culminating in neurocognitive dysfunction. Furthermore, miRNA expression can change over time post-TBI, so the miRNA, especially the above-mentioned 17 miRNAs, can be potentially used as molecular markers for TBI progress.

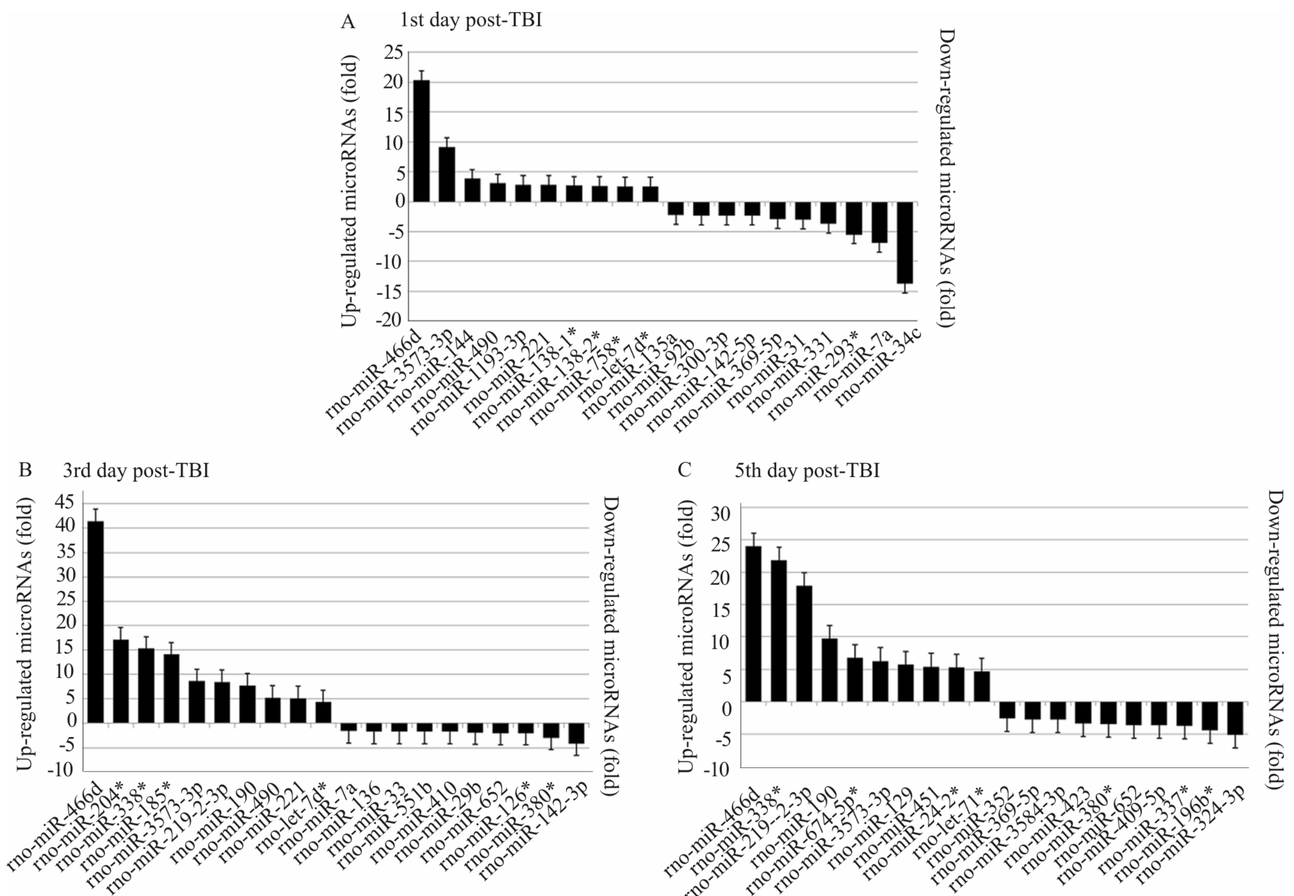

Fig. 2 MicroRNAs with significantly altered expression levels after TBI
The top 10 up-regulated and down-regulated microRNAs and their fold changes at day 1 (A), 3 (B) and 5 (C) after TBI respectively

Table 3 The sequences of U6, miR-142-3p and miR-221 primers

Gene name	Primer sequences
Reverse transcription reaction	
U6	5′-CGCTTCACGAATTTGCGTGTCAT-3′
miR-142-3p	5′-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCCATAAA-3
miR-221	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGAAACC-3′
Quantitative PCR	
U6	F: 5′-GCTTCGGCAGCACATATACTAAAAT-3′
	R: 5′-CGCTTCACGAATTTGCGTGTCAT-3′
miR-142-3p	GSP: 5′-GGGGGTGTAGTGTTTCCTA-3′
	R: 5′-CAGTGCGTGTCGTGGA-3′
miR-221	GSP: 5′-CGAGCTACATTGTCTGCTGGGT-3′
	R: 5′-GTGCAGGGTCCGAGGT-3′

GSP: gene-specific primer; F: forward primer; R: reverse primer

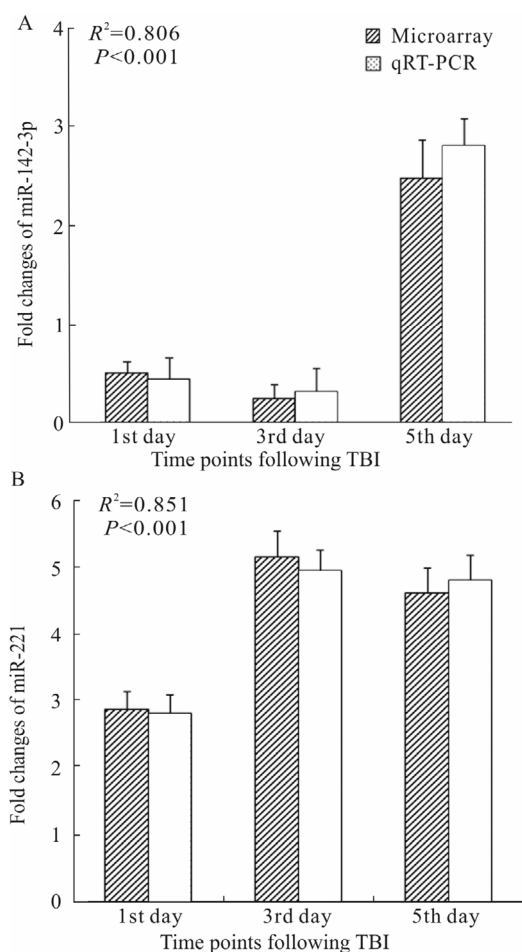

Fig. 3 Results of quantitative RT-PCR

Quantitative RT-PCR validation of miR-142-3p (A) and miR-221 (B) shows a strong correlation between the microarray and quantitative PCR data. The *r* represents correlation coefficient.

The microarray analysis data showed that miR-142-3p and miR-190 were the only two miRNAs that either increased or decreased at all three time points. Another notable microRNA is miR-221, which appeared to have a relatively restricted angiogenic activity at the early stage after trauma, and the manipulation of the miR-221 expression levels may provide a means for tempering post-injury vascular disruptions. Therefore, we chose miR-142-3p and miR-221 for further discussion (miR-190 was highly relevant to cancer[16, 17], thus we won't discuss this further in this article.).

In the present study, the fluctuation of miR-142-3p expression in the hippocampus was particularly significant, which showed a down-regulation at 1st and 3rd day followed by late significant up-regulation at 5th day post-TBI. Its changed trend was opposite, suggesting that miR-142-3p does not passively respond to injury. The relatively firm bounce of miR-142-3p expression level occurred in the later injury stages. It is likely that regulating miRNAs expression is an active approach taken by cells to cope with injury. Previous studies found that miR-142-3p regulates the production of cyclic AMP (cAMP) by controlling adenylyl cyclase (AC) 9 mRNA expression in $CD4^{+}CD25^{-}$ T cells and $CD4^{+}CD25^{+}$ T_{REG} cells[18]. cAMP has been confirmed to regulate diverse cellular functions such as regeneration, plasticity, and survival, promote the outgrowth and influence the morphology of neurites of neurons, as well as participate in the restoration of damaged tissue[19]. It revealed miR-142-3p may be actively involved in nerve growth, recovery, regeneration and synapsis remodeling in advanced stage post-injury through cAMP-pathway. And besides, in this study, miR-142-3p expression pattern was changed with the time post-injury, indicating temporal expression of miR-142-3p may be used as a molecular marker for assessment of TBI progression and inference of damage time in forensic clinical identification. Some of the previous scholars demonstrated that the expression level of miR-142-3p in peripheral blood mononuclear cells (PBMCs) could be used as a novel diagnostic marker for acute myeloid leukemia (AML)[20]. MiR-142-3p has also been documented to be a putative serum marker for risk assessment in early-stage lung adenocarcinoma patients[21]. The present research provides data basis for expanding possibility of miR-142-3p as biomarker to forensic medicine.

Another notable miRNA in this study is miR-221. We revealed persistently up-regulated expression of miR-221 in rat hippocampus at all the three time points post-injury. Similar results have been reported by Lei[22] that miR-221 was up-regulated in rat cerebral cortex at 4 time points post-TBI (6, 24, 48 and 72 h). MiR-221 is well known for its pro-proliferative and pro-migratory roles in different cell types[23–25], and it is also identified as anti-angiogenic miRNA[26, 27]. Meanwhile, miR-221 has been demonstrated to be a compensatory regulator of p27. $p27^{kip1}$ is a cell cycle inhibitor, regulated by platelet-derived growth factor (PDGF) signaling, and functions as a critical regulator in the proliferation of many cell types. High activity of miR-221 is required to maintain low $p27^{Kip1}$ levels, which is critical for PDGF-mediated induction of cell proliferation and migration[28, 29]. Therefore, we suggest that miR-221 may play a key role in regulating angiogenesis and cell proliferation mediated via PDGF signaling pathway at the early stage after trauma. Although it has not yet been studied whether miR-221 expression is altered in hippocampus after TBI, it actually has wide market foreground.

In conclusion, miRNAs have different expression levels at various time point post-TBI, suggesting miRNAs play key roles in the pathological process (transmission, feedback, regulation and control) after injury and work collaboratively like a complicated gene expression network. Specific miRNAs such as miR-142-3p and miR-221 may be potentially used as sensitive and informative biomarkers for TBI forensic assessment, and thus make some improvements in exploratory research for TBI-related cognitive disorder.

Conflict of Interest Statement

All authors have declared that no conflicts of interest exist.

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