Original Article

miR-148a-3p exhaustion inhibits necrosis by regulating PTEN in acute pancreatitis

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Abstract: Background: Acute pancreatitis (AP) is a necro-inflammatory disorder with high mortality rate. With advances in understanding the pathogenesis of AP, microRNAs (miRNAs) have been reported to play an essential role in AP progression. However, the mechanism that allows miR-148a-3p to regulate necrosis in AP remains unclear.

Methods: Caerulein treatment was used to induce AP in mice or cells. miR-148a-3p-/- mice or miR-148a-3p inhibition in wild type mice were used to investigate the effect of miR-148a-3p on AP. The expression of miR-148a-3p was detected by quantitative real-time polymerase chain reaction (qRT-PCR). The abundances of phosphatase and tensin homolog (PTEN) and hallmarks of necrosis or apoptosis were measured by qRT-PCR or western blots (WB). Cell necrosis, apoptosis, serum amylase or lipase activity and inflammatory cytokines levels were investigated by commercial assay kit. Inflammatory infiltration was analyzed by immunohistochemistry (IHC).

Results: miR-148a-3p was highly expressed in AP and knockout of miR-148a-3p inhibited water content, cell necrosis, amylase and lipase activity while inducing PTEN expression. Moreover, miR-148a-3p deletion attenuated inflammatory infiltration and necrosis by promoting apoptosis. In addition, miR-148a-3p knockdown protected against cell necrosis, amylase, and lipase activity in AP. Intriguingly, PTEN was a target of miR-148a-3p and interference of PTEN reversed the effect of miR-148a-3p deficiency on AP in vitro.

Conclusion: miR-148a-3p inhibition repressed necrosis by regulating PTEN expression in AP, providing a novel biomarker of therapeutics for AP treatment.

Keywords: Acute pancreatitis, necrosis, miR-148a-3p, PTEN

Introduction

Acute pancreatitis (AP) is a common necro-inflammatory disease with a rising incidence in clinical practice [1]. With the advance in understanding the pathogenesis of AP, the effective strategies for diagnosis and treatment of AP remain an open question [2]. Necrosis exacerbates the mortality risk in AP because of multiple complications [3]. Currently, many investigators show great promise in the regulation of balance of apoptosis and necrosis for AP treatment [4]. However, a novel driver participating in necrosis in AP progression is required.

MicroRNAs (miRNAs), a class of short non-coding RNAs, play pivotal roles in regulating cell proliferation, apoptosis, necrosis, and inflammation in varying disease processes, including AP [5]. miRNAs have been regarded as biomarkers of acinar cell injury in pancreas [6]. For example, miR-551b-5p has been reported to be critical for the inflammatory response and disease process in patients with AP [7]. miR-181b is associated with pancreatic injury, autophagy and apoptosis in AP [8]. Also, miR-7, miR-9, miR-122, and miR-141 may serve as biomarkers of diagnosis and prognosis for AP [9]. miR-148a-3p is a novel miRNA which is implicated in proliferation, invasion, apoptosis, autophagy and chemo-resistance in many cancers progression [10, 11]. Moreover, miR-148a-3p has been indicated to be involved in cell death in osteosarcoma [12]. Available evidence indicates that miR-148a-3p is expressed in mice with AP [13]. However, the mechanism that underlies that miR-148a-3p regulates necrosis in AP remains poorly understood.

Phosphatase and tensin homolog (PTEN) is a tumor suppressor and addresses tumor growth and metabolism in different cancer progressions [14]. Moreover, inhibition of PTEN has been reported to be beneficial for human dis-
PTEN may have an impact on cell apoptosis and necroptosis in human prostate cancer cells [16]. Notably, PTEN is low expressed and may play important role in AP process [17]. Intriguingly, bioinformatics analysis predicates the putative binding sites of miR-148a-3p and PTEN [18]. Thus, we assume PTEN is required for miR-148a-3p-mediated regulation of necrosis in AP. In the present study, we establish AP model by caerulein treatment in vivo and in vitro. Moreover, we investigated the effect of miR-148a-3p on necrosis in miR-148-3p-/ or miR-148a-3p inhibition mice as well as AR42J cells. Notably, we also probed the interaction between miR-148a-3p and PTEN.

**Materials and methods**

**Murine model of AP**

Wide type (WT) or miR-148a-3p knock out (miR-148-3p-) C57BL/6 mice (male, 6-week-old) were obtained from Shanghai Nanfang Research Center for Model Organisms (Shanghai, China). All mice were allowed access to water as well as food and acclimatized for one week prior to study. Mice were randomized and administered caerulein in PBS or PBS alone (50 μg/kg, Sigma, St. Louis, MO, USA) intraperitoneal injection every other day for four times (n = 5 per group). Pancreatic tissues and blood were collected at day 1, 3 or 7 after the final injection. Serum was obtained from blood by centrifugation at 3500 g for 20 min at 4°C. Mice treated for 1 d was used for further study. Every experiment was conducted under protocols approved by the Animal Research committee of Chang An Hospital.

**Pancreatic water content**

After treatment, pancreatic tissues were collected and immediately weighed for wet weight. Then the samples were dried for approximately 24 h at 80°C and weighed again to get dry weight. The pancreatic water content was calculated as (wet weight - dry weight)/wet weight × 100%.

**Primary cell preparation and necrosis assay**

Acinar cells were isolated from pancreas of treated mice following the established protocol [19]. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Carlsbad, CA, USA) media with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, and 100mg/ml streptomycin (Gibco) at 37°C with 5% CO₂. Cells necrosis was investigated by using lactate dehydrogenase (LDH) cytotoxicity assay kit (Thermo Fisher, Wilmington, DE, USA). In brief, acinar cells were maintained into 96-well plates at a density of 1 x 10⁴ cells/well. Subsequently, the cell medium supernatants were collected for release of LDH assay according to the manufacturer's instructions.

**Serum amylase, lipase and inflammatory cytokines assay**

Serum amylase activity was measured by using Amylase Activity Assay Kit (Sigma) and lipase activity was investigated by Lipase Activity Assay Kit (Sigma) according to the manufacturer's protocol. Cytokines IL-6 and TNF-α levels were detected in serum using commercial mice ELISA Kit (Thermo Fisher) following the manufacturer's instructions. The absorbance was detected at 450 nm with reference wave length at 620 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA was prepared by using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and then used for synthesis of cDNA via All-in-one mRNA or miRNA qPCR detection kit (GeneCopoeia, Rockville, MD, USA), followed by mixed in SYBR Green for qRT-PCR. U6 or GAPDH was used to normalize miR-140-3p or PTEN expression using 2^-ΔΔCT method, respectively. All primers were listed as follows: miR-148a-3p (5’TCA-GTGCACACTAGACTTGT-3’), U6 (5’TTCGTTGAGGAGGT-3’) and universal miRNA adapter PCR primer (5’-GCTGTCAACAGTACCGT-3’), PTEN (Forward, 5’-AAGGACCGACGGTGTA-3’; Reverse, 5’-CCTGAGGTGAGGT-3’), FADD (Forward, 5’-GGTGGCATTTGA-3’; Reverse, 5’TCCCTTTACCCTAGAT-3’), RIP3 (Forward, 5’-ATGTCTAAACTCT-3’; Reverse, 5’TCTTCCCTACGCACT-3’), GADPH (Forward, 5’-GCTGTCAACAGTACCGT-3’; Reverse, 5’-ACCCAGTAGACTCCAGAGGC-3’).

**Western blots (WB)**

Tissues or cells were treated in cell lysis buffer containing 1% protease inhibitor (Thermo Fisher) and then spun at 10,000 x g for 10 min at 4°C, followed by quantified with BCA assay.
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Then proteins were denatured in loading buffer (Thermo Fisher) at 98°C for 10 min and separated by SDS-PAGE gel. Subsequently, proteins were transferred to polyvinyliden difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and blocked with blocking reagent (Thermo Fisher) for 1 h. In turn, the membranes were incubated with primary antibodies against PTEN, RIP1, Cleaved RIP1, FADD, RIP3, caspase8, Cleaved caspase8, caspase3, Cleaved caspase3, or β-actin (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight and then developed with horseradish peroxidase (HRP) conjugated anti-mouse or anti-rat IgG (1:2000 dilution, Cell Signaling Technology) for 2 h at room temperature. The protein blots were investigated using enhanced chemiluminescence (ECL, Thermo Fisher) and β-actin was used to normalize band intensities.

Immunohistochemistry (IHC)

Tissues sections were prepared by fixed, embedded and sectioned. After blocking endogenous peroxidase using 3% H2O2, sections were incubated with primary antibodies against CD11b, F4/80 (1:100 dilution, Abcam, Cambridge, MA, USA) or Gr1 (1:100 dilution, R&D Systems, Minneapolis, MN, USA) for 2 h. Subsequently, cells were incubated with HRP-conjugated anti-mouse IgG for 30 min after being rinsed with PBS, followed by stained with DAB and hematoxylin (Sigma). The positive cells were examined under a light microscopy (Olympus, Tokyo, Japan).

TUNEL

To evaluate cell apoptosis in pancreas, sections were incubated with TUNEL staining detection kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions. In brief, sections were blocked with 3% H2O2 for 15 min after rinsed with PBS. Then sections were incubated in TUNEL reaction solution for 1 h at room temperature and subsequently with POD for 30 min. The sections were then incubated with DAB and hematoxylin counterstain, followed by examined under a light microscope.

miR-148-3p inhibition in vivo and cell transfection

miR-148a-3p inhibitor (anti-miR-148a-3p), inhibitor negative control (anti-NC), miR-148a-3p mimic (miR-148a-3p), mimic NC (NC), shPTEN or scramble were constructed by GenePharma (Shanghai, China). To establish miR-148a-3p inhibition model in vivo, anti-miR-148a-3p, anti-NC or vector alone was mixed with caerulein and then introduced into WT mice by intraperitoneal injection. To investigate the effect of miR-148a-3p on PTEN expression, miR-148a-3p, anti-miR-148a-3p or their NC were transfected into acinar cells. To evaluate the effect of miR-148a-3p and PTEN on AP in vitro, anti-miR-148a-3p or shPTEN was transfected into AR42J cells.

Luciferase activity assay

Promising binding sites of 3’ untranslated regions (3’-UTR) of PTEN and miR-148a-3p were predicated by TargetScan software online. Wild or mutant type of 3’-UTR of PTEN was amplified and then inserted into pGL3 vector (Promega, Madison, WI, USA) to generate luciferase reporting vectors (PTEN-wt or PTEN-mut), respectively. Luciferase reporter vectors and miR-148a-3p or NC were co-transfected in acinar cells for 48 h. Then the luciferase activity was evaluated using luciferase assay kit (Promega).

Statistical analysis

The results were analyzed by using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Data were expressed as the mean ± standard error of the mean (SEM). Difference between two groups was investigated by Student’s t test and p values < 0.05 was regarded as significant.

Results

Deletion of miR-148a-3p inhibited AP in caerulein-treated mice

Since miR-148a-3p is required for AP, the expression of miR-148a-3p was measured in WT mice after PBS or caerulein treatment. As a result, the abundance of miR-148a-3p was obviously enhanced at day 1 after caerulein injection and reached its peak, followed by being progressively reduced at day 3 and 7 (Figure 1A). Moreover, we investigated the effect of miR-148a-3p knockout on AP in caerulein-treated mice. Caerulein treatment induced edema in pancreas compared with PBS treatment, while miR-148a-3p knockout reduced the water content of pancreas (Figure 1B). In addition, acinar cells showed less necrosis in
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Figure 1. Deletion of miR-148a-3p inhibited AP in miR-148a-3p−/− mice. A. The expression of miR-148a-3p was detected in PBS or caerulein-induced pancreatitis in WT mice at day 1, 3 or 7 after caerulein treatment. B. Pancreatic water content was measured in WT or miR-148a-3p−/− mice. C. Cell necrosis was investigated in acinar cells from pancreas of WT or miR-148a-3p−/− mice. D and E. Serum amylase or lipase activity was analyzed in PBS or caerulein-treated mice. F-H. The abundance of PTEN was examined at mRNA and protein levels in pancreatitis of WT or miR-148a-3p−/− mice after PBS or caerulein treatment. *P < 0.05.
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miR-148a-3p-/- mice than that in WT mice after caerulein treatment (Figure 1C). Furthermore, the levels of amylase and lipase were markedly inhibited in serum of miR-148a-3p-/- mice (Figure 1D and 1E). Caerulein led to a great loss of PTEN mRNA expression, whereas miR-148a-3p deletion supported the abundance of PTEN mRNA (Figure 1F). A similar trend was displayed relative to PTEN protein level in miR-148a-3p-/- mice compared with WT mice after caerulein treatment (Figure 1G and 1H).

miR-148a-3p knockout blocked inflammatory infiltration in AP

Inflammation played important role in AP and inflammatory infiltration participated in the pathophysiology of AP. Therefore, we probed whether knockout of miR-148a-3p might regulate inflammatory cell infiltration. After caerulein treatment, CD11b positive cells were present in pancreas of WT mice at day 1 and day 2, while the number of CD11b positive cells was lowered in response to miR-148a-3p knockout (Figure 2A). As for IHC with F4/80, results revealed lower macrophages were infiltrated to pancreas of miR-148a-3p-/- mice compared with WT mice (Figure 2B). Similarly, reduced neutrophil infiltration was observed in pancreas of miR-148a-3p-/- mice, uncovered by less Gr1 positive cells than that in WT mice after caerulein treatment (Figure 2C). Moreover, the production of inflammatory cytokines was measured in serum of WT or miR-148a-3p-/- mice. Results showed that caerulein protected TNF-α and IL-6 secretion in WT mice, but deletion of miR-148a-3p attenuated the expression of TNF-α and IL-6 (Figure 2D and 2E).

Abrogation of miR-148a-3p inhibited necrosis in AP

To explore whether necrosis was implicated in AP progression, apoptosis and necrosis were investigated in mice treated with caerulein at day 1. TUNEL assay revealed that caerulein treatment induced apoptosis in WT mice compared with PBS treatment, and miR-148a-3p knockout exacerbated apoptosis (Figure 3A). Moreover, we evaluated the protein expressions of PTEN, RIP1, Cleaved RIP1, FADD and RIP3 in pancreas, which are the part of necroosome. Caerulein treatment promoted these proteins abundance in pancreas of WT mice (Figure 3B). However, miR-148a-3p deletion blocked the expressions of FADD and RIP3 (Figure 3B). In addition, miR-148a-3p-/- group indicated little change of RIP1 level compared with WT group, while miR-148a-3p knockout induced Cleaved RIP1 expression (Figure 3B). Furthermore, the related protein levels in apoptosis pathway were investigated in pancreas of WT or miR-148a-3p-/- mice after caerulein treatment. Results showed that the abundance of total caspase8 was decreased in miR-148a-3p-/- mice, while the Cleaved caspase8 was...
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Figure 3. Abrogation of miR-148a-3p inhibited necrosis in AP. A. Apoptosis was investigated in the pancreas of WT or miR-148a-3p−/− mice treated with PBS or caerulein for 1 d. B. The hallmarks of necrosis were detected in mice with AP. C. Apoptosis-related protein expressions were examined in pancreatic tissue. *P < 0.05.

Figure 4. Knockdown of miR-148a-3p protected against AP in WT mice. A. The expression of miR-148a-3p was detected in mice with or without AP after miR-148a-3p inhibition. B and C. The effect of miR-148a-3p knockdown on PTEN protein abundance was measured in caerulein-treated mice. D. Necrosis was investigated in acinar cells after anti-miR-148a-3p or control treatment. E and F. The effect of miR-148a-3p inhibition on serum amylase and lipase activity was investigated in WT mice with PBS or caerulein treatment. *P < 0.05.

elevated compared with that in pancreas of WT mice (Figure 3C). Knockout of miR-148a-3p induced higher expression of caspase3 and cleaved caspase3 (Figure 3C).

Knockdown of miR-148a-3p protected against AP in caerulein-treated WT mice

Seeing the amelioration of AP by miR-148a-3p deletion, we next investigated whether inhibition of miR-148a-3p might also effectively address caerulein-induced AP in WT mice. As a result, administration of anti-miR-148a-3p strikingly suppressed miR-148a-3p expression after caerulein treatment (Figure 4A). Moreover, knockdown of miR-148a-3p resulted in a notably elevated PTEN protein level compared with anti-NC treatment (Figure 4B and 4C). Compared with either anti-NC or vehicle, miR-148a-3p exhaustion hindered necrosis in acinar cells (Figure 4D). Further, the introduction of anti-miR-148a-3p protected against the levels of amylase as well as lipase in serum of WT mice after caerulein treatment (Figure 4E and 4F).

PTEN was bound to miR-148a-3p

Since miR-148a-3p and PTEN were abnormally ectopic in AP, we probed the potential interaction between miR-148a-3p and PTEN in caerulein-treated acinar cells. TargetScan online
indicated the promising binding sites of PTEN and miR-148a-3p, uncovering that PTEN might be a direct target of miR-148a-3p (Figure 5A). Hence, luciferase activity assay was conducted to validate the prediction in caerulein-treated acinar cells. Luciferase activity was greatly impaired in cells co-transfected with PTEN-wt and miR-148a-3p compared with the NC group, whereas there was little change of the activity in response to PTEN-mut (Figure 5B). Moreover, overexpression of miR-148a-3p led to a great loss of PTEN mRNA level in caerulein-treated acinar cells, while inhibition of miR-148a-3p effectively enhanced PTEN expression compared with their counterparts, respectively (Figure 5C). Consistent with gene expression, the PTEN protein showed a similar trend of abundance with respect to miR-148a-3p or anti-miR-148a-3p in caerulein-treated acinar cells (Figure 5D and 5E).

Abrogation of PTEN overturned the effect of miR-148a-3p knockdown on necrosis in caerulein-treated AR42J cells

To further the effect of PTEN on miR-148a-3p-mediated necrosis in AP, anti-miR-148a-3p or (and) shPTEN were transfected into AR42J cells with caerulein treatment. Upon caerulein treatment, miR-148a-3p knockdown inhibited cell necrosis, whereas interference of PTEN ablated the inhibitory effect on necrosis (Figure 6A). Moreover, an obvious increase of PTEN mRNA level was observed in caerulein-treated cells with anti-miR-148a-3p transfection, while shPTEN administration blocked PTEN abundance compared with scramble group (Figure 6B). In addition, absence of miR-148a-3p suppressed the caerulein-induced expressions of FADD and RIP3, whereas PTEN deficiency supported the mRNA levels of FADD and RIP3 (Figure 6C and 6D). Similarly, alterations of PTEN, FADD and RIP3 expressions displayed same trends at the protein level in caerulein-treated AR42J cells with anti-miR-148a-3p or (and) shPTEN transfection (Figure 6E-H).

Discussion

AP is a common necro-inflammatory disease threatening many people. miRNAs have been reported to serve as biomarkers for diagnosis and prognosis of AP [20]. miR-148a-3p has been reported to be highly expressed in mice with AP [13]. However, a broader view of miR-148a-3p participating in AP progression is required. A number of investigators have used caerulein to induce AP in mice [19, 21]. In our
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Figure 6. Interference of PTEN reversed knockdown of miR-148a-3p-mediated inhibitory effect on necrosis in caerulein-treated AR42J cells. A. Deficiency of PTEN supported caerulein-induced necrosis. B-D. The mRNA levels of PTEN, FADD and RIP3 were detected in caerulein-treated AR42J cells after anti-miR-148a-3p and shPTEN transfection. E-H. The protein abundances of PTEN, FADD and RIP3 were measured in AR42J cells. *P < 0.05.
study, we also established an AP model by caerulein treatment in mice or in vitro. After the treatment, miR-148a-3p was enhanced in AP, which is in agreement with former work [6, 13]. Notably, the abundance of miR-148a-3p reached its peak at day 1 after caerulein treatment, suggesting the severity of caerulein-induced AP might be severest at day 1, which is consistent with previous study [19, 22]. Here we first provided the view of the underlying mechanism of miR-148a-3p regulating necrosis in AP.

Necrosis raises the risk of morbidity and mortality in AP through triggering organ failure and complications [3]. Elevated lipase and amylase levels are diagnostic biomarkers of AP in the clinic [23]. Hence, we investigated the effect of miR-148a-3p on necrosis, lipase and amylase activity in vivo. Results suggested miR-148a-3p exhaustion inhibited cell necrosis and AP processes in miR-148a-3p−/− or WT mice treated with caerulein. Furthermore, inflammatory cell infiltration is required for many acute inflammatory disorders, including AP [19, 24]. For example, CD11b, F4/80 and Gr1 have been regarded as markers of monocytes, macrophages and neutrophil, which plays essential roles in inflammatory infiltration [19]. We also found an infiltration of inflammatory cells in mice with AP and inhibition of miR-148a-3p suppressed the infiltration in the present study. Intriguingly, we further found that miR-148a-3p depletion decreased necrosis by inducing apoptosis, which is also in consistent with previous effort which demonstrated that AP is positively correlated with necrosis but negatively with apoptosis [25]. Similarly, miR-19b deficiency also inhibited AP progression by decreasing cell necrosis in rat and in AR42J cells [26]. Moreover, miR-21 expression was enhanced and its deficiency promoted apoptosis and inhibited cell necrosis in vivo [19]. Conversely, miR-29a might contribute to apoptosis by regulating tumor necrosis factor receptor superfamily member 1A (TNFRSF1A) in AR42J cells [27].

Functional miRNAs were realized in AP by regulating target gene expression [28]. Several such reports suggested that miR-148a-3p, as a targeting miRNA, is implicated in regulation of varying genes in many conditions. The available evidence indicated that miR-148a-3p might enhance thrombospondin-4 expression and angiogenesis via regulating kruppel-like factor 6 in tendinopathy development [29]. Moreover, miR-148a-3p inhibited epithelial ovarian cancer progression through regulating the hepatocyte growth factor receptor tyrosine-protein kinase Met expression [10]. Besides, lysine specific demethylase 6b, as a target of miR-148a-3p, inhibited the differentiation of adipocyte and osteoblast [30]. Also, PTEN was negatively correlated with caerulein-induced AP in our study, which is also in agreement with a former finding [17]. PTEN was a tumor suppressor which played multiple biological roles through targeting of relevant miRNAs in many cancers and diseases [31]. Moreover, PTEN has been reported to be associated with apoptosis and necrosis in human prostate cancer cells [16]. On the basis of previous demonstrations, miR-148a-3p notably regulated rabbit preadipocyte differentiation and glomerular cell proliferation by inhibiting PTEN expression [18, 32]. However, these data might not directly support whether PTEN is required for miR-148a-3p-mediated role in AP because of the different microenvironment. Hence, we validated the interaction between miR-148a-3p and PTEN and probed the attenuation of PTEN to the function of miR-148a-3p on necrosis in AP. However, there was an absence of clinical data on miR-148a-3p participating in AP in our study. Moreover, we also found TNF-α and IL-6 levels were elevated in serum of AP and miR-148a-3p knockout inhibited the secretion of inflammatory cytokines. This in consistent with a previous study [33]. The work also suggested that caerulein induced AP with systemic complications and might injure remote organ systems, such as lung. Therefore, the effect of miR-148a-3p on AP progression in patients and the remote organ systems needs investigation in future.

In the current study, miR-148a-3p was up-regulated in AP and its deficiency inhibited necrosis, AP progression and inflammatory infiltration while inducing apoptosis in vivo. Moreover, PTEN was a target of miR-148a-3p and its interference reversed knockdown of miR-148a-3p-mediated necrosis in vitro. These data suggested miR-148a-3p inhibition protected against necrosis in AP by targeting PTEN, providing a novel therapeutic avenue for AP treatment.

Disclosure of conflict of interest

None.
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