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A RARE INSULMAME-MEDIATED PYROPTOSIS CURE THAT MAY BE USED TO ADDRESS ACUTE PANCREATITIS

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ABSTRACT

This study aims to examine the role of membrane-associated ring-CH-type finger 9 (MARCH9) in the regulation of acute pancreatitis (AP). Methods: Ten healthy persons and fifteen AP patients hospitalized to Changzhou Second People's Hospital Nanjing, China, took part in the research. We tested the individuals' serum samples for MARCH9 expression. Inducing rat pancreatic acinar (PA) cells with ceruletide and then transfecting them with a MARCH9 overexpression vector allowed for the establishment of an AP cell model. We found the molecular structure and level of MARCH9, which are associated with pyroptosis and inflammatory cytokines. Caspases 1 activity and cell survival rate were measured. The results showed that both the serum of AP patients and ceruletide-induced PA cells have low levels of MARCH9. The survival rate of ceruletide-induced PA cells was boosted and the levels of inflammatory cytokines and components associated with pyroptosis were lowered when MARCH9 was overexpressed. In ceruletide-induced PA cells, caspase 1 activity was shown to be decreased when MARCH9 was overexpressed. Furthermore, when ceruletide was used to elevate PA cell levels of IL6, p-STAT3/STAT3, and p-JAK2/JAK2, the overexpression of MARCH9 exhibited a negative regulatory impact. Results: MARCH9 overexpression inhibits NLRP3-induced pyroptosis in PA cells via controlling the IL6/JAK/STAT3 pathway; this finding may have implications for the treatment of AP. Pyroptosis, acute pancreatitis, AR42J cells, Ceruletide.

INTRODUCTION

Acute pancreatitis (AP) is an acute abdominal disease and the mortality rate can reach 47 to 69 % [1]. Blood amylase and lipase are significantly increased in AP patients, which can also be accompanied by an increase in blood sugar [2]. Systemic inflammation brings a second strike to the patients by increasing the burden on organs and exacerbating the severity of AP [3]. Therefore, seeking effective treatment for AP is currently a hot topic in research. Pyroptosis is a form of cell death involving members of the caspase family [4,5]. The release of pro-inflammatory cytokines induced by activating NLRP3 inflammatome is one of the causes of pyroptosis. [6]. For instance, the activation of receptor-associated factor 6 induces pyroptosis through the caspase 1/3 signaling pathway, thereby contributing to the progression of AP [7]. Therefore, inhibiting the occurrence of pancreatic cell pyroptosis is one of the ways to alleviate AP. Membrane associated ring-CH-type finger (MARCH) includes 11 family members, from MARCH1 to MARCH11 and they play a role in immune regulation, cell polarity, and toll like receptor signal transduction [8]. MARCH9, a member of the MARCH protein family, may be related to cellular immune regulation [9]. In addition, it was recently shown that MARCH9 regulates the pancreatic cells pyroptosis induced by NLRP3 inflammasome in AP [10]. This indicates that MARCH9 participates in the regulation of AP

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occurrence. The study of MARCH9 in AP is still however immature.

The potential function and possible mechanisms of MARCH9 in AP was discussed in the current study, with the intent being to provide new ideas for the treatment of AP.

METHODS

Clinical samples

Venous blood (10 ml) were collected from patients with AP admitted in Changzhou Second People's Hospital Nanjing, China (AP group, n = 15, 6 females and 9 males) and from 15 healthy individuals (normal group, n = 15, 8 females and 7 males) were collected. Healthy individuals were those who participate in physical examinations in outpatient clinics. AP patients were hospitalized and did not suffer from any disease other than AP. Serum was separated from blood sample and kept at - 80 ℃. All participants signed informed consent. The study was approved by the Ethics Committee of The Affiliated Changzhou No. 2 People's Hospital with Nanjing Medical University (approval no. [2023]KY106-01) and complied with guidelines of World Medical Association Declaration of Helsinki [11].

The amylase and lipase levels in the patient's serum were assayed using an amylase assessment kit and a lipase assessment kit. All kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

Cells culture

Rat PA cells (AR42J cells, ATCC, Manassas, VA, USA) were cultured in DMEM medium (5 % CO2, 37 °C). Streptomycin (100 μg/ml), fetal bovine serum (10 %) and penicillin (100 U/ml) (all reagents: Gibco, Waltham, MA, USA) were contained in the culture medium. AR42J cells were separated into ceruletide, control, ceruletide + NC, and ceruletide + MARCH9 groups. To establish an AP cell model, AR42J cells were cultivated in the medium complemented with ceruletide (100 nmol/L, MCE, China) for 24 h. MARCH9 overexpression vector (MARCH9) and the negative control (NC) were got from GenePharma (Shanghai, China). The cells were harvested 24 h after transfecting by Lipofectamine 2000.

Western blotting and RNA extraction and quantitative real time-PCR (qRT-PCR)

The western blotting and qRT-PCR procedures are based on previous research. The relative expressions of interleukin-6 (IL-6), MARCH9, tumor necrosis factor α (TNF-α) and IL-1β were measured,and their primers sequences were listed in Table 1. The primary antibodies (Abcam) are as below: toll-like receptor 4 (TLR4, 1:1000), MARCH9 (1:1000), P65 (1:500), NLRP3 (1:1000), p-P65 (1:500), IL-1β (1:1000), gasdermin D (1:1000), IL-6 (1:1000), caspase 1 (1:1000), IL-18 (1:1000), Janus kinase 2 (JAK2, 1:5000), signal transducer and activator of transcription 3 (STAT3, 1:1000), p-JAK2 (1:500), p-STAT3 (1:1000) and GAPDH (1:2000).

Table 1: Primer sequences used in PCR

Variable	Primer	Sequence (5'-3')
MARCH 9	Reverse	GCAGCAATCTGGACCTTCT CGA
	Forward	AATCCGCTGGATCAGTGAG AGG
TNF-α	Reverse	GGGAGTAGACAAGGTACA AC
	Forward	TCTCATCAGTTCTATGGCC C
IL-6	Reverse	TGTACTCCAGGTAGCTATG G
	Forward	GTTCTCTGGGAAATCGTGG A
IL-1β	Reverse	CATCATCCCACGAGTCACA G
	Forward	CAGCAGCATCTCGACAAGA G
GAPDH	Reverse	GGCCATCCACAGTCTTCTG AG
	Forward	CCTGCACCACCAACTGCTT A

Immunofluorescence (IF)

AR42J cells were fixed with paraformaldehyde solution (4 %, Sigma Aldrich, Darmstadt, Germany) and then blocked with PBS, Triton (0.3 %) and normal goat serum (5 %) for 1 hour.

Samples were cultivated (4 °C) overnight with an anti-MARCH9 primary antibody (Abcam) diluted at a ratio of 1:100. After that, samples were cultivated with a cy3-conjugated secondary antibody (Abcam) diluted at a ratio of 1:500 and DAPI staining (1:1000, Sigma Aldrich) conducted for one hour at room temperature. The images were collected by a fluorescence microscope (UY203i; Aopu, Chongqing, Sichuan, China).

Cell counting kit-8 (CCK-8) assay

The cell survival rate was evaluated by CCK-8 method and then planted in 96-well plates (1 × 103 cells/well) for 12 h. Each well was mixed with CCK-8 solution (10 μL). One hour later, a microplate

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reader (A51119500C; Thermo Fisher) was used to read the absorbance at 450 nm.

Determination of LDH level

Lactic dehydrogenase (LDH) levels in the AR42J cells were determined using LDH assay kit (Sigma Aldrich).

Flow cytometry

To test pyroptosis in the AR42J cells, caspase 1 detection kit (Beyotime, Shanghai, China) measured the active levels of caspase 1. The active caspase 1 enzyme was labeled with FAM-YVAD-FMK and determined using flow cytometry. Pyroptosis was defined as being double positive for propidium iodide (PI) staining and FAM-YVAD-FMK.

Statistical analysis

The data was analyzed through SPSS 20.0 (Chicago, IL, USA) and displayed as SD. The statistical significance is assessed by one-way-ANOVA followed by Tukey post hoc test, *t*-test. $P < 0.05$ was deemed statistically significant. All assays were repeated as independent experiments in at least triplicate.

RESULTS

Low expression of serum MARCH9 in AP patients and AP cell models

Serum samples from AP patients were collected and MARCH9 expression was determined. Figure 1 A demonstrated that AP patients showed higher levels of serum amylase and lipase. qRT-RCR and western blotting results revealed that MARCH9 was lowly expressed in serum from AP patients (Figure 1 B and C). Moreover, AP cell models were established using ceruletide and it was found that MARCH9 expression decreased in the ceruletide group (Figure 1 D and E).

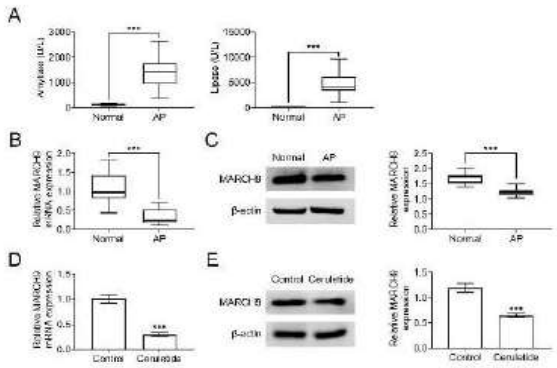

Figure 1: MARCH9 expression in serum of AP patients and AP cell models. (A) Levels of serum amylase and lipase. B) MARCH9 mRNA expression. (C) MARCH9 protein level. (D) MARCH9 mRNA expression. (E) MARCH9 protein expression. ***$P < 0.05$, vs normal or control

Overexpression of MARCH9 enhanced the activity of AR42J cells induced by ceruletide

Here, AP cell models were transfected with MARCH9 overexpression vector. The results showed that MARCH9 level increased in AR42J cells induced by ceruletide after transfection with MARCH9 overexpression vector (Figure 2 A). In the ceruletide + MARCH9 group, there was a remarkable increase in cell survival rate (Figure 2 B), and a rapid reduction in LDH levels (Figure 2 C).

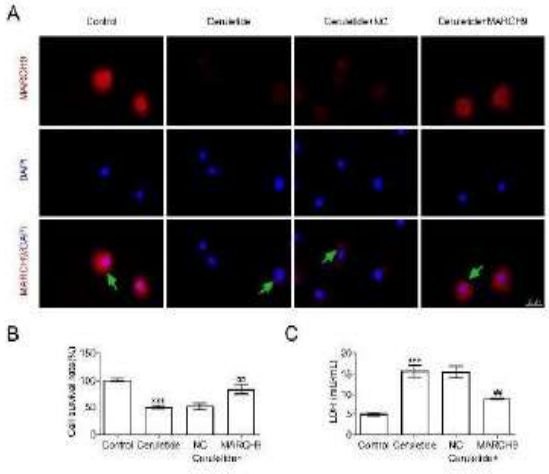

Figure 2: The role of MARCH9 overexpressed in activity of AR42J cells induced by ceruletide. AR42J cells were induced with ceruletide and then transfected with MARCH9 overexpression vector. (A) Immunofluorescence (IF) images. (B) Cell survival rate. (C) the levels of LDH. ***$P < 0.05$, vs control; ##$p < 0.05$, vs ceruletide + NC **Over-expression of MARCH9 inhibited inflammation in AR42J cells induced by ceruletide**

As shown in Figure 3 A, the levels of inflammatory cytokinesin ceruletide + MARCH9 group were remarkably lower than ceruletide + NC group ($p < 0.05$). TLR4 and p-P65/P65 expressions were down-regulated in AR42J cells induced by ceruletide after transfection with MARCH9 overexpression vector (Figure 3 B).

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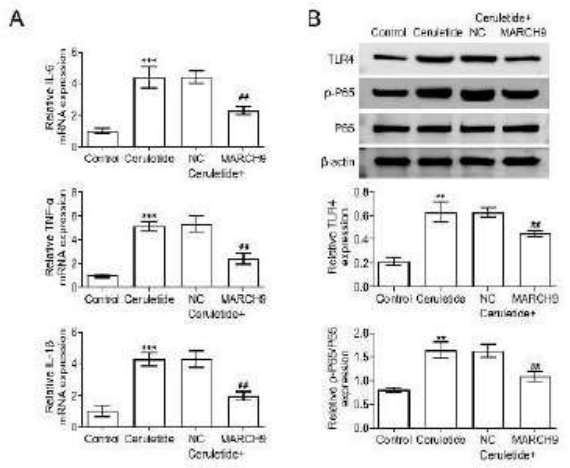

Figure 3: Effect of MARCH9 overexpressed in inflammation in AR42J cells induced by ceruletide. (A) TNF-α, IL-6 and IL-1β levels. (B) TLR4 and p-P65/P65 expressions. ***$P < 0.05$ or **$p < 0.05$, vs control; ##$p < 0.05$ or ###$p < 0.05$, *vs* ceruletide + NC

Overexpression of MARCH9 inhibited the pyroptosis of AR42J cells induced by ceruletide

Western blotting results revealed that MARCH9 down-regulated the expressions of NLRP3, GSDMD-N, caspase 1, IL-1β and IL-18 in AR42J cells induced by ceruletide (Figure 4 A). As shown in Figure 4 B, the overexpression of MARCH9 regulated the activity of caspase 1 in ceruletide + MARCH9 group, compared with ceruletide + NC group.

Over-expression of MARCH9 inhibited IL-6/JAK/SATA3 pathway

To further investigate the regulatory mechanism of MARCH9 in the pyroptosis of AR42J cells induced by ceruletide, the expression of related proteins was determined using western blotting. The results showed that MARCH9 overexpression repressed the expressions of IL-6, p-JAK2/JAK2 and p-STAT3/STAT3 in AR42J cells induced by ceruletide (Figure 5).

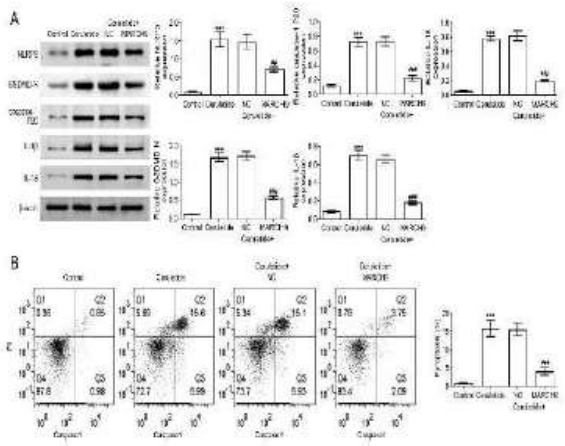

Figure 4: Effect of MARCH9 overexpressed in the pyroptosis of AR42J cells induced by ceruletide. (A) Expressions of NLRP3, GSDMD-N, caspase 1, IL-1β and IL-18. (B) Activity of caspase 1. $P < 0.05$, vs control; ##$p < 0.05$, vs ceruletide + NC

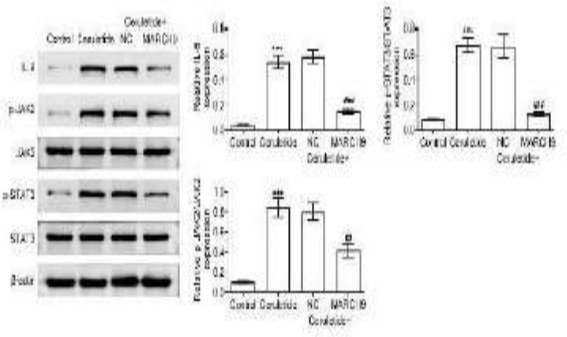

Figure 5: Effect of MARCH9 overexpressed in IL-6/JAK/SATA3 levels. Expressions of IL-6, p-JAK2/JAK2, and p-STAT3/STAT3. ***$P < 0.05$, vs control; ###$p < 0.05$ or ##$p < 0.05$, vs ceruletide + NC

DISCUSSION

Recent studies showed that inhibiting pyroptosis alleviates pancreatic injury and significantly affects the progression of AP [12]. This study showed that MARCH9 expression is downregulated in AP patients' serum and has the ability to regulate pyroptosis in PA cells.

Research has shown that MARCH9 participates in cancer progression. In lung adenocarcinoma, the overexpression of MARCH9 attenuates the carcinogenic effect of intercellular adhesion molecule 1 (ICAM-1) [13]. Furthermore, studies have found that MARCH9 is related to immune regulation in the

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bod [9], and participates in innate immunity by catalyzing the polyubiquitination of key immune factors [14]. Another recent study found that MARCH9 was related to the occurrence of AP and the overexpression of MARCH9 inhibits the NLRP3 inflammasome-dependent pancreatic cell pyroptosis by mediating the ubiquitination of NADPH oxidase-2, which may regulate the progression of AP [10]. The NLRP3 inflammasome is involved in regulating cellular inflammatory responses through the modulation of downstream pro-inflammatory factors [15]. One way to effectively mitigate these inflammatory responses is to target NLRP3 [16]. In addition, NLRP3 is also closely related to the occurrence of pyroptosis. For example, isoliquiritin ameliorates depression symptoms by suppressing miRNA-27a-mediated pyroptosis induced via NLRP3 inhibition [17]. Furthermore, cisplatin activates NLRP3/caspase 1 pathway, contributing to trigger pyroptosis in triple-negative breast cancer cells [18].

In the present study, MARCH9 was lowly expressed in the serum of AP patients, which suggests that MARCH9 was related to the development of AP. To further explore the role of MARCH9 in AP, an *in vitro* AP cell model was established by inducing PA cells with ceruletide and investigated for its effect on PA cells pyroptosis by overexpressing MARCH9. As expected, the upregulation of MARCH9 suppressed the inflammation in ceruletide-induced PA cells, as well as NLRP3 inflammasome-mediated pyroptosis, leading to enhanced viability of the PA cells. In addition, this study revealed a potential association between MARCH9 and the IL6/JAK/STAT3 pathway. However, this study is only a preliminary worK at the cellular level and has not been validated in animal models. We will supplement this in the follow-up study.

CONCLUSION

The results confirm that MARCH9 is low expressed in AP patients and its overexpression contributes to the inhibition of NLRP3-induced pyroptosis in PA cells by regulating IL6/JAK/STAT3 pathway. Thus, MARCH9 is a potential biomarker for AP treatment.

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