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BRIEF ARTICLE

Plasma miR-216a as a potential marker of pancreatic injury in a rat model of acute pancreatitis

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Abstract

AIM: To study the potential value and specificity of plasma miR-216a as a marker for pancreatic injury.

METHODS: Two rat models were applied in this article: L-arginine-induced acute pancreatitis was used as one model to explore the potential value of plasma miR-216a for detection of pancreatic injury; nonlethal sepsis induced in rats by single puncture cecal ligation and puncture (CLP) was used as the other model to evaluate the specificity of plasma miR-216a compared with two commonly used markers (amylase and lipase) for acute pancreatitis. Plasmas were sampled from rats at indicated time points and total RNA was isolated. Real-Time Quantitative reverse transcriptase-polymerase chain reaction was used to quantify miR-216a in plasmas.

RESULTS: In the acute pancreatitis model, among five time points at which plasmas were sampled, miR-216a concentrations were significantly elevated 24 h after arginine administration and remained significantly increased until 48 h after operation (compared with 0 h time point, $P < 0.01$, Kruskal-Wallis Test). In the CLP model, plasma amylase and lipase, two commonly used biomarkers for acute pancreatitis, were significantly elevated 24 h after operation (compared with 0 h time point, $P < 0.01$ and 0.05 respectively, Pairwise Bonferroni corrected *t*-tests), while miR-216a remained undetectable among four tested time points.

CONCLUSION: Our article showed for the first time that plasma miR-216a might serve as a candidate marker of pancreatic injury with novel specificity.

Key words: MiR-216a; Plasma miRNA; Pancreatic injury; Acute pancreatitis; Biomarker

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INTRODUCTION

MicroRNAs (miRNAs) are endogenous small (18-25 nt), non-coding RNAs that repress expression of mRNAs by either cleavage or translational repression through perfect or imperfect binding to the 3' untranslated regions of target mRNAs. Since the discovery of the founding member named *lin-4* in *Caenorhabditis elegans* (*C. elegans*), miRNAs have now been shown to be involved in multiple important biological processes, including development, differentiation, and cancer, *etc*. The most recent release of the miRBase Registry (version 14, released on September 2009) lists 721 different miRNAs identified in humans[1]. It is estimated that miRNAs may contribute to the regulation of more than one third of all human genes[2].

In 2008, Jeyaseelan *et al*[3] first identified the existence of miRNAs in the circulation and suggested their possible use as biomarkers for stroke and related pathologies. This principle was validated in a series of literature reports and analyzing miRNAs levels in circulation is forecast to be a promising field for identifying biomarkers of cancer. For instance, miR-92 has been established to be a potential noninvasive molecular marker for colorectal cancer screening[4]. Most recently, two articles reported that miR-208, 122, 133a, and 124 held promise as biomarkers for injury of heart, liver, muscle, and brain respectively, indicating that tissue-specific miRNAs can be exploited as circulating accessible biomarkers for tissue injury[5,6].

In this study, we evaluated the hypothesis that pancreas-specific miRNA (miR-216a) might leak into the circulation from the injured pancreatic cells and this miRNA might serve as a good biomarker for pancreatic injury. Hence we used an arginine-induced pancreatitis model to study whether pancreas-specific miRNA can be detected in the circulation in the setting of pancreatic injury. As miR-216a is strictly expressed in pancreas, we further used the cecal ligation and puncture (CLP) model, a model simulating perforation, intestinal strangulation, sepsis, and multiple organ dysfunction syndrome (MODS), to evaluate the specificity of miR-216a to pancreas injury compared with the two most commonly used biomarkers for acute pancreatitis.

MATERIALS AND METHODS

Study design

This study was divided into three phases: Phase Ⅰ (Identification of miR-216a's specificity to pancreas): In this phase, thirteen different tissues including heart, liver, spleen, lung, kidney, thyroid gland, pancreas, small intestine, large intestine, brain, skeletal muscle, testis, and blood vessel were collected from healthy Sprague Dawley (SD) rats. reverse transcriptase-polymerase chain reaction (RT-PCR) was used as the means to quantify relative concentrations of miR-216a in various tissues. Specific expression of miR-216a in pancreas was identified for further analysis in phase Ⅱ. Phase Ⅱ (Validation of miR-216a's eligibility as a biomarker for pancreatic injury): In this phase, plasma samples were collected from SD rats at 5 different time points (0, 12, 24, 48, 72 h after induction of acute pancreatitis model) before they were sacrificed. Plasma miR-216a was quantified using RT-PCR and its differential expression between different time points was compared to testify its potential as a biomarker for pancreatic injury. Phase Ⅲ (Validation of miR-216a's specificity for pancreatic injury): Nonlethal sepsis induced in rats by single puncture CLP[7], a model simulating perforation, intestinal strangulation, sepsis, and MODS, will lead to non-specific hyperamylasemia and hyperlipasemia. We quantified plasma miR-216a in this model to see if it is more specific compared with amylase and lipase, two common laboratory markers used to establish the diagnosis of acute pancreatitis[8,9].

Animals and setup of two models

Male SD rats (200 ± 10 g) were purchased from the Experiment Animal Center of the Second Military Medical University (Shanghai, China). The rats were maintained in a temperature-controlled room on a 12-h light/12-h dark cycle and fed standard rat chow and tap water ad libitum. All animal experiments were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University, Shanghai.

Acute pancreatitis was induced in SD rats (n = 50, 10 at each time point) by injecting 2 × 250 mg/100 g body weight of L-Arginine (Sigma) intraperitoneally in a 1-h interval, as a 20% solution in 0.15 mol/L NaCl. After sampling caval vein blood, rats were sacrificed at 0, 12, 24, 48, and 72 h following arginine administration. Acute pancreatitis was confirmed by plasma amylase and lipase level elevations and typical inflammatory features observed microscopically.

The induction of nonfulminant sepsis was performed under chloral hydrate anesthesia (300 mg/kg of body weight) using cecal ligation with a single, 18-gauge puncture as previously described[7,10]. After surgery, animals were fluid resuscitated with 40 mL/kg of subcutaneously administered sterile saline and were given free access to water but not food. At 0, 6, 12, and 24 h following single-puncture CLP operation, animals were reanesthetized with a 300 mg/kg intraperitoneal injection of chloral hydrate. Vena caval blood was collected for further RNA, amylase and lipase analysis.

Plasma amylase concentrations were measured with an automatic analyzer (Hitachi 7600-120); Plasma lipase concentrations were measured by using a Cobas-mira (Roche, USA).

Blood processing and isolation of plasma

All peripheral blood samples were collected in 2 mL BDVacutainer spray-coated K2 EDTA tubes (BD Diagnostic Systems). Samples were allowed to sit at room temperature for a minimum of 30 min and a max of 2 h. Separation of the blood sample was accomplished by centrifugation at 1200 × g at 4℃ for 20 min. Each plasma sample (300 μL at least) was removed into a 1.5 mL Ep-

pendorf tube, leaving enough plasma in the original tube such that the lowest point of the meniscus did not touch the clot. Then the samples were stored at -80℃ waiting for further extraction for total RNA isolation.

RNA isolation

All plasma samples were thawed on ice and 100 μL of each sample was transferred to a tube containing 750 μL of TRI Reagent BD and 20 μL of acetic acid (5 mol/L). Five microliters of synthetic *C. elegans* miRNAs (cel-miR-39, 50 pmol/L, synthesized by Qiagen) was added to each denatured sample as the spiked-in control[11,12]. RNA was isolated using the TRI Reagent® BD (cat. No. TB 126) following the manufacturer's protocol for RNA isolation. Each obtained RNA pellet was resuspended in 40 μL nuclease-free water and stored at -80℃.

Real-time quantitative RT-PCR analysis

A TaqMan miRNA real-time RT-PCR kit (Applied Biosystems) was used to detect and quantify the mature miRNA existing in total RNA extracted from tissues or plasmas. Briefly, 100 ng of tissue-derived total RNA or 2 μL of plasma-derived total RNA (from 5 μL of plasma) was reverse transcribed by TaqMan® MiRNA RT Kit. Negative controls were included with every real-time RT-PCR assay, and no amplification of the signal was detected when nuclease-free water was added instead of RNA or cDNA sample. Data were analyzed with 7500 software v.2.0.1. (Applied Biosystems), with the automatic Ct setting for adapting baseline and threshold for Ct determination. RT-PCR assays were performed in triplicate on each cDNA sample. Tissue expression levels of miR-216a were normalized to RNU6B[4,5], whereas cel-miR-39 was used to normalize the expression levels of miRNAs in plasma as described previously[11,12].

To relatively quantify miR-216a's concentrations in different tissues, we conducted RT-PCR with a known amount of synthetic miR-216a (Shanghai GenePharma Co., Ltd., Shanghai, China). In the presence of 0.67 amol (Ct = 35) to 48 fmol (Ct = 17.8) of synthetic miR-216a, we observed an excellent linearity (r^2 = 0.997) between the logarithm of the amount of input miR-216a and Ct value, suggesting that Taqman PCR assay is capable of detecting miR-216a at a detection limit equivalent to a Ct value of 35. Of note is that no miR-216a signal was detected in the plasma of healthy rats at all; no signal was detected even after 45 cycles of real-time PCR. The amount of miRNA not detected after 45 cycles of a real-time PCR was regarded in the present study as a Ct equivalent to 45. We set 35 as the baseline because the limit for reliably detecting synthetic miR-216a was 0.67 amol (Ct = 35).

Statistical analysis

With SPSS 13.0 software, data was compared between groups using analysis of variance (ANOVA), Kruskal-Wallis Test and Pairwise Bonferroni corrected *t*-test methods. The *P* value of less than 0.05 was defined as statistically significant.

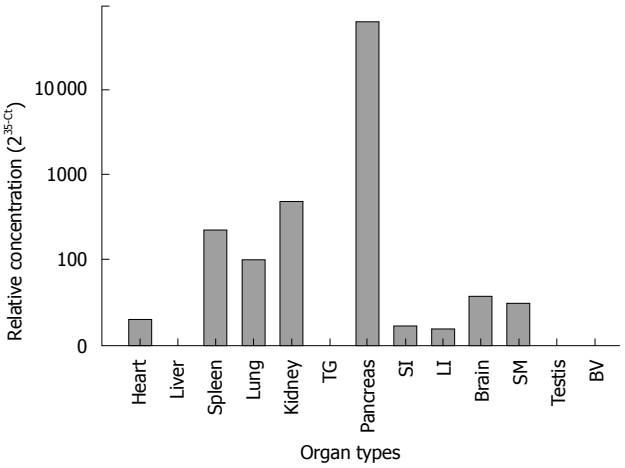

Figure 1 Expression of miR-216a in healthy rat tissues. Measurements of miR-216a in different samples were achieved by conducting real-time reverse transcriptase-polymerase chain reaction assay by means of 2^{35-Ct} as the relative expression level. Three rats were used in this experiment. Bars represent mean values of plasma miR-216a's quantification. TG: Thyroid gland; SI: Small intestine; LI: Large intestine; BV: Blood vessel.

RESULTS

Identification of miR-216a as a pancreas-specific miRNA

Various miRNA array analyses demonstrated that miR-216a was highly specific to the pancreas[13-15]. To verify that it is indeed produced specifically and abundantly to serve as a good biomarker candidate for pancreatic injury, we quantified the concentrations of miR-216a in 13 different tissues sampled from normal rats (Figure 1). As expected, pancreas tissue had the highest concentration of miR-216a among these samples, 128-fold higher than in kidney, which listed the next highest concentration.

Concentrations of plasma miRNAs in a pancreas-injury model

Arginine-induced pancreatitis was used as the pancreas injury model to investigate our hypothesis. We measured the plasma concentrations of amylase, lipase, and miR-216a. As miR-16 had been reported to be stably expressed across normal tissue types[16] and could be detectable at modest levels in normal plasmas[11], we tested its plasma concentrations to see if pancreas injury might lead to general elevation of plasma miRNAs.

As shown in Figure 2, plasma amylase and lipase concentrations were significantly elevated 24 h after intraperitoneal injection of arginine ($P < 0.01$, Kruskal-Wallis Test). Light micrographs of the pancreas showed interstitial edema, inflammatory infiltrate, acinar cell necrosis, and adipose tissue in interstitial spaces (Figure 3). Both laboratory tests and microscopic demonstrations supported our model of pancreatic injury. We further quantified plasma miR-216a using 35-Ct as its relative concentration. As shown in Figure 2A, though undetectable at baseline (Ct > 35), miR-216a concentrations in plasma were significantly elevated 24 h after arginine administration and remained significantly increased until 48 h after administration ($P < 0.01$, Kruskal-Wallis

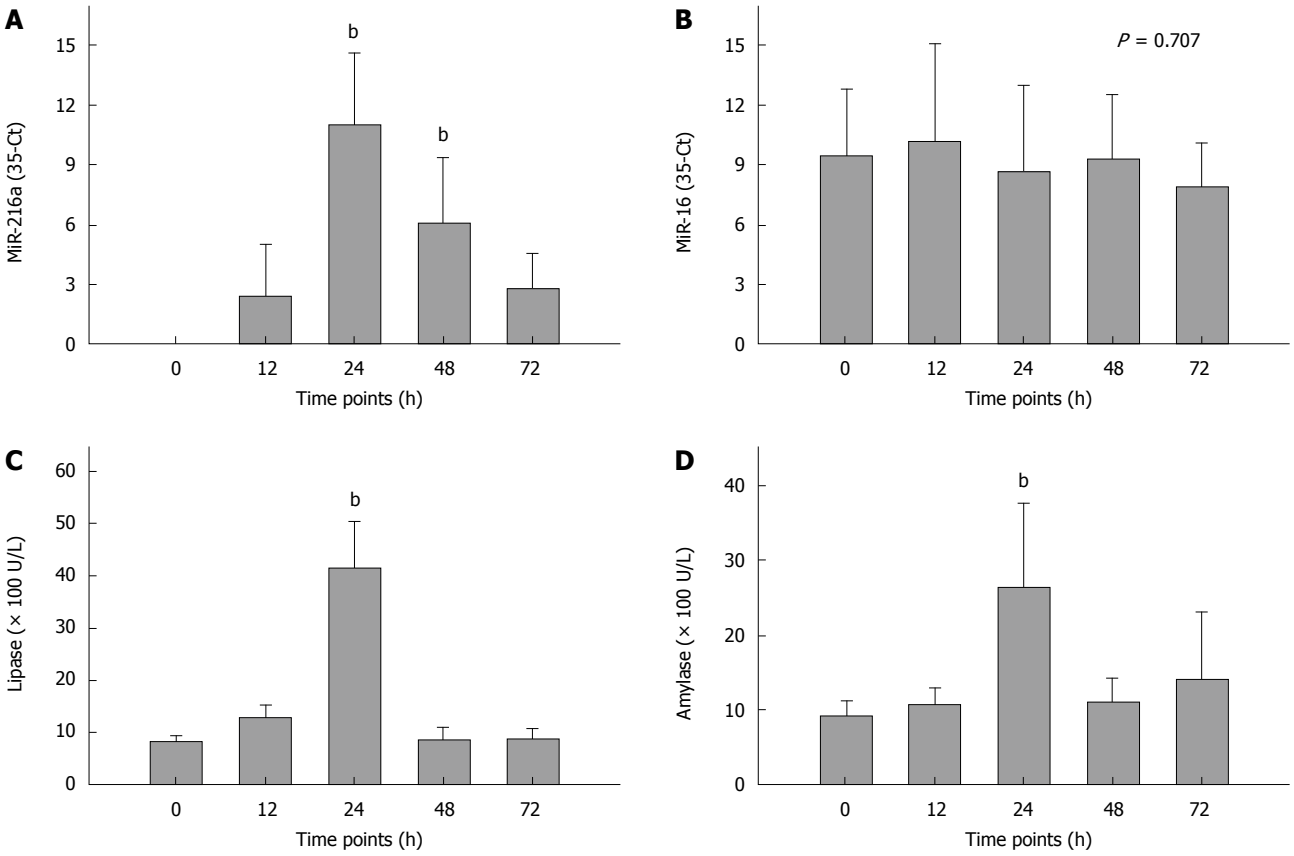

Figure 2 Plasma levels of miR-216a, miR-16, lipase, and amylase at different time points after induction of acute pancreatitis. A: Production of plasma miR-216a was significantly increased 24 h after L-arginine administration and remained significantly higher until 48 h after administration (Kruskal-Wallis Test); B: The amount of plasma miR-16 remained unchanged across all time points (One-way ANOVA); C and D: Plasma lipase and amylase levels were significantly elevated 24 h after administration (Kruskal-Wallis Test). Data are presented as the mean and SD. Ten rats were studied at each time point. $^{b}P < 0.01$ *vs* 0 h time point.

Test). Furthermore, we did not identify significant elevations of plasma miR-16 at any time points ($P = 0.707$, one-way ANOVA), indicating that pancreas injury did not lead to general increase of plasma miRNAs.

MiR-216a may be more specific than amylase and lipase as a biomarker for acute pancreatitis

As miR-216a is pancreas-specific and various pathologic conditions may lead to nonspecific hyperamylasemia and hyperlipasemia, we hypothesized that miR-216a might be more specific than amylase and lipase in diagnosing acute pancreatitis. In the CLP model of our experiment, plasma amylase and lipase were significantly elevated 24 h after operation ($P < 0.01$ and 0.05 respectively, Pairwise Bonferroni corrected *t*-tests, Figure 4B), while miR-216a remained undetectable. Microscopic examination showed no sign of pancreatic injury (Figure 4A), which further consolidated our hypothesis that miR-216a might be a reliable biomarker for pancreatic injury with novel specificity.

DISCUSSION

Accumulating evidence suggests that circulating miRNAs may be good biomarkers for specific tissue injury. For example, Jeyaseelan *et al*[3] provided evidence that some of the miRNAs that were highly expressed in the ischemic brain could be detected in blood samples; Kai Wang's exploration demonstrated that specific miRNA species, such as miR-122 and miR-192, exhibited dose- and exposure duration-dependent changes at a significantly early stage of drug-induced liver injury[17]. Most recently, two articles reported that miR-208, 122, 133a, and 124 held promise as biomarkers for injury of heart, liver, muscle and brain respectively, indicating that tissue-specific miRNAs could be exploited as circulating accessible biomarkers of tissue injury[5,6]. Furthermore, Ai *et al*'s[18] results, which revealed that circulating miR-1 might be a novel, independent biomarker for diagnosis of acute myocardial infarction, extended the principle of circulating miRNAs' eligibility as biomarkers into clinical settings.

Our data show for the first time that the plasma concentration of miR-216a, which is produced exclusively in pancreas, increases in the model of arginine-induced acute pancreatitis. This result provides clues that plasma miR-216a may be a good biomarker for pancreatic injury. Furthermore, the undetectable concentration of miR-216a in the control group compared with the extremely high concentrations seen in the acute pancreatitis model with histologically documented toxicity highlights the signal-to-noise ratios seen with miR-216a, suggesting that this miRNA may serve as a good biomarker to monitor the injury to pancreas.

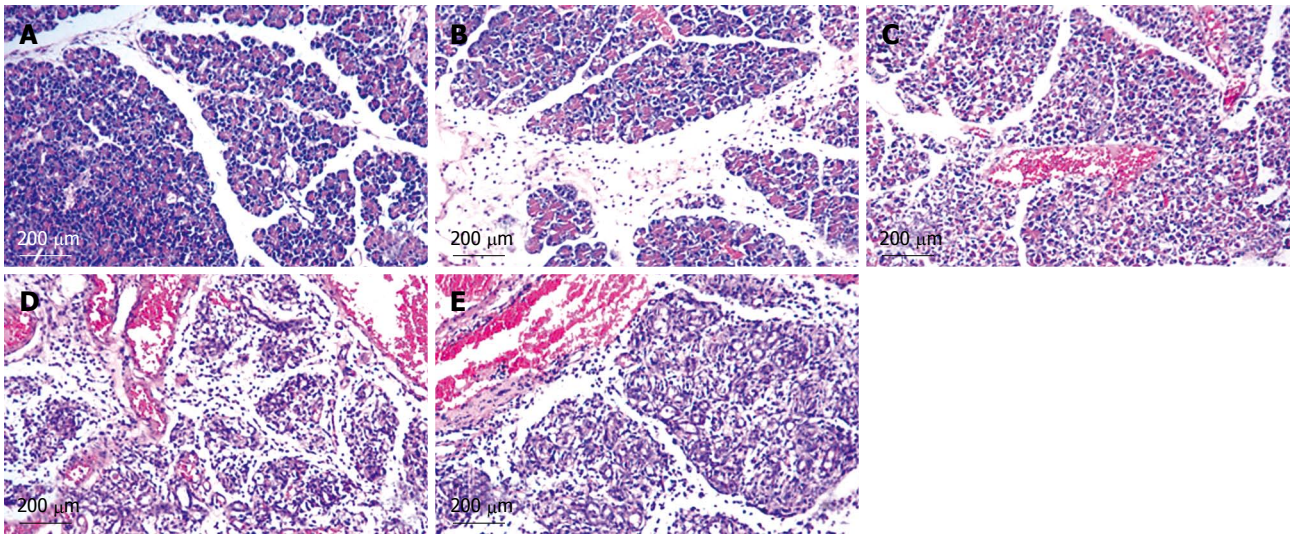

Figure 3 Light micrographs of the pancreas at 0 h (A), 12 h (B), 24 h (C), 48 h (D) and 72 h (E) after arginine injection. A: Neither interstitial edema nor acinar cell necrosis is seen; B: Interstitial edema and slight cellular infiltration in the interstitium can be seen; C: The acinar structures are partially destroyed. Interstitial edema and inflammatory infiltrate are greater in degree than at 12 h; D: The acinar architecture is markedly disrupted; E: Most pancreatic acinar cells show signs of degeneration or necrosis. Adipose tissue can be seen in the interstitial spaces.

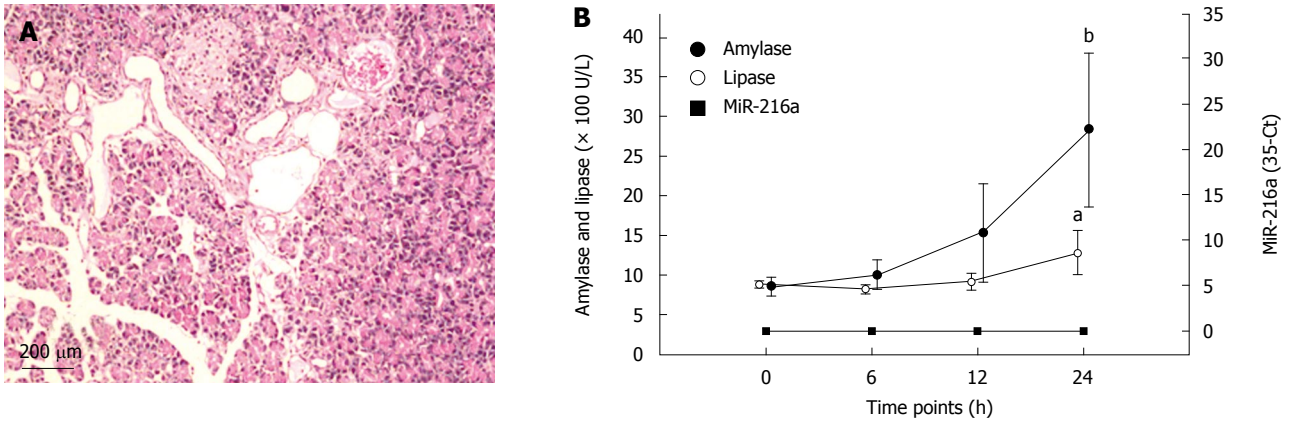

Figure 4 Amylase, lipase levels and light micrographs of pancreatic tissue in cecal ligation and puncture model. A: Pancreatic tissue was normal in the cecal ligation and puncture (CLP) model; B: Plasma amylase and lipase were significantly elevated 24 h after induction of this model, while plasma miR-216a remained undetectable. Data are presented as the mean ± SD. Eight rats were studied throughout the experiment with the CLP model. [a]$P < 0.05$ *vs* 0 h time point; [b]$P < 0.01$ *vs* 0 h time point. Pairwise Bonferroni corrected *t*-tests.

As miR-216a is specifically expressed in pancreas (Figure 1), we hypothesized that plasma miR-216a might be more specific than those commonly used in clinical settings. Although amylase and lipase are the two most common laboratory markers used to establish the diagnosis of acute pancreatitis[8,9], nonspecific hyperamylasemia and hyperlipasemia may occur under various conditions. For example, in diabetic ketoacidosis nonspecific elevations of amylase and lipase occur in 16%-25% of cases. Diagnosis of acute pancreatitis based solely on elevated amylase or lipase, even > 3 times normal, is not justifiable[19]. Furthermore, hyperamylasemia in the background of non-pancreatic diseases has been reported in a series of literature[20,21] whereas different groups have identified elevations of lipase in a number of conditions such as acute cholecystitis, intestinal infarction, duodenal ulcer, obstruction or inflammatory bowel disorders, liver diseases, and abdominal trauma[22-24]. Our article demonstrated that nonspecific elevations of amylase and lipase would occur in the CLP model while the plasma concentration of miR-216a remained undetectable, indicating that plasma miR-216a might be useful in justifying whether the elevated concentration of amylase or lipase was due to pancreatic injury in certain complex pathologic settings.

In this article, we validated for the first time the eligibility of a pancreas specific miRNA as a biomarker for pancreatic injury and its potential advantage of specificity over two previously confirmed markers of acute pancreatitis in certain pathology courses. The next question is whether elevation of plasma miR-216a has clinical significance and whether it offers advantages over measurement of amylase and lipase in human. As miR-216a is strictly conserved across species and its assessment (e.g. qRT-PCR) is simple and universally applicable, the discovery-validation pipeline for miRNA biomarkers will be more efficient than traditional proteomic biomarker discovery-

validation pipelines. Furthermore, highly sensitive PCR will possibly lower detection limits for plasma miR-216a compared to amylase and lipase. Future clinical assessments in humans are warranted to test its feasibility in patients.

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COMMENTS

Background

MicroRNA (miRNA) is a kind of small, noncoding RNA which can repress expression of target mRNAs. Various groups validated that miRNA could remain stable in circulation and served as a novel biomarker for different physiological or pathological conditions. More recently, several studies showed that certain miRNAs were strictly expressed in some tissues and these tissue-specific miRNA could leak into circulation, holding potential as non-invasive biomarkers with novel specificity. Nonetheless, there is no study concerning the diagnostic value of pancreas-specific miRNA in circulation for acute pancreas injury.

Research frontiers

The potential of pancreas-specific miRNA, miR-216a, as a biomarker for pancreatic injury has never been investigated previously.

Innovations and breakthroughs

This is the first report that pancreas-specific miRNA, miR-216a, could leak into the circulation to serve as a biomarker for pancreatic injury. Furthermore, this article showed that the specificity of circulating miR-216a is significantly higher than amylase and lipase, two most commonly used biomarkers in diagnosing acute pancreatitis.

Applications

Amylase and lipase are the two most commonly used biomarkers for pancreatitis detection. However, non-specific hyperamylasemia and hyperlipasemia will occur in plenty of clinical settings, which greatly lowers the specificity of these two biomarkers. MiR-216a is specifically expressed in pancreas and circulating miR-216a exhibited higher specificity than amylase and lipase. Furthermore, polymerase chain reaction is a highly sensitive technique which will possibly lower detection limits for plasma miR-216a than amylase and lipase.

Peer review

This paper reports, for the first time, that circulating miR-216a is a specific biomarker for pancreas injury. The study is nicely designed and the manuscript is pretty good.

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