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Chronic pancreatitis and pancreatic cancer demonstrate active epithelial–mesenchymal transition profile, regulated by miR-217-SIRT1 pathway

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ABSTRACT

Epithelial–mesenchymal transition (EMT) is supposed to be an inflammation induced response which may take a central role in tumorigenesis. Since recent evidence indicates that microRNAs may be involved in EMT, the present study set out to reveal the miRNA which might regulate the EMT in CP (chronic pancreatitis) and PC (pancreatic cancer) and its potential mechanism. Firstly, we provided evidence that both CP and PC tissues demonstrated active EMT profile. Consistently, miR-217 was obviously down-regulated in CP, PC and TGF-β1 treated PC cells, while negatively correlated to its direct target SIRT1. Moreover, either ectopic expression of miR-217 or inhibition of SIRT1 remarkably induced mesenchymal to epithelial transition (MET) in TGF-β1 treated PC cells. On the contrary, miR-217 inhibitor promoted EMT in PC cells but not in SIRT-knockdown PC cells. Clinical information from a cohort of 54 PC patients demonstrated that down-regulated miR-217 was positively correlated with late tumor stage, lymphatic invasion, vascular infiltration and distant metastasis. These results suggest that the overexpressed TGF-β1 in inflammation triggers the deregulation of the miR-217–SIRT1 pathway and then promotes the EMT process, which might be involved in the tumorigenesis of PC. Additionally, miR-217 may function as a novel target and predictor for PC prevention and therapy.

Introduction

Pancreatic cancer is the fourth leading cause of cancer deaths in the USA in both sexes with <5% survival after 5 years [1]. Recently, epidemiological and experimental data demonstrated close connection between chronic pancreatitis (CP) and pancreatic cancer (PC) [2–4]. However, the mechanism of tumorigenesis from CP to PC is far from defined. Recent research suggested that the epithelial to mesenchymal transition (EMT) might take center stage as the convergence point between chronic inflammation and cancer [5]. The EMT process is characterized as a change in cell shape from epithelial to mesenchymal-like, with the down-regulation of epithelial markers and the up-regulation of mesenchymal markers [6]. The cell acquires the capacity to degrade the basement membrane and migrate through the extracellular matrix either during embryonic development or cancer progression [7,8]. Hotz et al. showed that the EMT transcription factors Snail and Slug were expressed in pancreatic cancer but not in normal pancreas, suggesting a role in the progression of human pancreatic tumors [9]. Javle et al. also revealed that the EMT markers were associated with poor survival in surgically resected pancreatic adenocarcinoma [10]. Moreover, TGF-β1, the important inflammatory cytokine which overexpressed both in chronic pancreatitis and pancreatic cancer [11,12], was regarded as promoter of tumor progression and the activator of EMT [7]. Therefore, these results suggested that EMT might be involved in the tumorigenesis from CP to PC. However, the EMT profiles in CP have yet to be fully characterized. Therefore, the present study set out to first explore whether the active EMT process also exists in CP to further reveal whether EMT is involved in the tumorigenesis from CP to PC.

MicroRNAs (miRNAs) are small non-coding RNAs that bind the 3'UTR of mRNAs, which result in the degradation or translation inhibition of the target mRNAs. It was revealed that miRNA expression could be dynamically regulated in response to specific immune and inflammatory stimuli under the inflammatory microenvironment. Aberrantly expressed miRNA is frequently associated with

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a variety of cancers, including pancreatic cancer [13–15]. Among those miRNAs, miR-217 was remarkably down-regulated both in pancreatic cancer with microRNA microarray analysis [16]. Azevedo-Pouly et al. revealed that pancreatic acinar ductal trans-differentiation complicated with decreased miR-217 [17]. Therefore, these results suggested that miR-217 might be involved in the tumorigenesis of PC from CP. Given that miRNA has potential functions in carcinogenesis of PC from CP, it is of prime interest to confirm whether both CP and PC existing active EMT process and is regulated by miRNA.

Since a single miRNA can target multiple mRNAs, we applied the online software, MicroRNA to predict the downstream target genes of miR-217. The results showed that silent information regulator 1 (SIRT1) might be a potential target of miR-217. SIRT1 is a mammalian NAD+-dependent class III histone deacetylase (HDAC) which connects with crucial stress-responsive signal transduction pathways including inflammation [18,19]. Our previous research showed that SIRT1 was overexpressed in PC and might function as a promoter in tumorigenesis of PC [4]. Moreover, Wauters et al. also proved that overexpressed SIRT1 regulated acinar to ductal metaplasia and supported cancer cell viability in pancreatic cancer [20]. Furthermore, other evidence demonstrated that SIRT1 served as a promoter in EMT process by cooperating with EMT transcription factors in prostate cancer cell [21].

Therefore, we hypothesize that the down-regulated miR-217 might up-regulate the expression of SIRT1 and then facilitate the EMT in CP and PC. We detected the miR-217–SIRT1 signaling pathway both in the CP and PC tissues to reveal whether this pathway was deregulated and correlated with the EMT profile. Then, we further explored whether the miR-217–SIRT1 pathway could regulate the EMT process of pancreatic cancer cells in an in vitro EMT model which was induced by TGF-β1. Finally, correlation between miR-217 expression and clinicopathological characteristics of patients with PC were evaluated.

Materials and methods

Patients and tissue samples

Fifty-four pancreatic cancer samples (PC) were collected from patients with pancreatic ductal adenocarcinoma (PDAC) who had not received any radiotherapy or chemotherapy before excision. Chronic pancreatitis samples (CP) were obtained from 26 patients with pancreatic duct stones or pancreatic calcification. Twenty-seven normal pancreas samples (NP) were taken from peripheral tissues of serous cystadenoma or insulinoma. The original histopathological reports were obtained from each case and the diagnosis of pancreatic cancer was confirmed. Immediately after surgical removal, tissue samples were either snap-frozen in liquid nitrogen or fixed in 10% buffered formalin solution and then embedded in paraffin, respectively. The patients were all informed that the samples might be applied for further scientific research. All participants provided their written consent to participate in this study before operation and the study protocol was approved by the ethics committee of Huazhong University of Science and Technology.

Cell culture

The human pancreatic cancer cell lines BxPC-3, PANC-1 were cultured as described previously [15]. Cells were treated with 10 ng/ml human TGF-β1 to induce EMT [22].

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) were performed according to the manufacturer's protocol (TaKaRa) and the corresponding data analysis was described earlier [15]. The expression of mRNAs and miRNAs was normalized with reference to GAPDH and U6 small nuclear RNA respectively. Primers sequence used to amplify genes are listed in the Supplementary Table S1.

MicroRNA transfection and RNA interference

A final concentration of 50 nM miR-217 precursor, miR-217 inhibitor, siSIRT1 and their matched control was used in microRNA transfection and RNA interference. Detailed materials and procedure for microRNA transfection and RNA interference are given in the Supplementary Methods.

Luciferase reporter assay

The pmiR-RB-ReportTM-SIRT1 (wild type SIRT1 3'UTR, WT) and pmiR-RB-ReportTM–Control (mutant type SIRT1 3'UTR, MUT) was purchased from RIBOBIO (Guangzhou, China). PANC-1 cells were seeded in 24-well plates (70–80% confluence) and transfected with pmiR-RB-ReportTM-SIRT1 or pmiR-RB-ReportTM–Control (200 ng/well) (RIBOBIO) by Lipofectamine 2000 (Invitrogen). Cells were co-transfected with 50 nM miR-217 or 50 nM miR-NC respectively. Luciferase activity was determined using the dual luciferase assay system (Promega; Madison, WI) after 48 h of transfection. Luciferase activity was normalized to Renilla luciferase activity.

Western blot analysis, immunohistochemistry and immunofluorescence

Cells were lysed in the presence of 50 mM Tris, pH 7.5, 150 mM NaCl, and 1% NP-40 on ice. Equal amounts of protein were denatured in SDS sample buffer and separated on 8%, 10%, 12% polyacrylamide gels and transferred to PVDF membranes. The blots were then probed with various antibodies, such as antibodies against β-catenin, Vimentin, MMP-2, GAPDH, and E-cadherin. Immunohistochemistry and immunofluorescence are performed as described [9]. Detailed data for antibodies and procedures of western blot, immunohistochemistry and immunofluorescence were given in the Supplementary Methods.

Cell invasion and migration assay, wound healing assay

Cells (2×10^4 to 6×10^4) were plated in 200 μl RPMI 1640 medium with 0.1% fetal bovine serum into the upper chamber. The lower chamber was filled with 700 μl RPMI 1640 medium with 30% fetal bovine serum. Migration assay were done using Transwell chambers without coating of ECM gel. Detailed wound healing assay methods are also given in the Supplementary Methods.

Soft agar colony formation assay

Cells (1250 cells/well) were suspended in 10% FBS-RPMI 1640 containing 0.3% agar. The cells were then placed into a 24-well culture plate containing a hard agar base composed of 10% FBS-RPMI 1640 and 0.5% agar. The cultures were returned to the incubator and fed every 2 days with 250 μl of growth medium. The plates were incubated for 2 weeks. And then, the cells were stained with 0.05% crystal violet overnight at 37 °C. Colonies were visualized and counted by light microscopy. Soft agar assay were performed in triplicate.

Statistical analysis

Data are presented as the mean ± standard deviation. The data were analyzed using the SPSS 13.0 Windows version software. Statistical analyses were done by analysis of variance (ANOVA) or Student's t test. P value <0.05 was considered statistically significant.

Results

Both CP and PC tissues demonstrated active EMT profiles

As no comprehensive data set describing EMT profiles in normal pancreas (NP), chronic pancreatitis (CP), and pancreatic cancer (PC) tissues is currently available, we first sought to characterize the EMT profiles in these three types of pancreatic tissues. The expression of epithelial marker (E-cadherin), mesenchymal marker (N-cadherin), EMT inducing transcription factors (Snail and ZEB1) in CP and PC tissues were compared with NP tissue by qPCR and IHC. As shown in Fig. 1A, compared to NP tissues, E-cadherin mRNA was significantly down-regulated in CP and PC tissues ($p<0.05$), whereas there was no significant difference between CP and PC. On the contrary, mRNAs of N-cadherin, Snail and ZEB1 in CP and PC tissues were significantly up-regulated ($p<0.05$). On the other hand, IHC results showed that 75.6% CP, 70.3% PC and 33.3% NP tissues demonstrated positive N-cadherin expression, respectively. Snail was positively expressed in 79.3% CP, 80.3% PC and 46.2% NP samples. Similarly, ZEB1 expression was also higher in CP (72.0%) and PC (83.3) than that in NP (30.0%) samples. Whereas, only 19.2% CP and 25.6% PC samples showed positive E-cadherin expression, which was lower than that in NP tissues (60.3%) (Fig. 1B and C).