Extracellular vesicles microRNA analysis in type 1 autoimmune pancreatitis: increased expression of microRNA-21

Short title: miR-21 and type 1 AIP

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Author Contributions

Koh Nakamaru and Takashi Tomiyama designed the study and wrote the initial draft of the manuscript. Sanshiro Kobayashi, Manami Ikemune , Satoshi Tsukuda, Takashi Ito, Toshihiro Tanaka, Kazushige Uchida, Takashi Yamaguchi, Yugo Ando, Tsukasa Ikeura, Toshiro Fukui, Akiyoshi Nishio, Makoto Takaoka, Patrick S.C. Leung, M.E. Gershwin, and Kazuichi Okazaki contributed to analysis and interpretation of data, and assisted in the preparation of the manuscript. All other authors have contributed to data collection and interpretation, and critically reviewed the manuscript.

Abstract

Background

The molecular basis of type I autoimmune pancreatitis (AIP) remains unclear. Recent attention on the role of extracellular vesicles microRNA (EV miRNA) in immune homeostasis has prompted us to perform an extensive miRNA screening of serum-derived EV in type 1 AIP patients.

Methods

Serum EV miRNA expression was analyzed using microarrays in type 1 AIP patients (n = 10) and healthy adults (n = 10). Differences in signals >3 times or < 1/3 represented significant differences in expression.

Results

The signals of eight miRNAs (miR-659-3p, -27a-3p, -99a-5p, -21-5p, -205-5p, -100-5p -29c-3p, and -125b-1-3p) were significantly higher and two miRNAs (miR-4252 and -5004-5p) were significantly lower in type 1 AIP patients than in healthy adults. Quantitative reverse-transcription polymerase chain reaction revealed that EV miR-21-5p was significantly upregulated in type 1 AIP patients compared with that in healthy adults (P = 0.04). In situ hybridization study of miR-21-5p using tissue specimens revealed high miR-21 expression in various cell populations such as pancreatic acinar, and inflammatory cells in the pancreas of type 1 AIP patients.

Conclusions

Circulating extracellular vesicles exhibit altered patterns of miRNA expression with elevated miRNA-21-5p in type 1 AIP patients compared with that in healthy adults. Further, miRNA-21-5p was highly expressed in various cell populations of the pancreas in type 1 AIP patients. Our data suggests that miRNA-21-5p levels may be involved in the regulation of effector pathways in the pathophysiology of AIP and potentially a disease marker for monitoring disease interventions.

Keywords

microarray; RT-PCR; in situ hybridization; inflammatory disease

Abbreviations: EV, extracellular vesicle; AIP, autoimmune pancreatitis; HC, healthy control; CP, chronic pancreatitis**;** RNA, ribonucleic acid; miR, micro RNA; RT-PCR, reverse transcription polymerase chain reaction; rpm, rotations per minute; Cel-miR-39, *Caenorhabditis elegans* miRNA-39 ; TAPA-1, Target of the Antiproliferative Antibody 1; PBS, phosphate buffered saline; TBS, Tris buffered saline; WBC, white blood cell; IgG, immunoglobulin G; CRP, C-reactive protein; P-Amy, pancreatic Amylase; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; IL-4, Interleukin-4; Gata3, GATA binding protein 3; PDCD4, Programmed cell death 4; NF-κB, nuclear factor κB; ISH, in situ hybridization; PTEN, Phosphatase and Tensin Homolog Deleted from Chromosome 10; FOXO1, Forkhead box protein O1; N/A, not available

Introduction

Extracellular vesicles (EVs) are secreted by various cell populations into body fluids such as serum, urine, airway secretions, and breast milk [1-6]. These vesicles contain nucleic acids, including microRNA, and proteins derived from the cells from which they were secreted. EVs engage in cell-to-cell communication by transporting their contents to recipient cells [1, 2]. EVs not only maintain immune homeostasis [3-6] but are also involved in the pathophysiology of several autoimmune diseases [7-11].

Type 1 autoimmune pancreatitis (AIP) is a distinctive type of pancreatitis characterized by the diffuse irregular narrowing of the main pancreatic duct, sausage-like diffuse swelling of the pancreas, and immune-cell infiltration with high serum titers of IgG and IgG4[12]. It has been more than three decades since the initial case reports of chronic pancreatitis with hypergammaglobulinemia [13], and subsequently the specific diagnosis of AIP was described by Yoshida et al [14]. More recently, AIP has been classified into two subtypes: type 1 AIP, lymphoplasmacytic sclerosing pancreatitis (LPSP), and type 2 AIP or idiopathic duct-centric chronic pancreatitis (IDCP) with granulocytic epithelial lesions (GEL) [15]. Type 1 AIP is regarded as a pancreatic manifestation of IgG4-related disease (IgG4-RD) [16]. The fibroinflammatory processes underlying type 1 AIP respond well to steroids and/or immunosuppressive

agents [17]; therefore, an abnormal immune response is considered the core for the pathogenesis of disease[18]. Although studies have indicated that the loss of tolerance via the adaptive immune system [19] and epigenetic changes [20] contribute to the development of AIP, there are still major void in the understanding of the effector mechanisms underlying this uncommon autoimmune disease . To address this, we performed a comparative analysis of a well-defined cohort with AIP compared to age and sex-matched controls to examine miRNA expression in circulating EVs.

Methods

Clinical samples

This study included patients with type 1 AIP (n = 23) diagnosed according to the international consensus diagnostic criteria [15], patients with chronic pancreatitis (CPs) (n = 23) diagnosed according to Japanese clinical diagnostic criteria [21], and age and sex-matched healthy controls (HCs) (n = 23). The clinical characteristics of all subjects are listed in Table 1. All study subjects provided written informed consent approved by the ethics committee of Kansai Medical University for this study. AIP patients have not been treated with any steroids or immunosuppressive agents before any assays. Ten subjects from each three groups were subjected to microarray analysis. Ten subjects other than microarray from each three groups

were subjected to RT-PCR. The surgical specimens of type 1 AIP patients (n=3) and chronic pancreatitis patients (n=3) were used for in situ hybridization. Surgical specimens in normal lesion of epidermoid cyst was study in parallel as healthy control (n=3).

Serum EV isolation.

EVs were isolated using the ExoQuick precipitation kit, according to the manufacturer's instructions (System Biosciences, CA, USA). Briefly, 500 μL of serum was mixed with ExoQuick and incubated for 30 min at 4°C. After centrifuging at 13,000 rpm for 4 min and removing the supernatant, the EV pellet was stored at -80˚C until use for further analysis. The isolated EVs were characterized by analysis of CD9, CD63 ,CD81 and GM130 expression using western blotting (Fig. 1).

miRNA microarray analysis

Samples from patients with type 1 AIP, HC, and CP (n=10 of each) were used for microarray analysis, RNA extraction, and miRNA expression profiling. Total RNA was extracted from liquid samples using 3D-Gene RNA extraction reagent (Toray Industries Inc., Tokyo, Japan) and the miRNeasy mini kit (QIAGEN, Germany) according to the manufacturer's instructions. Extracted total RNA was labeled using the 3D-Gene miRNA labeling kit (Toray Industries Inc., Tokyo, Japan). Labeled RNAs were hybridized onto the 3D-Gene Human miRNA Oligo chip (Toray Industries Inc., Tokyo, Japan). The

annotation and oligonucleotide sequences of the probes were confirmed using the miRBase (http://microrna.sanger.ac.uk/sequences/). After cycles of stringent washes, fluorescence signals were scanned using the 3D-Gene Scanner (Toray Industries Inc., Tokyo, Japan) and analyzed using 3D-Gene Extraction software (Toray Industries Inc., Tokyo, Japan). The relative expression level of a given miRNA was calculated by comparing the signal intensities of the valid spots for all microarray experiments. The data were globally normalized per array, such that the median of the signal intensity was adjusted to 25.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

EV RNAs were quantified in an additional 10 samples, each, of type 1 AIP, HC, and CP. Total RNAs were purified using the miRNeasy Serum/Plasma Kit (Qiagen, Germany). Because a standard internal control miRNA for EVs is not available, *Caenorhabditis elegans* miRNA (Cel-miR-39) was used as a standard control [22]. EVs were resuspended in 200 µl of PBS and thereafter 1,000 µl of QIAzol Lysis Reagent was added, followed by chloroform extraction and ethanol precipitation. The total RNAs were measured using the NanoDrop 2000 and and Cel-miR-39 ($5.6x10^8$ copies) was added. Reverse transcription reaction was performed using the miScript Ⅱ RT kit (Qiagen, Germany). The miRNAs were quantified by real-time PCR using the miScript SYBER Green PCR kit (Qiagen, Germany) and the Rotor-Gene Q2 system (Qiagen, Germany). The expression of EV miRNA was normalized to that of cel-

miR-39 and determined using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used were as follows: 5'-UCACCGGGUGUAAAUCAGCUUG -3'(Cel-miR-39), 5'- UAGCUUAUCAGACUGAUGUUGA -3'(miR-21-5p), 5'- UUCACAGUGGCUAAGUUCCGC -3' (miR-27a-3p), 5'-UAGCACCAUUUGAAAUCGGUUA -3' (miR-29c-3p), 5'- AACCCGUAGAUCCGAUCUUGUG -3' (miR-99a-5p), 5'- AACCCGUAGAUCCGAACUUGUG -3' (miR-100-5p), 5'-ACGGGUUAGGCUCUUGGGAGCU -3' (miR-125b-1-3p), 5'- UCCUUCAUUCCACCGGAGUCUG -3' (miR-205-5p), 5'- CUUGGUUCAGGGAGGGUCCCCA -3' (miR-659-3p), 5'-GGCCACUGAGUCAGCACCA -3' (miR-4252) and 5'- UGAGGACAGGGCAAAUUCACGA -3' (miR-5004-5p) (miScript Primer Assays(Qiagen, Germany)).

In situ hybridization

Tissue sections (6 μm) were de-paraffined with xylene and rehydrated by an ethanol series and PBS. The sections were fixed with 10% formalin in PBS for 30 min at 37℃ and washed with distilled water. Thereafter, the sections were placed in 0.2 M HCl for 10 min at 37℃, washed with PBS, treated with 4 μg/ml Proteinase K in PBS for 10 min at 37℃, and washed with PBS again. Then, the sections were heat-treated with PBS for 5 min at 80℃, cooled immediately in cold PBS, and washed with 1x Standard Saline Citrate. Hybridization was carried out with digoxygenin labeled probes at concentrations of 18 nM in

probe diluent (Genostaff Co., Ltd.) for 16 h at 60°C. The hybridization sequences were as follows: 5‘-TCAACATCAGTCTGATAAGCTA-3’ (miR-21-5p) and 5’-GTGTAACACGTCTATACGCCCA-3’ (scramble) (Qiagen, Germany). After hybridization, the sections were washed 3 times with 50% formamide in 2x Standard Saline Citrate for 30 min at 50°C and washed 5 times in TBST (0.1% Tween 20 in TBS) at room temperature. After treatment with 1x blocking reagent (Genostaff Co., Ltd.) for 15 min at room temperature, the sections were incubated with anti-DIG AP conjugate (Roche Diagnostics) diluted 1:2000 with x50 blocking reagent (Genostaff Co., Ltd.) in TBST for 1 h at room temperature. Next, the sections were washed twice with TBST and then incubated in 100 mM NaCl, 50 mM MgCl2, 0.1% Tween 20, and 100 mM Tris-HCl (pH 9.5). Coloring reactions were performed with NBT/BCIP solution (Sigma-Aldrich) overnight and then the sections were washed with PBS. The sections were mounted with G-Mount (Genostaff Co., Ltd.).

Statistical analysis

Mann–Whitney U test and Pearson’s correlation test were used for data analysis; P values < 0.05 were considered to represent statistically significant differences.

Results

EV miRNA expression profiles

We analyzed EVs from patients with type 1 AIP and HCs using microarray analysis. In total, 165 miRNAs were found to be differentially expressed in patients with type 1 AIP relative to those in HCs. Further, 132 miRNAs were upregulated and 33 were downregulated in type 1 AIP patients compared with those in HCs, respectively (Fig. 2). As shown in Table 2, 8 miRNAs (miR-659-3p, -27a-3p, -99a-5p, -21-5p, -205-5p, -100-5p, -29c-3p, and -125b-1-3p) were significantly upregulated (>3-fold) and 2 miRNAs (miR-4252 and -5004-5p) were significantly downregulated (<1/3 or lower) in type 1 AIP patients when compared with HCs. These tendencies were found between AIPs and CPs but not between CPs and HCs (Table2). Microarray expression profiling is available on NCBI's GEO (https://www.ncbi.nlm.nih.gov/geo/); the data accession number, GSE128508.

Quantitative evaluation of EV miRNA expression level by RT-PCR

qRT-PCR was used to verify the data obtained from microarray analysis. The relative expression levels of 10 miRNAs were analyzed using the miScript SYBER Green PCR kit and normalized to those of spike-in cel-miR-39 (Fig. 3). The expression level of miR-21-5p was significantly higher in type 1 AIP patients than in HCs (P=0.04) and tended to be higher in type 1 AIP patients than in CP patients (P=0.05). The miR-27a-3p and miR-100-5p expression level in type 1 AIP patients was higher than that in CP patients

(miR-27a-3p; P=0.58, miR-100-5p; P=0.21) or HCs (miR-27a-3p; P=0.48, miR-100-5p; P=0.19), respectively; however, the difference was not statistically significant. There was no significant difference in the expression levels of miR-29c-3p between type 1 AIP, CP, and HCs (P= 0.21; type 1 AIP vs CPs) (P=0.28; type 1 AIP vs HCs). We were unable to obtain normal amplification curves for miR-659-3p, miR-99a-5p, miR-205-5p, miR-100-5p, miR-125b-1-3p, miR-4252, and miR-5004-5p.

Relationship between miR-21 expression level and clinical features

There were no positive correlation between miR-21-5p and clinical data on WBC, IgG, IgG4, IgE, CRP, P-Amy, CEA, and CA19-9 (data not shown).

Diffuse pancreatic distribution of miR-21 in type 1 AIP

The high expression of miR-21 in circulating EVs of type 1 AIP patients prompted us to examine miR-21 expression in the pancreas of type 1 AIP patients by in situ hybridization (ISH) using resected specimen of type 1 AIP patients. ISH revealed that the expression of miR-21 in pancreatic duct epithelium was similar between HCs, type 1 AIP and CP patients (Fig. 4A). However, miR-21 was highly expressed in the pancreatic acinar cells (Fig. 4B) in type 1 AIP patients but not HCs. Interestingly, the expression of miR-21 at pancreatic acinar cells and inflammatory cells was similar in both AIP and CP patients (Figure 4B-C). However, the ratio of miR-21 positive inflammatory cells per inflammatory site was significantly

1 higher in AIP patients than that in CP patients (Figure 4D). Hematoxylin and eosin staining of serial

2 section revealed that inflammatory cells in type 1 AIP specimens were mainly consist of lymphocytes

3 characterized by large nucleus and small cytosol (Fig. 4C). The expression patterns of miR-21 in HCs,

4 AIP and CP patients are summarized in Table 3.

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Discussion

Extracellular vesicles play vital roles in the intercellular regulation of various biological processes [1, 2, 23]. miRNA, which functions in the regulation the gene expression at the post-translational level, is one of the key constituents of EVs [24]. Alterations in miRNA expressions are involved in the initiation or modulation of multiple human diseases [25-28].

In this study, we demonstrated an altered pattern of EV miRNA population in AIP, with elevation of miR-21-5p in AIP when compared with HCs. Furthermore, ubiquitous high expression miR-21 is evident in pancreas of AIP patients including pancreatic ducts, acinar cells, and inflammatory cells. miR-21expression is also elevated in acinar cells of mice with acute pancreatitis and the expression level correlates with the severity of disease [29]. In mice with chronic pancreatitis, miR-21 are exported in pancreatic stellate cells derived EV [30]. It is well known that aberrant expression of miR-21 in pancreatic ductal adenocarcinoma cells contribute to tumor progression and refractoriness leading to poor prognosis [31, 32]. In pancreatic duct, however, our findings showed that miR-21 expression was also detected in non-pathogenic pancreata. Previous microarray study reported that miR-21 expression was approximately 3-fold higher in pancreatic cancer cells than in normal pancreas [33], implying that normal pancreatic ductal

cells modestly but constitutively express miR-21. Thus, we speculate that high expression of miR-21 in circulating EVs of type 1 AIP is not due to invariable expression of miR-21 at pancreatic ductal epithelial cells. Indeed, our data indicated that miR-21 was highly expressed in acinar cells and inflammatory sites but not in non-pathogenic pancreata. Alcoholic chronic pancreatitis is featured by progressive destruction of pancreatic parenchyma with fibrosis while inflammatory cell infiltration is generally sparse [34]. In contrast, type 1 AIP is histologically accompanied with a dense inflammatory cell infiltration denominated as lymphoplasmacytic sclerosing pancreatitis [35]. Although miR-21 expressing patterns of inflammatory cells were comparable between type 1 AIPs and CPs, the number of inflammatory cell in pancreatic tissues of type 1 AIP was enormous. Taken together, our findings suggest that high expression of miR-21 in circulating EVs was the consequence of over expression of miR-21 at inflammatory cells in type 1 AIP. It is of interest to note that serum EV mir-21-5p was recently reported to be three fold higher in diabetic children than healthy children [36].

It is unlikely that the pathophysiology of type 1 AIP involves only a single specific mechanism [18-20, 37]. Both Th1 and Th2 cells are differentiated from CD4+ naïve T cells, which are regulated by cytokine. Earlier work described a predominance of Th2 cytokines in Type 1 AIP, including IL-4, IL-5 and IL-13 [38],

indicating that Th2 immune response plays a key role in the progression of type 1 AIP, although the underlying mechanism of Th2 polarization in type 1 AIP has not been fully elucidated. miR-21 promotes Th2 differentiation from Th0 and up-regulates Th2-related genes such as *Gata3 and Il4* [39]. Programmed cell death 4 (Pdcd4), which is one of the miR-21 targets, induces activation of nuclear factor κB (NF-κB) and suppresses IL-10 [40]. Moreover, miR-21 targets suppress intrinsic IL-12 secretion by dendritic cells [41]. These signal and cytokine regulations together thereby can enhance Th2/Th1 ratio in type 1 AIP. In chronic pancreatitis, however, EV miR-21 was not elevated compared with its level in type 1 AIP, while a high expression was found in pancreatic tissues in our study. This discrepancy might be explained by the difference in the pathophysiology between type 1 AIP and chronic pancreatitis. Pathophysiology of type 1 AIP is characterized by lymphoplasmacytic sclerosing pancreatitis (LPSP) with the dominant infiltrating inflammatory cells being plasmacytes and CD4+ helper T cells. However, in chronic pancreatitis, cell-mediated cytotoxicity by CD8+ cytotoxic T cells and perforin-expressing CD56+ cells play an important role in the pathogenesis of alcoholic chronic pancreatitis although the populations of CD4+ T and CD8+ T cells are almost equivalent [42]. Therefore, we speculate that the high expression of miR-21 in tissues, as well as the resulting inflammatory milieu of the pancreas, is not disease-specific as it is caused by all pancreatitis types, including type 1 AIP and CP. However, the high miR-21 expression in type 1 AIP

probably participates in the crosstalk between EVs in tissue and the periphery as miR-21 is also highly expressed in circulating EVs, which together account for the Th2 predominance in type 1 AIP.

Pancreatic cancer is one of the most lethal malignant neoplasms with poor prognosis because most patients are not indicated for surgery at diagnosis. Thus, a relevant biomarker has been long desired. miR-21 is one of the most representative oncomiRs that have been frequently reported in various malignant diseases [43-46] including adenosquamous carcinoma of the pancreas [47] and pancreatic ductal adenocarcinoma [48]. In pancreatic cancer, miR-21 expression is not only locally upregulated in the pancreatic tumor site [49] but also in the periphery [50]. Studies have suggested that peripheral miR-21 as a novel candidate predictor not only for diagnosis [48] but for prognosis [51] and chemosensitivity [52]. Carcinogenesis by miR-21 is orchestrated by targeting of tumor suppressors, cell cycle regulators, and cellular apoptosis proteins such as PTEN, PDCD4, Sprouty, FOXO1, and SMAD7 [53]. In our study, miR-21 expression was diffusely detected in the pancreatic tissue of both type 1 AIP and CP patients, while a high expression of miR-21 in circulating EVs was detected in only type 1 AIP patients. Chronic pancreatitis is risk factor of pancreatic cancer [54]. Interestingly, in addition to its association with pancreatic cancer, type 1 AIP has also been reported in patients with other cancer types [55, 56]. With the

circulatory nature of miR-21, these findings are consistent with the variations in the distribution of miR-21 among type 1 AIP, CP, and pancreatic cancer. However, it is likely that the magnitude of miR-21 expression in the periphery and tissue of type 1 AIP patients is lower than that in those of pancreatic cancer patients [33, 49, 50]; hence we speculate that miR-21 more likely function as a modulator of the Th2 balance in type 1 AIP rather than as an oncomiR.

The present study reveals that circulating extracellular vesicles of patients with AIP contain high level of miR-21, which might polarize Th2 immune balance. In addition, miR-21 are diffusely expressed in pancreatic tissue especially at inflammatory sites and acinar cells of type 1 AIP. Together, these findings suggest that miR-21 in pancreatic tissue, released to periphery via exosomes, is key pathway of pathophysiology of type 1 AIP.

Conflicts of interest

The authors have no competing interests to declare.

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Figure legends

Fig. 1. Immunoblotting of extracellular vesicle markers in the circulating EVs from HCs and type 1 AIP patients.

Circulating EVs collected from type 1 AIP and CP patients and HCs were analyzed by western blotting with representative EV markers including CD81 (top), CD63 (upper-middle) and CD63 (lower-middle) and negative marker including GM130 (bottom). These findings verified that circulating EVs have exosome-like character.

Fig. 2 Scatter plot of microRNA microarray for AIP patients and HCs.

Scatter plot shows the 3-fold upregulated and downregulated microRNAs (the upper and lower dots compared with the linear border, respectively) in type 1 AIP patients compared to those in HCs.

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Fig. 3. Quantification of microRNA in circulating EVs by RT-PCR.

Ratio of miRNA expression to Cel-miR-39 expression is shown for each miRNA selected from the result of microarray analysis in AIPs (n=10), CPs (n=10) and HCs (n=10). Data are expressed as the mean ± SEM. Significant difference was found between AIPs and HCs in miR-21-5p (P=0.04).

Fig. 4. miR-21 expression determined by in situ hybridization of pancreatic tissues in HCs, CP, and type 1 AIP. In situ hybridization of miR-21 in (A) pancreatic duct, (B) acinar cells, and (C) H &E staining of type 1 AIP and CP . Bars indicate 50 μm, Note the positive hybridization in the pancreatic duct (A) and acinar cells (B) and presence of inflammatory sites with fibrosis (C) . H&E staining of type 1 AIP and CP indicates the enormous inflammatory cells in type 1 AIP (C). The number of inflammatory cells expressed miR-21 in each inflammatory site of type 1 AIP and CP (D) are shown as mean ±SD. Data were obtained from 3 independent experiments.

1 Table 1 Clinical features

	AIP (n=23)	CP (n=23)	HC (n=23)
Age (range)	63.0 (49-80)	63.0 (50-81)	61.0 (33-83)
Gender (Female/Male)	4/19	8/15	10/13
Pancreatic swelling (Diffuse/Focal)	6/17	-	-
Other organ involvement (Sialoadenitis/Retroperitoneal fibrosis/None)	7/2/14	-	-
Serum IgG4 level (mg/dl, range)	584.5(32-1490)	-	-

2

Table 2. The ratio of 10 miRNAs in Table 2 in comparison among AIPs (n = 10), CPs (n = 10), and HCs (n = 10).

	Ratio		
miRNA	AIP vs. HC	AIP vs. CP	CP vs. HC
upregulated			
hsa-miR-659-3p	13.04	27.10	N/A
hsa-miR-27a-3p	4.96	2.98	1.78
hsa-miR-99a-5p	4.37	11.49	0.15
hsa-miR-21-5p	3.57	3.66	0.66
hsa-miR-205-5p	3.46	4.27	0.35
hsa-miR-100-5p	3.23	N/A	0.16
hsa-miR-29c-3p	3.04	3.35	N/A
hsa-miR-125b-1-3p	3.00	2.37	N/A
downregulated			
hsa-miR-4252	0.17	N/A	0.10
hsa-miR-5004-5p	0.33	0.50	N/A

N/A; not available

Table 3. miR-21 positive cells at pancreatic ducts and acinar cells

	AIP			CP			HC		
	1	2	3	1	2	3	1	2	3
Pancreatic duct	+++	+	+++	++	+++	-	+++	++	+++
Acinar cell	+++	+	+++	+++	+++	-	-	+	-

MiR-21 positive sites: -, 0/9sites; +, 1-3/9sites; ++, 4-6/9sites; +++,7-9/9sites