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Dysregulated miRNA Associated with Transcription Factors of Insulin Gene Expression in Chronic Pancreatitis

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Abstract

Background/Aim: MicroRNAs with regulatory functions in gene expression are implicated in different diseases. The present study investigated differentially expressed miRNAs that possibly influence transcription factors involved in insulin gene expression in Chronic Pancreatitis (CP) employing bioinformatics approaches. ***Methods***: Pancreatic tissues were collected from CP patients undergoing partial pancreatectomy (n = 16) and controls (n = 15) undergoing resections for non-pancreatic malignancies. MiRNA profiles obtained using microarrays were validated by qRT-PCR. Target search involving miRWalk and TarBase as well as functional annotation employing KEGG (Kyoto encyclopedia of genes and genomes) and DAVID (Database for Annotation) databases were performed. Ingenuity pathway analysis (IPA) was used to construct networks relating miRNAs to their target genes. mRNA and proteins related to insulin gene transcription factors and hormones were evaluated by qRT-PCR and western blotting followed by confirmation upon immunofluorescent staining. ***Results***: Microarray data revealed 10 up-regulated and 15 down-regulated miRNAs in CP as compared to controls (Log2 FC > 2). Bioinformatic analysis showed 8399 target genes and KEGG pathway analysis suggested a role for the dysregulated miRNAs in modulating cytokine signaling, fibrosis, JAK-STAT signaling and insulin synthesis. IPA analysis suggested a simplified network attributing dysregulated miRNAs to NFκB-dependent cytokine signaling. Further, associations could be noted between miRNA 200b with Maf A, 138-1 with Neuro D and 27b with FoxO1. Decreases in mRNA levels of Pdx1, Neuro D and increases of Maf A and FoxO1 transcription factors could be noted ($P < 0.01$) in CP. These results were confirmed by western blotting and immunofluorescence staining. ***Conclusion***:

Our results identified dysregulation of miRNAs 138-1, 27b and 200b which were found to be associated with insulin gene transcription factors Neuro D, FoxO1 and Maf A respectively.

Keywords

MicroRNAs, Transcription Factors, Networks, β-Cell Dysfunction

1. Introduction

Chronic pancreatitis (CP) is a progressive inflammatory disorder ultimately culminating in exocrine and endocrine insufficiency contributing to associated maldigestion, abdominal pain and clinical diabetes. Endocrine insufficiency and clinical diabetes are popularly believed to occur as a result of fibrosis and β-cell destruction (apoptosis) in CP [1]. Several investigators demonstrated insulin secretory defects in patients with long standing CP [2]. It is held that insulin secretory defects arise initially as a result of β-cell dysfunction and overt clinical diabetes manifests from β-cell destruction much later in the course of the disease. Such findings suggested a need to study the mechanism of β-cell dysfunction under conditions of chronic inflammation in CP. Diabetes mellitus, secondary to chronic pancreatitis, is now categorized as Type 3C DM [3]. The complex pathophysiology of CP involving secondary diabetes presents distinct clinical and laboratory features, in contrast to autoimmunity and insulin resistance associated respectively with Type 1 and Type 2 DM. Currently it is reported that 5% - 10% in western population and 15% - 20% of diabetics in South East Asia have Type 3C DM [4]. Despite vast knowledge on Type 1 and Type 2 DM, Type 3C diabetes is relatively the less studied form of diabetes. Interestingly, our earlier reports demonstrated reduced islet response to glucose stimulation even in non-diabetic CP patients [5].

MicroRNAs are small, endogenously expressed noncoding RNAs (~21 - 25 nucleotides) known to influence basic cellular functions by regulating gene expression. miRNAs bind to the 3'-untranslated region of mRNAs and lead to either transcriptional repression or mRNA degradation [6] [7]. Altered miRNA profiles have been implicated in different diseases such as diabetes, inflammatory diseases and various malignancies [8] [9] [10]. A few studies have also established basic functional role of miRNAs in islets (hormone secreting cell clusters) including miRNA375 in islet development, miRNA9 in regulating insulin secretory response, miRNA124 in regulating intracellular signaling and miRNA30d in induction of insulin production by targeting MAP4K4 [11] [12] [13] [14]. Further, it was shown that miRNA21, 34a and 146 act as negative regulators of insulin signaling [15]. Increased expression of miRNA29 family in pre-diabetic NOD mice was shown to be associated with cytokine mediated β-cell dysfunction [16].

Islet response to circulating nutrients involves tight coordination and regula-

tion between synthesis and secretion of pancreatic hormones. Expression of insulin gene in response to secretagogues is known to be controlled by various transcription factors such as Pdx1, neuro D, Maf A that bind to enhancer elements in the promoter region of insulin gene [17]. Even though several investigators have reported altered miRNA profiles in CP and pancreatic cancer [18], relatively much less is known about the influence of aberrant miRNA(s) on β-cell function in CP; putative interactions between miRNAs and mRNAs of transcription factors of insulin gene expression have also not been elucidated under inflammatory conditions of CP. The established role of miRNAs in inflammation [9] as well as the reported role of miRNAs in β-cell dysfunction in Type 2 DM [19] and in prediabetic NOD mice [16] have led us to hypothesize that aberrant miRNA expression in CP may also have an effect on insulin gene transcription, which may contribute to β-cell dysfunction.

The present study was thus conducted to (a) evaluate altered pattern of miRNAs in CP and identify their putative targets including genes coding for islet hormones and associated transcription factors; (b) identify association of aberrant miRNAs with signaling pathways involved in insulin gene expression and establish possible network(s) between miRNAs and target genes in CP using bioinformatics approaches.

2. Patients and Methods

Patients: Pancreatic tissues were obtained either from CP patients (n = 16) who had undergone partial pancreatectomy/lateral pancreaticojejunostomy for pain relief or from patients who had undergone pancreaticoduodenectomy for ampullary/periampullary pathology without any history of CP (controls; n = 15), Histological confirmation of CP involved H&E staining of the resected specimens by a single pathologist experienced in pancreatic pathology who was blinded to the patient groups. Patients with CP having pancreatic malignancies, endocrine tumors, acute on CP were excluded. The study was initiated after the protocols were approved by the Institutional Ethics Committee of Asian Institute of Gastroenterology and all the patients had given informed consent.

MicroRNA profiling and validation of dysregulated miRNAs

Total RNA from the pancreatic tissues was extracted and purified using miRvana RNA isolation kit following the manufacturer's instructions (Applied Biosystems/Ambion, Austin, TX). Qualitative and quantitative assessment of total RNA was obtained using Agilent 2100 bioanalyzer as per the prescribed protocol of the manufacturer (Agilent Technologies, USA). Good quality RNA samples with RNA Integrity Number (RIN) > 7 were subjected to microarray analysis for miRNA profiling and to qRT-PCR for validation of identified miRNA. MicroRNA profiling, microarray data analysis and validation with qRT-PCR were performed as described earlier [20]. The raw data were submitted to the ArrayExpress database (accession ID: E -MTAB-3882).

Target gene prediction and pathway analysis of dysregulated miRNAs

Putative target genes for the differentially expressed miRNAs were identified and retrieved from miRWalk 2.0 database [21], which compiles and compares the predictions of other databases. Target genes that were experimentally validated for dysregulated miRNAs were retrieved from Diana Lab Tarbase v.6 [22]. The putative and validated targets of dysregulated miRNAs were subjected to KEGG pathway annotation using DAVID functional annotation tool [23]. A two sided Fisher's exact test and Chi-square test were used to classify the enrichment (Re) of pathway and false discovery rate (FDR) was calculated. A p value of <0.01 and an FDR of <0.05 were chosen for identification of enriched pathways.

IPA analysis to deduce network of altered miRNAs and target genes

Differentially expressed miRNAs and the respective target genes were uploaded into QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City), which uses pooled genes as queries and then retrieves all other gene objects related to query genes/miRNAs stored in knowledge base. Based on the related genes a set of networks were generated with different scores. Networks that are considered to be best fit (implied by the score derived from the p-value) indicate the maximum likelihood of users' list of genes in a network being found.

Evaluation of the network of transcription factors of insulin gene in CP

One μg of total RNA was subjected to cDNA synthesis using oligodT, and M-MuLV Reverse transcriptase enzyme (Invitrogen). qRT-PCR for islet hormones (insulin, glucagon, somatostatin, polypeptide) and transcription factors (Pdx1, NeuroD, MafA, FoxO1) was performed using SYBR Green PCR Master Mix employing Step One Real Time PCR (Applied Bio systems). Primers were designed spanning multiple exons using IDT (Integrated DNA Technologies, Singapore) software available online. All RT-PCRs included no template controls and RT minus controls. For the relative quantification, 10 μL of master mix was prepared with 0.5 μL RT product, 0.2 μL volume each of appropriate forward and reverse primers, 5 μL SYBR green PCR master mix and 4.1 μL RNase free water. Samples were run in duplicates and data were normalized to human GAPDH. mRNA levels were relatively quantified by using ΔΔCT method (List of used Primers is given in **Supplementary Table S1**).

Western blot analysis and immunofluorescent staining for expression of Pdx1, Neuro D and MafA

Proteins were isolated from minced pancreatic tissue using 500 μl of RIPA lysis buffer with protease cocktail inhibitor (Sigma Aldrich, St Louis, USA) and probing the western blots either with rabbit anti-Pdx1 or rabbit anti-NeuroD or rabbit anti-MafA (Invitrogen, Shanghai, China) or anti β-actin (Santa cruz Biotechnology, USA) as per standard protocols [24].

Paraffin embedded pancreatic tissues obtained from CP patients and controls were subjected to immunofluorescent staining. The sections were permeabilized with 0.2% Triton X-100 for 5 minutes at 4˚C and stained with primary antibo-

dies for hormones; insulin and glucagon (guinea pig anti-insulin and mouse anti-glucagon at 1:200; Sigma Aldrich), somatostatin, polypeptide: (Invitrogen, Shanghai, China) and transcription factors (pancreatic duodenal homeobox; PDX-1, (rabbit anti PDX-1, rabbit anti MafA and Mouse Neuro D at 1:100 dilution) followed by overnight incubation at 4˚C. After repeated washings with PBS, sections were treated with secondary antibodies tagged with fluorescent dyes guinea pig Alexa 488 for insulin and goat anti mouse Alexa 546 for glucagon at 1:200 dilutions; Invitrogen, Shanghai, China (goat anti rabbit Alexa 546 for PDX-1 and goat anti guinea pig Alexa 488 for insulin; Invitrogen, China, donkey anti goat Alexa 546 for Neuro D).Fluorescence images were captured using a Bioimager (CARV II, BD BioSciences, CA, USA).

Statistical analysis: The data were analyzed using statistical package for social sciences (SPSS version 20.0, Chicago, USA). A two tailed students P value of < 0.05 was considered statistically significant.

3. Results

Clinical characteristics of patients

A total of 16 patients with CP (male n = 12, mean age 34.44 ± 15.85 years), as confirmed by clinical, endoscopic and imaging (CT, MRI, EUS and ERCP) investigations were recruited for the study. All of the CP patients had abdominal pain for 36 ± 21 months with radiological findings revealing the presence of intraductal calculi and main duct dilatation. Histopathological examination confirmed intra and interlobular acinar atrophy and fibrosis in these patients. The control group (n = 15; male = 10, mean age 50.53 ± 9.53 years) were ascertained to be without CP.

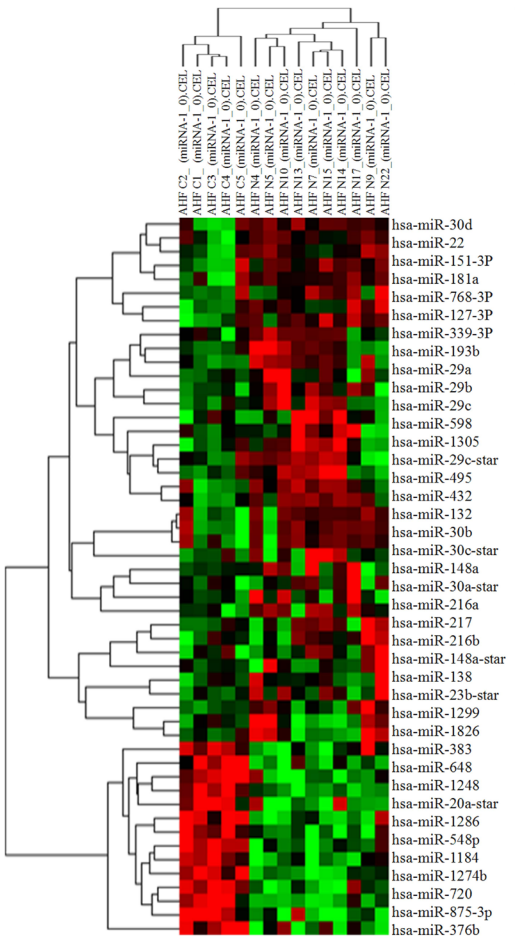
Microarray experiments demonstrated differential expression profiles of miRNA in CP

Significance analysis of Microarray (SAM) identified 52 differentially expressed miRNAs in CP tissues as compared to control tissues (**Supplementary Table S2**). Hierarchical clustering of the normalized expression data showed two distinct clusters of miRNAs (**Figure 1(a)**). Further refinement with t-test and Bonferroni adjustment revealed a total of 25 differentially expressed miRNAs that includes 10 up-regulated and 15 down-regulated miRNAs with high significance ($P < 0.05$). The differentially expressed miRNAs detected by highest logarithmic fold changes between CP and adjacent control tissues are shown in **Figure 1(a)**. Among the up-regulated ones, miR-138-1 (3.12 fold change) and among the down regulated miRNA-217, miR-216a (−4.85, −4.21) were highly significant (**Figure 1(b)**).

qRT-PCR analysis corroborated microarray miRNA expression profiles in CP

Microarray data identifying the three up-regulated miRNAs (miR-138, miR-767,

(a)



(b)

Mature miRNA	Score	Log 2 Fold changes	Accession No	Chr Location	miRNA Family	miRNA cluster
has-miR-138-1-3p	4.22	3.12	MIMAT0004607	3p21.32	miR-138	
has-miR -376b-5p	3.96	2.84	MIMAT0022923	14q32.31	miR-368	
has-miR-1285-5p	3.87	2.79	MIMAT0022719	7q21-q22	miR-1285	
has-miR-615-3p	3.73	2.68	MIMAT0003283	12q13.13	miR-615	
has-miR-767-3p	3.44	2.62	MIMAT0003883	Xq28	miR-767	has-miR-105-1
has-let-7g-5p	3.36	2.57	MIMAT0000414	3p21.2	let-7g	
has-miR-942-3p	3.35	2.55	MIMAT0026734	1p13.1	miR-942	
has-miR-767-5p	3.32	2.51	MIMAT0003882	Xq28	miR767	has-miR-105-2
has-miR-518b	3.13	2.3	MIMAT0002844	19q13.42	miR-515	has-miR-526b,519b,525,523,518f,520c,524
has-miR-516b-5p	2.97	2.12	MIMAT0002859	19q13.42	miR-515	has-miR-526a-2,518e,518a,518d,520h,517
has-miR-217	-4.85	-4.64	MIMAT0000274	2p16.1	miR217	has-miR-216a
has-miR-216a	-4.21	-3.84	MIMAT0000273	2p16.1	miR216	has-miR-217
has-miR-375	-4.08	-3.64	MIMAT0000728	2q35	miR-375	
has-miR-216b	-3.82	-3.32	MIMAT0004959	2p16.1	miR216	
has-miR-148a-5p	-3.78	-3.18	MIMAT0004549	7p15.2	miR-148	
has-miR-22-3p	-3.12	-2.74	MIMAT0000077	17p13.3	miR-22	
has-miR-30a-5p	-3.03	-2.56	MIMAT0000087	6q13	miR-30	
has-miR-200c-3p	-2.98	-2.47	MIMAT0000617	12p13.31	miR-8	miR-141
has-miR-30d-5p	-2.89	-2.40	MIMAT0000245	8q24.22	miR-30	miR-30b
has-miR-200b-3p	-2.73	-2.32	MIMAT0000318	1p36.33	miR-8	has-miR-200a,429
has-miR-151-3p	-2.65	-2.25	MIMAT0000757	8q24.3	miR-28	
has-miR-130b-3p	-2.64	-2.25	MIMAT0000691	22p21.6	miR-130	has-miR-301
has-miR-29c-5c	-2.52	-2.18	MIMAT0004673	1q32.2	miR-29	has-miR-29b2
has-miR-29a-3p	-2.26	-2.06	MIMAT0000086	7q32.3	miR-29	has-miR-29b1
has-miR-27b-3p	-2.21	-2.02	MIMAT0000419	9q22.32	miR-27	has-miR-23b,3074,24-1

(c)

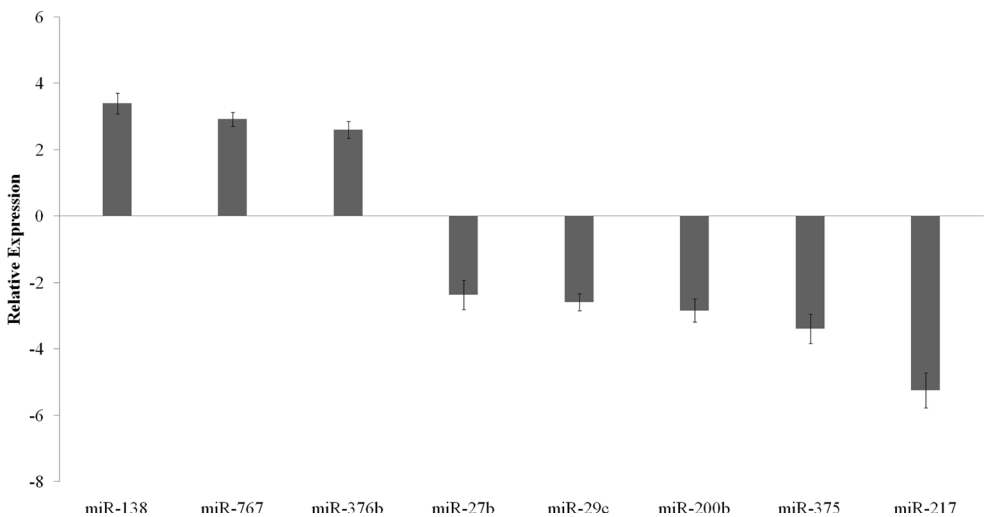


Figure 1. Differentially expressed miRNAs in CP tissues *vs.* normal pancreatic tissues. (a) Clustered heat maps illustrate miRNA differential expression profiles across 5 CP samples and 10 controls; (b) 25 miRNAs significantly ($P \leq 0.05$) differentially regulated (fold change ± ≥2) in CP patients compared with normal pancreatic tissues are listed in order of their fold change. The accession number is for the mature miRNA sequence; Chr, Chromosome; (c) Quantitative real-time PCR analysis of dysregulated miRNA expression in CP (n = 16) compared with normal pancreatic tissues (n = 15). The miRNA abundance was normalized with U6SnRNA as internal control. Relative expression was analyzed by $2^{\wedge -\Delta\Delta CT}$ method.

miR-376b) and five down-regulated miRNAs (miR-217, miR-200b, miR-27b, miR-29c and miR-375) miRNAs was validated by qRT-PCR. The results were in accordance with the microarray data indicating differential expression of these miRNAs in CP ($P < 0.05$). Of the seven miRNAs, differential expression of miR-138, miR-767, miR-375 and miR-217 was significant ($P < 0.001$). The relative expression of differentially expressed miRNAs (log2 folds) is shown in Figure 1(c).

Target gene prediction of aberrant miRNA revealed fibrosis and insulin signaling pathways in CP

In silico analysis was performed to predict target mRNAs of the 25 differentially expressed miRNAs. The number of mRNA targets predicted for each of the differentially expressed miRNAs varied substantially. A three-way intersection of the three algorithms (Targetscan, miR Walk and miRNaDa) revealed 9577 target transcripts corresponding to 8399 unique official gene symbols. Functional annotations of target genes using DAVID identified 12/53 significant pathways of which TGFβ, T cell receptor and cytokine-cytokine receptor interaction pathways were related to inflammation and fibrosis. Similarly, pathways related to insulin synthesis, calcium signaling and Type2DM could be related to β cell specific pathways as shown in the Table 1.

KEGG, ingenuity pathway analysis and putative networks in CP

Fibrosis, inflammation, insulin signaling pathways and malignancy were found to be enriched on pathway analysis utilizing KEGG database. IPA analysis of 25 dysregulated miRNAs and respective target genes (predicted /validated) generated ten functional networks. Four of these networks that are relevant to CP include those involved in (i) inflammatory disease, fibrosis and cancer (ii) cytokine signaling, insulin synthesis and β cell function (iii) inflammatory response and cell morphology and (iv) immunological disease and cell cycle. Since CP is an inflammatory disease wherein islets have to function in an inflammatory milieu, we conducted further analysis relating miRNA expression to cytokine signaling and insulin gene transcription. Consequently, the final network generated involved cytokines, transcription factors and miRNA. It depicts insulin gene transcription to be coordinately regulated by the three crucial transcription factors (Pdx1, NeuroD, MafA) and FoxO1 which are the confirmed targets of mir19, 130b, 30 family for Neuro D and miR 27 family for FoxO1 respectively. None of the altered miRNA targeted Pdx1 directly. This network also identifies a putative link between miR 200 family and MafA. Inflammatory cytokines (IL1 family, IL10, IFNγ, IL6) were found to be targets of differentially expressed miRNAs 191, 27, 29 and 191 respectively as reflected in the network (Figure 2). Validated targets of networks are shown in Supplementary Table S4.

Glucose responsive MafA expression increased and Pdx1, Neuro D expression decreased in CP

mRNA levels of the transcription factors of insulin gene were noted to be al-

Table 1. Putative targets and pathways that are associated with dysregulated microRNAs in chronic pancreatitis.

Term	Count	P Value	Genes	Bonferroni	Benjamini	FDR
hsa05200: Pathways in cancer	209	5.31E−07	STAT5A, STAT5B, CTNNB1, CUL2, RARA, RARB, FAS, CCNA1, WNT10A, PLD1, WNT10B, BCR, RXRA, VEGFA	1.05E−04	1.05E−04	6.64E−04
hsa04910: Insulin signaling pathway	91	7.26E−05	PHKB, PRKAG2, PDE3B, FOXO1, AKT1, PDPK1, SHC1, PRKACA, INSR, IRS4, IRS2, SOCS2, MAPK3, MAPK9	0.01	0.0047	0.020
hsa04020: Calcium signaling pathway	114	1.08E−04	SLC8A3, ADCY1, TNNC1, ITPKA, PRKX, ATP2B1, GRIN2D, PRKACA, EGFR, HTR4, GRIN2A, CHP2, MYLK2, CAMK4, CACNA1I, GRIN1, TACR3, PHKB, EDNRA, EDNRB, PLCB4, PLCB1, PLCB2, HTR5A, PRKCA, PTGER3	0.02	0.005	0.013
hsa04510: Focal adhesion	127	2.10E−04	PDGFA, CHAD, VCL, CTNNB1, ILK, CDC42P2, PDGFC, PDGFD, RAPGEF1, EGFR, ROCK1, ACTN4, ROCK2, PIK3CD, ACTN1, MYLK2, PPP1CB, VEGFB, MAPK1, LAMC3, VEGFA, MAPK3, COL1A2, PDGFRA, MAPK9	0.04	0.008	0.026
hsa04520: Adherens junction	55	2.98E−04	WASF3, LOC100271831, WASF1, WASF2, LMO7, IQGAP1, CTNNB1, VCL, ACVR1C, MAP3K7, CDC42, ACVR1B, CSNK2A1, CDC42P2, INSR, PTPRJ, EGFR, PTPRM, PTPRF, ACTN4, BAIAP2, LEF1, ACTN1, CTNNA1, FARP2, MAPK1, EP300, MAPK3, WASL, FGFR1, ERBB2, CTNND1, CDH1, ACP1, TCF7L1, SRC, PVRL1, SORBS1, PVRL2, RAC1, SSX2IP, YES1, PTPRB, ACTB, TCF7, TGFBR1, NLK, MET, CREBBP, TGFBR2, SMAD4, SMAD2, SNAI2, CSNK2A1P, SNAI1, TJP1, FYN, PTPN1	0.057	0.009	0.037
hsa04310: Wnt signaling pathway	98	3.19E−04	PPP2R5B, BTRC, PRKX, CTNNB1, WNT2, MAP3K7, WNT4, CSNK2A1, PRKACA, PRKACB, WNT6, WNT10A, WNT10B, VANGL1, ROCK1, ROCK2, VANGL2, CHP2, SKP1, DVL1L1, EP300, MAPK9, WNT5A, WNT5B	0.061	0.008	0.039
hsa04722: Neurotrophin signaling pathway	82	4.52E−04	FASLG, FOXO3, LOC442113, AKT1, LOC440917, CDC42, MAP3K5, GAB1, CDC42P2, NGFRAP1, SHC1, CSK, FOXO3B, RAPGEF1, FRS2, MAP2K7, AKT3, SHC4, AKT2, MAP2K5, IRAK2, IRS4, IRAK1, IRS2, PIK3CD, TP53	0.085	0.01	0.045
hsa04350: TGF-beta signaling pathway	60	6.47E−04	NOG, E2F4, ACVRL1, E2F5, LOC100271831, GDF5, TGFB3, RPS6KB2, RPS6KB1, ACVR1C, ACVR1B, CDKN2B, ZFYVE9, ZFYVE16, LOC643778, IFNG, MYC, PITX2, PPP2R1B, ROCK1, RBL2, ROCK2, SKP1, INHBB, MAPK1, INHBA, ACVR2A, ACVR2B, EP300, MAPK3, INHBC, ACVR1, BMPR2, DCN, PPP2CA, THBS1, THBS2, TFDP1, BMP2, SMAD9, SMAD7, SMAD6, TGFBR1, CREBBP, TGFBR2, SMAD4, SMAD2, SMAD1, SP1, ID2, ROCK1P1, ID1, SMURF2, ID4, SMURF1, ID3, BMPR1B, BMP7, CHRD, BMP8B, BMP6, BMPR1A, BMP8A	0.12	0.01	0.048

Continued

hsa04010: MAPK signaling pathway	158	0.002587266	MEF2C, FGF19, PDGFB, PDGFA, GNA12, FGF11, TGFB3, PRKX, PRKACG, MAP3K7, MAP3K6, MAP3K5, LOC100132771, MAP3K8, CDC42P2, PRKACA, FAS, PRKACB, RAPGEF2, MAP2K7, IL1A, MAP2K6, MAP2K5, EGFR, CHP2, PTPRR, FGF23, STK4, DDIT3, STK3, MAP4K3, MAP4K4, MAPK1, ARRB1	0.40	0.02	0.031
hsa04930: Type II diabetes mellitus	34	0.004333089	HK2, PDX1, KCNJ11, LOC652797, HK2P1, SLC2A4, SLC2A2, HK3, PIK3R5, IRS4, IRS2, SOCS2, SOCS3, SOCS1, PIK3CD, LOC100131098, SOCS4, MAPK10, PRKCE, ADIPOQ, IRS1, MAPK1, GCK, PKM2, PKLR, MAPK3, CACNA1G, MAPK9, CACNA1E, MafA, IKBKB, CACNA1C, CACNA1D	0.57	0.036	0.045
hsa04660: T cell receptor signaling pathway	68	0.008734447	CD8A, CD8B, PDCD1, IL10, MAP3K7, AKT1, PAK6, CDC42, FOS, IFNG, CDC42P2, PAK1, AKT3, AKT2, BCL10, CHP2, CDK4, CARD11, MAPK1, PRKCQ, NCK2, NCK1, MAPK3, NFKBIE, GRB2, KRAS, RASGRP1, SOS1, ICOS, ZAP70, NFAT5, CD4	0.82	0.067	0.039
hsa04060: Cytokine-cytokine receptor interaction	151	0.01225817	PDGFB, IL6ST, PDGFA, IL18, GDF5, TNFSF15, TGFB3, IL15, CXCL11, CXCL12, TNFSF18, IL10, CXCL10, IL11, ZFP91, IFNG, CCR10, IL15RA, CSF3R, PDGFC, FAS, IFNK, IL1A, EGFR, LIFR, IL25, IL26, TNFRSF14	0.913023416	0.086	0.030

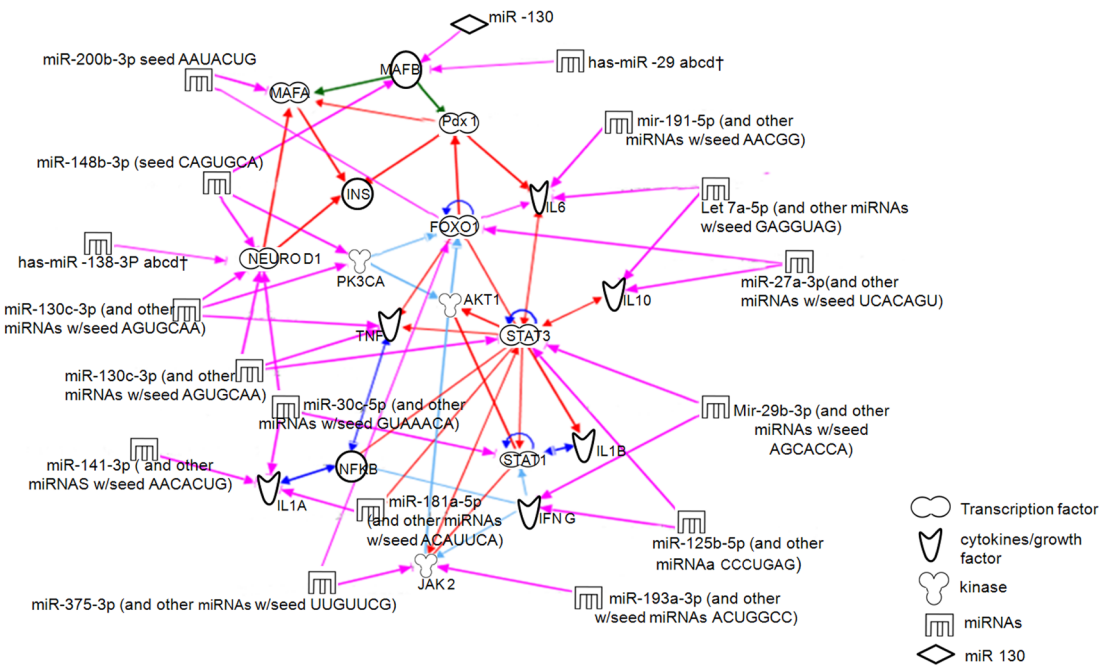


Figure 2. Functional network analysis of differentially expressed miRNA targets in CP. Curated network generated involving cytokines, transcription factors, insulin and miRNA depicts, crucial transcription factors (Pdx1, NeuroD, MafA) that co-ordinately regulates insulin gene transcription and FoxO1.These are confirmed targets of mir19, 130b, 30 family; miR 27 family for neuroD and FoxO1 respectively.

tered in CP. While the expression of Pdx1 and neuro D decreased by 5.02, 2.96 fold, that of MafA increased by more than 2.41 folds in CP. On the other hand FoxO1, a transcription factor known to be increased under conditions of oxidative stress, showed two fold increase in CP in comparison to controls. Similarly expression of genes coding for islet hormones namely insulin, glucagon, somatostatin, and pancreatic polypeptide (PP-1) indicated 4.3, 2.8, 4.4 and 1.9 log2 fold decrease respectively in CP patients as compared to control tissues (**Figure 3(a)**). Results obtained with qRT-PCR were akin to those obtained with western blot analysis, indicating increased MafA (~3 times) and decreased NeuroD and Pdx1 in CP as compared to controls (**Figure 3(b)**). These observations were validated upon immunofluorescence staining of the paraffin embedded sections for insulin, glucagon, somatostatin, NeuroD and Pdx1 (**Figure 3(c)**).

Differentially expressed miRNA negatively correlated with NeuroD, MafA and FoxO1 expression in CP

The IPA network indicated three putative links between differentially expressed miRNAs and β-cell specific transcription factors: miR-200b (−2.75) and MafA (2.41); miR-138 (2.76) and Neuro D (−2.965); and miR-27b (−2.24) and FoxO1 (2.21). miRNA targets of the transcription factors were confirmed by matching the seed sequences of miRNAs and genes coding for β cell specific transcription factors as shown below. Pearson correlation analysis revealed a significant ($P < 0.05$) negative correlation between mRNA levels with miRNA levels; NeuroD and miR-138 ($r = -0.617$, $P = 0.012$), MafA and miR-200b ($r = -0.597$, $P = 0.015$), FoxO1 and miR-27b ($r = -0.571$, $P = 0.021$) (**Figure 4**).

4. Discussion

Micro RNAs are known to play a pivotal role in controlling basic cellular functions by regulating gene expressions. We conducted this study to explore whether there is any dysregulation of pancreatic miRNAs under the inflammatory conditions prevalent in CP and to identify putative links between differentially expressed miRNAs and transcription factors that coordinately regulate insulin gene expression contributing to secondary diabetes occurring in the disease. Our results demonstrate a network of three miRNAs (miR200b, 138 and 27b) regulating insulin gene transcription factors along with cytokines in CP.

The dysregulated profiles of miRNA obtained on microarray and validated on qRT-PCR indicate that inflammation in CP is associated with altered miRNA expression. The observed expression profiles of dysregulated miRNA in the present studies were similar to earlier studies which examined miRNA patterns in the progression of chronic pancreatitis to pancreatic ductal adenocarcinoma [18] which reported up-regulation of miR-339 and down-regulation of miR-34, miR-181, miR-375, miR125b and miR-99. Further, the down regulation patterns (Supplementary **Table S3**) were akin to those reported in a study comparing the abundance of circulating and tissue miRNAs in CP [25]. Subtle differences

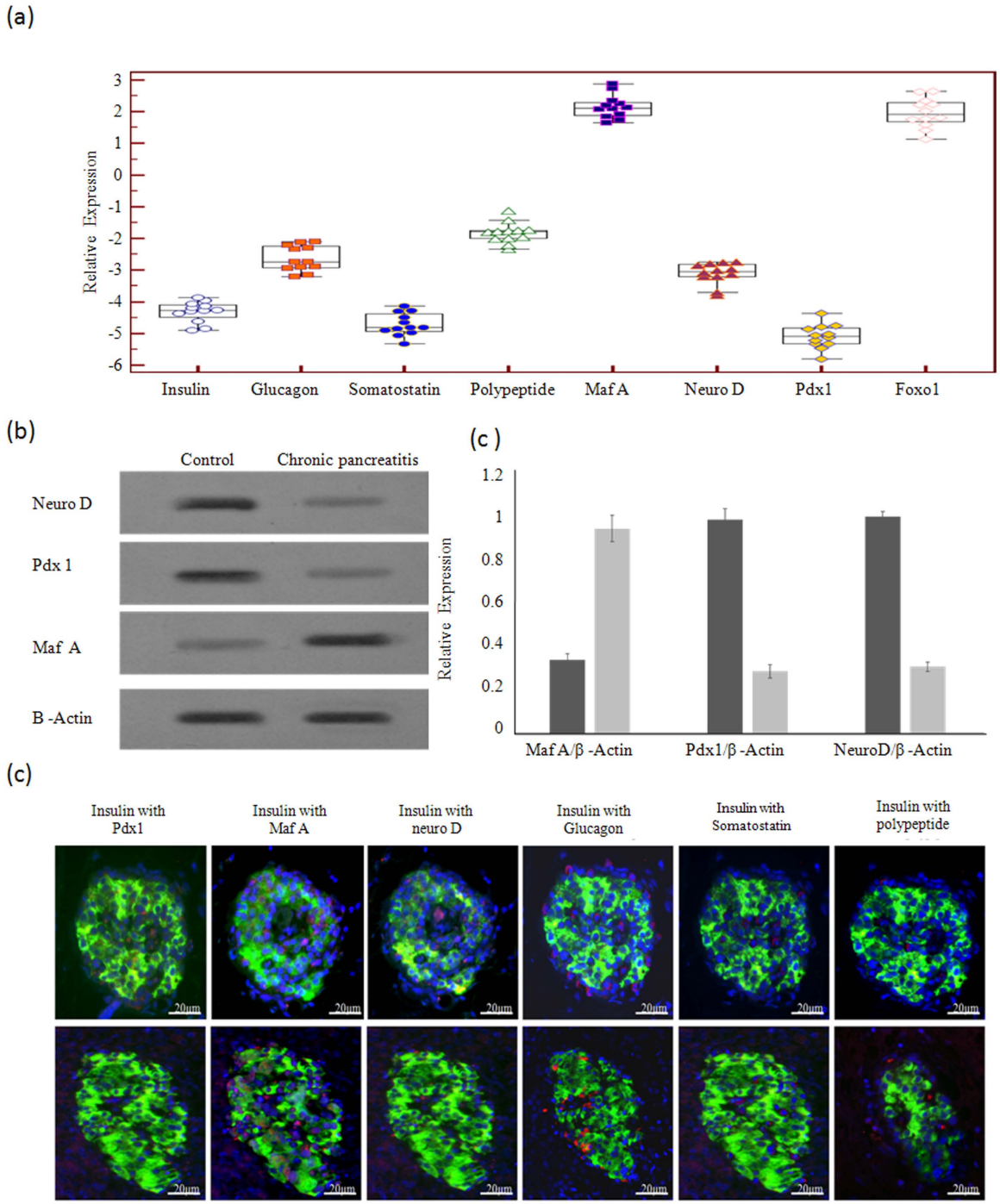
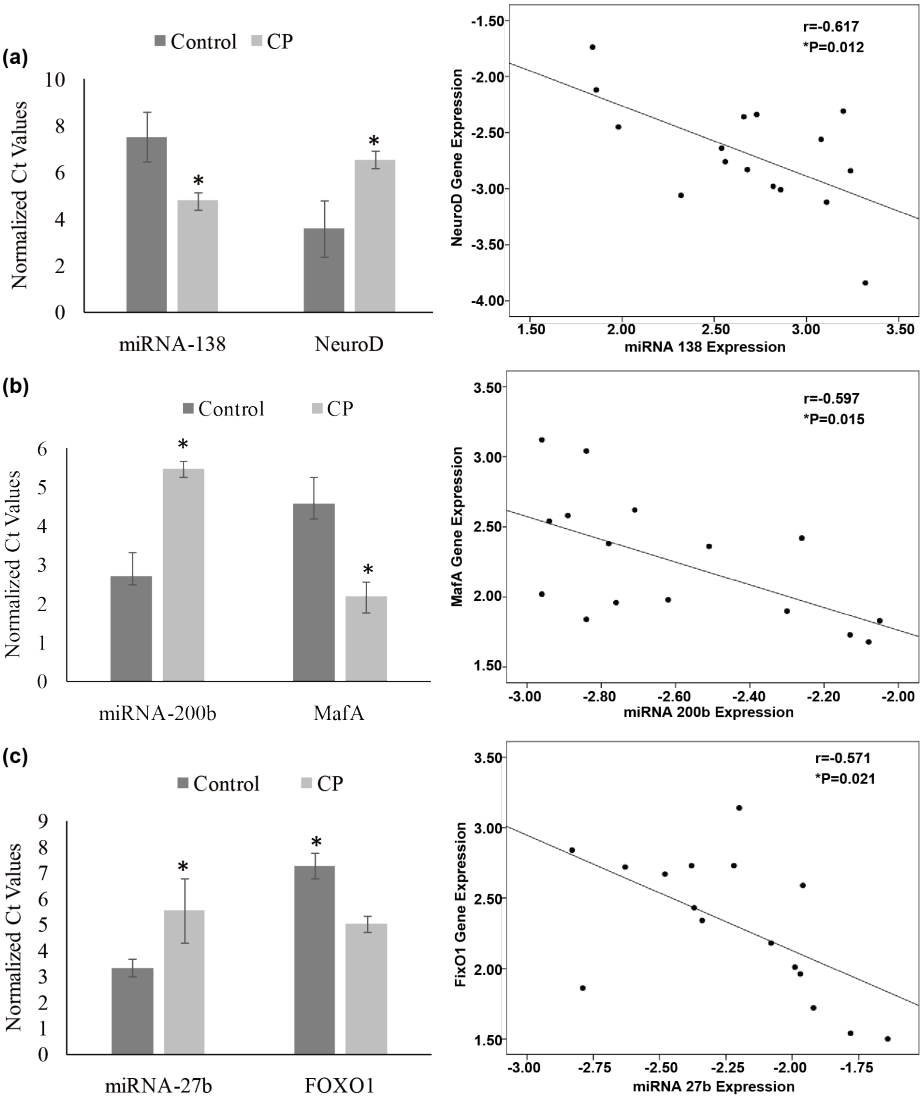


Figure 3. Islet Hormones and transcription factors in CP. (a) Altered expression patterns of islet hormones and transcription factors in CP tissues identified by qRT-PCR. The mRNA transcript abundance was normalized to that of GAPDH. Relative expression was analyzed by $2^{\wedge -\Delta\Delta CT}$ method; (b) Protein expression of β-cell specific transcription factors (NeuroD, PDX1 and MafA) in CP by western blot normalized to β-Actin as reference control. The bar graph represents the expression levels as ratio of target protein expression with β-actin; (c) Immunofluorescence staining of islets in CP compared to control tissues confirms altered expressions of islet hormones and transcription factors. Islets from control group and CP patients were stained with fluorescent antibodies to visualize insulin (green), PDX-1, MafA, NeuroD, Glucagon, Somatostatin, Polypeptide (red), and nuclei (blue). All images were obtained at original magnification (400×).



(d)

```
hsa-miR-138      3' ccggacuaaguguug UGGUCGa 5'
                                    | | | | | |
NEUROD1          5'aaauauauauuuuucaACCAGCa 3'

hsa-miR-200bc    3' aguaguaaugguccGUCAUAAu
                                  | | | | | | |
MAF A            5' gaaaaaguaaugcaCAGUAUUu

hsa-miR-27b/27a  3' cgucuuGAAUCG -- GUGACACUu 5'
                       : | |   | :     : | | | | | | |
FOXO1            5' acucuuUUUUGUAAUACUGUGAu 3'
```

Figure 4. Inverse and negative correlation between miRNA and transcription factors. (a) Pearson correlation analysis showed negative correlation of miRNA-target gene pairs (a) miR-138 with NeuroD ($r = -0.617$, $P = 0.012$); (b) miR-200b with MafA ($r = -0.597$, $P = 0.015$); (c) miR-27b with FoxO1 ($r = -0.571$ $P = 0.021$). In Graphs aligned towards left vertical axis indicates delta Ct values and are opposite to actual expression values obtained after normalizing with reference internal control genes (U6SnRNA for miRNAs and GAPDH for mRNAs). In Graphs aligned towards right r represents Pearson correlation coefficient; (d) Sequence matching of the predicted binding sites shows miR-138 binds to the 3' UTR region of NeuroD1, miR-200b/200c binds to MafA and miR-27b/27a binds to FoxO1 mRNA. The miRNA seed sequences are highlighted in red and sequences complementary to seed region in 3'UTRs of mRNA are highlighted in green. NeuroD1, Neurogenic differentiation1; MafA, Musculoaponeurotic Fibrosarcoma Oncogene Homolog A; FoxO1, Forkhead box protein O1.

between these two studies in miRNA expressions may be ascribed to differences in etiopathogenesis of CP (largely alcoholic in Western countries and idiopathic in tropical countries). Although these studies focused on miRNA expression patterns during progression of CP to pancreatic cancer, no study has been conducted to find an association between dysregulated miRNA and transcription factors involved in regulating insulin gene expression in CP. Such an identification of networks relating aberrant pancreatic miRNA expression to insulin gene transcription is of relevance in unraveling the mechanism of endocrine dysfunction in CP.

Target gene prediction analysis of differentially expressed miRNAs using bioinformatics approaches (miRbase, Target scan and miRwalk) yielded several transcripts (8399) related to various signaling pathways with ≈200 overlapping targets f or each miRNA (**Figure S1**). It is known that paired miRNA genes present within 10 kb distance (usually named as miRNA clusters) are usually co-expressed and have functionally significant role in repressing the target genes [26]. Among the 25 differentially expressed miRNA, a few clusters of miRNA (miR-29a/29b/29c, miR-200a/200b/200c/141, miR-216/217, miR-30a/30b/30c, miR-27a/27b/24/23b) were noted to be associated with target genes of specific pathways involved in fibrosis, inflammation, epithelial-mesenchymal transition (EMT), oxidative stress and dysregulated insulin signaling. Multiple target genes of miR-29 cluster that include collagens (COL7A1), fibrillins (FBN1), and matrix metalloproteins (MMPs) identified in this study were earlier implicated in cardiac, pulmonary and renal fibrosis [27] [28] [29]. It is also known that transforming growth factor (TGF)-β-mediated down-regulation of miR-29 would enhance fibrosis and role of TGF-β in fibrogenesis during progression of CP is well established [30]. In addition, Wang *et al.* showed that the miR-200 cluster (mir200 a, b and mir141) contributes directly to fibrosis by regulating of TGF-β2 [31]. Reporter gene assays have also confirmed TGF-β2 to be the direct target of miR-141/miR-200a [32]. Our results are in agreement with these studies suggesting that down regulation of miR200 and miR29 may be contributing to fibrosis in CP [33]. Decreased expression of miR-216/217 cluster which has been shown to be involved in EMT also suggests its role in fibrogenesis [34]. Decreased expression of miRNA-27 cluster is known to target PPARγ ligand, which modulates NF-κB dependent pro-inflammatory cytokine production and pancreatic stellate cell activation [35]; this in turn would contribute to increased oxidative stress and inflammation in CP. It is noteworthy that evidences are increasingly indicating that prolonged oxidative stress may be a contributing factor for pathogenesis and progression of CP [36]. Results of the present study as well as an earlier report by our group [37] also denote down-regulation of miR-30 family in CP; the miR-30 family is implicated in induction of insulin synthesis by targeting MAP4K4 regulation [38] [39] [40] these observations indicate that miRNA clusters might play a crucial role in inflammation, fibrosis

and aberrant β-cell functions associated with CP.

Although comprehensive search was conducted for target gene prediction, networks were generated restricting the analysis to target genes that are associated with oxidative stress, cytokine signaling, Inflammation and insulin signaling since they are highly relevant to the progression of CP and manifestation type 3CDM. An attempt was also made to understand the intricate relations between the dysregulated miRNAs, target genes, and β-cell dysfunction in CP employing Ingenuity Pathway Analysis. A curated network was generated involving miRNAs, target genes of cytokine signaling, oxidative stress, insulin synthesis, β cell function. Interestingly, the resultant network revealed a web consisting of various proinflammatory cytokines (IL6, IFNγ, IL1β, and TNFα), JAK2, NFκB signaling pathways, statins (STAT1 and STAT3), effectors of β-cell function (MafA, NeuroD and Pdx1), β-cell development (MafB), β cell oxidative stress protective transcription factor (FoxO1) and insulin. Earlier results from our laboratory and that of others have shown increased expression of proinflammatory cytokines [41] in CP and a role for NFκB [42] and oxidative stress was implicated in the inflammation in CP [43]. The network established in the present study has also identified links between cytokines and miRNAs (IL6 -miR191; IL10-miR27; IFNγ miR125b and 29C; IL1 miR141; TNFα miR 130 and miR19b) involving putative links between miR27-IL10 [44], miR29-IFNγ [45], TNFα-miR130 [46], that were previously established. In the network, NFκB and STAT proteins have network links with IFNγ, IL1A, IL1B TNFα, IL6 and IL10), while transcription factors and miRNAs (NeuroD-miR138, 19b, 130a, 30c, 148b; MafA-200b; MafB-miR130b, miR29; FoxO1-miR27a; STAT3-miR29b, 130a; STAT1-miR30c) were associated. Interestingly, none of the differentially expressed miRNA targeted Pdx1 (crucial β cell transcription factor). Results of this study established possible links between miRNAs, cytokines, oxidative stress and transcription factors of insulin gene and β-cell functions in CP.

Further analysis using target scan, matching the sequences of differentially expressed miRNAs with the target genes identified sequence complementarities between transcription factors of insulin gene and miRNAs. Amongst the 25 differentially expressed miRNA, three miRNAs 200b, 138-1 and 27b were identified to have binding sites in 3'UTRs of MafA, NeuroD and FoxO1 respectively. In addition to this, expression levels of these miRNAs were negatively correlated with Maf A, Neuro D and FoxO1, suggesting a role for these miRNA in regulating the expression of the transcription factors of insulin gene. Decreased mRNA levels of Pdx1, Neuro D, and increased expression of Maf A were confirmed upon probing the proteins on western blot analysis. Although previous studies have demonstrated differential expression of 200b, 27b, 138-1, their role in β cell function were not studied under conditions of inflammation. *In vitro* assays by earlier investigators demonstrated that FoxO1 inhibits Pdx1 transcription in β cell cultures by competing with FoxA2 (transcription factor) for a common

binding site in the Pdx1 promoter [47]. FoxO1 and Pdx1 have also been reported to show mutually exclusive nuclear localization [48] [49] [50]. Increased oxidative stress, arising from inflammation and decreased miR 27b could result in raised FoxO1 and diminished Pdx1 expression in CP as observed in this study could be due to raised FoxO1. Transgenic mice with FoxO1 gain of function in the pancreas exhibited glucose intolerance [51]. Conversely, FoxO1 ablation in β-cells resulted in increased insulin secretion [52]. Further, *in vitro* studies showed suppression of miRNA-27 resulted in increased FoxO1 protein levels and a consequent decrease in cell number [53]. Hence we believe that increased FoxO1 resulting from increased oxidative stress leads to dysregulation in β cell functions.

Insulin gene transcription is known to be coordinately regulated by transcriptional activators such as Neuro D, Maf A and Pdx1 [54] [55] [56] [57]. To our knowledge, this is the first report to show increased expression of Maf A in CP both at mRNA and protein levels. We had earlier shown that reduced expression of Pdx1 is associated with decreased β-cell function [5]. It is well established that Pdx1, Neuro D and Maf A bind to the insulin promoter to initiate insulin gene transcription. Even though FoxO1 is known to activate gene expression (Maf A) in response to oxidative stress via acetylation [58], decrease in Pdx1 and Neuro D in CP might result in loss of coordinated transcription of insulin gene resulting in decreased β cell function. Despite acetylated FoxO1 acting as a protective factor to β cells in response to oxidative stress under acute metabolic change, it fails to preserve β cell function in chronic conditions [58].

5. Conclusion

In summary, present study identified dysregulated miRNAs and indicated the pathways they target in CP. Dysregulated miRNA target genes are significantly enriched in pathways related to fibrosis and EMT (miR-29, 200, 217 miRNA clusters), inflammation/cytokines (miR-27 family) and insulin signaling pathways (miR-30 family and miR-375). Putative links were also identified upon bioinformatic analysis between miRNAs (miR-138, 200b, 27a/27b) and transcription factors of insulin gene (Neuro D, Maf A, and FoxO1) suggesting that miRNAs, such as miR138-1, 27b and 200b, might be important regulators of crucial transcription factors involved in insulin gene expression in CP.

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Supplementary Tables and Figures

Table S1. Primers list.

Gene	Forward primer	Reverse primer	Product Size	Annealing Temp
Insulin	CTAGTGTGCGGGGAACG	CACGCTTCTGCAGGGAC	148 bp	58
Somatostatin	ACTCCGTCAGTTTCTGCAG	CTGGGACAGATCTTCAGGTTC	136 bp	58.5
Glucagon	CATTGCTTGGCTGGTGAAAG	GCGGCAAGATTATCAAGAATGG	135 bp	58
Polypeptide	GTATGCAGCTGATCTCCGTAG	GGCATTATAAGTCCAGCGGG	149 bp	57
MafA	GAGAAGTGCCAACTCCAGAG	GCCAGCTTCTCGTATTTCTCC	101 bp	60
Pdx-1	TGAAGTCTACCAAAGCTCACG	GGAACTCCTTCTCCAGCTCTA	132 bp	58
NeuroD	CCAGGGTTATGAGACTATCACTG	TCCTGAGAACTGAGACACTCG	149 bp	60
FoxO1	GCAATGGCTATGGCAGAATG	AGTGTAACCTGCTCACTAACC	240 bp	57.5
GAPDH	AATCCCATCACCATCTTCCAG	AAATGAGCCCCAGCCTTC	122 bp	60

Table S2. Differentially expressed miRNAs identified by SAM (52). (a) Up regulated (14); (b) Down regulated (38).

(a)

Mature miRNA	Score(d)	Fold Change (Log2)	Chromosomal Location
hsa-miR-138-1-3p	4.22	3.12	Chr3 NC_000003.12
hsa-miR-376b	3.96	2.84	Chr14 NC_000014.9
hsa-miR-1285	3.87	2.79	Chr7 NC_000007.14
hsa-miR-615-3p	3.73	2.68	Chr12 NC_000012.12
hsa-miR-767-3p	3.44	2.62	ChrX NC_000023.11
hsa-let-7g-5p	3.36	2.57	Chr3 NC_000003.12
hsa-miR-942	3.35	2.55	Chr1 NC_000001.11
hsa-miR-767-5p	3.32	2.51	ChrX NC_000023.11
hsa-miR-518b	3.13	2.3	Chr19 NC_000019.10
hsa-miR-516b	2.97	2.12	Chr19 NC_000019.10
hsa-miR-519b-5p	2.92	1.86	Chr19 NC_000019.10
hsa-miR-1233	2.82	1.84	Chr15 NC_000015.10
hsa-miR-516b*	2.71	1.83	Chr19 NC_000019.10
hsa-miR-875-3p	2.71	1.82	Chr8 NC_000008.11

(b)

Mature miRNA	Score(d)	Log2 Fold Change	Chromosomal Location
hsa-miR-217	−3.35	−4.64	2p16.1
hsa-miR-216a	−3.32	−3.84	2p16.1
hsa-miR-375	−3.48	−3.64	2q35
hsa-miR-216b	−2.92	−3.32	2p16.1
hsa-miR-148a	−3.36	−3.18	7p15.2
hsa-miR-22-3p	−3.87	−2.74	17p13.3
hsa-miR-30a-5p	−3.47	−2.56	6q13
hsa-miR-200c-3p	−2.36	−2.47	12p13.31
hsa-miR-30d-5p	−4.57	−2.40	8q24.22
hsa-miR-200b-3p	−2.16	−2.32	1p36.33
hsa-miR-151-3p	−3.96	−2.25	8q24.3
hsa-miR-130b-3p	−2.67	−2.25	22q11.21
hsa-miR-29c-5p	−3.44	−2.18	1q32.2
hsa-miR-29a-3p	−2.82	−2.06	7q32.3
hsa-miR-27b-3p	−3.3	−2.06	9q22.32
hsa-miR-19b-3p	−2.12	−1.79	13q31.3
hsa-miR-191-5p	−2.56	−1.74	3p21.31
hsa-miR-152	−2.97	−1.56	17q21.32
hsa-miR-339-3p	−3.73	−1.51	7p22.3
hsa-miR-181a-5p	−3.66	−1.47	9q33.3
hsa-miR-99a-5p	−2.29	−1.47	21q21.1
hsa-miR-125b-5p	−2.19	−1.43	11q24.1
hsa-miR-141-3p	−2.26	−1.40	12p13.31
hsa-miR-30a-3p	−3.99	−1.36	6q13
hsa-miR-193b-3p	−4.48	−1.32	16p13.2
hsa-miR-34a-5p	−2.16	−1.32	1p36.33
hsa-miR-28-3p	−2.71	−1.22	3q28
hsa-miR-127-3p	−2.4	−1.22	14q32.2
hsa-miR-29b-3p	−2.44	−1.09	7q32.3
hsa-miR-1184	−2.6	−1.06	Xq28
hsa-miR-181b-5p	−2.29	−1.06	19q32.1

Table S3. Comparison of dysregulated miRNA in CP with other studies.

Differentially expressed microRNAs commonly found in S.Bauer CP data and AIG CP data					
Mature MicroRNA	S.Bauer *et al* CP Data		AIG CP Data		Ttest P value
	Log Median	Ttest_adjp value	up/downregulated	Fold Change	
hsa-miR-200c-3p	0.76	0.002	down regulated	−2.47	0.002
hsa-miR-130b-3p	1.38	0.004	down regulated	−2.25	0.004
hsa-miR-200b-3p	0.59	0.005	down regulated	−2.32	0.004
hsa-miR-148a-5p	0.76	0.022	down regulated	−3.18	0.001
hsa-miR-193a-3p	0.55	0.023	down regulated	−1.32	0.02
hsa-miR-30d-5p	0.37	0.028	down regulated	−2.40	0.002
hsa-miR-216b	1.1	0.028	down regulated	−3.32	0.004
hsa-miR-216a-5p	1.26	0.031	down regulated	−3.84	0.001
hsa-miR-339-3p	0.45	0.038	down regulated	−1.51	0.01
hsa-miR-151-3p	0.51	0.038	down regulated	−2.25	0.012
hsa-miR-29c-5p	0.68	0.042	down regulated	−2.18	0.009
hsa-miR-30a-3p	0.26	0.045	down regulated	−1.36	0.05
hsa-miR-217	1.42	0.045	down regulated	−4.64	0.0009
hsa-miR-200a	0.83	0.048	down regulated	−1.31	0.054

Table S4. Interactions of dysregulated miRNAs and target genes: relevance to CP.

IPA Network (Figure)	miRNA		miRNA target		Regulatory feedback loop	Confirmed biological functions	Proposed role in CP	Ref. nos.
	Gene	FC	mRNA	FC				
Figure 2	miR130b	decrease	MafB	decrease	miR-130b —MafB	MicroRNA fingerprints during human megakaryocytopoiesis.	Maf family transfription factors may be involved in beta cell dysfunction	23863625
Figure 2	miR-27b	decrease	FoxO1	increase	miR-27b —FoxO1	Coordinate regulation of FOXO1 by miR-27a, miR-96, and miR-182 in breast cancer cells.	FoxO1 gain of function in pancreas causes glucose intolerance, polycystic pancreas, and islet hyper vascularization.	19574223, 22384192
Figure 2	miR-29 family	decrease	IFN G	increase	miR-29 —IFN g	miR29 controls innate and adaptive immune responses to intracellular bacterial infection bytargeting interferon-γ.	Interferon γ and glycemic status in diabetes associated with chronic pancreatitis and its role in fibrogenesis	21785411, 22487478, 20971881
Figure 2	miR375	decrease	JAK2	Not known	miR375 —JAK2	miR-375 may function as a tumor suppressor to regulate gastric cancer cell proliferation potentially by targeting the JAK2 oncogene, implicating a role of miR-375 in the pathogenesis of gastric cancer	JAK2 mediated pathways were shown to stimulate replication and survival of β-cells	20548334, 22045263

Continued

Figure 2	miR-217	decrease	SIRT1	increase	miR217 —SIRT1 —EMT	Chronic pancreatitis and pancreatic cancer demonstrate active epithelial-mesenchymal transition profile, regulated by miR-217-SIRT1 pathway	miR217 is highly pancreas specific and it is shown to involve in progression of CP to pancreatic cancer	25172416
Figure 2,	miR-191	decrease	IL1	increase	miR191 —IL1	hsa-miR-191 Is a Candidate Oncogene Target for Hepatocellular Carcinoma Therapy	No proposed role of miR191, however elevation of proinflammatory cytokines (IL1a, IL1b) were reported in CP	20924108, 22487478
Figure 2	miR-181	decrease	SMAD	increase	miR-181 —TGFB —SMAD	miR-181a can affect the phosphorylation of Smad2 through activation of TGF-β signaling	Inhibition of transforming growth factor beta decreases pancreatic fibrosis and protects the pancreas against chronic injury in mice.	22714950, 15502860
Figure 2	miR-200b	decrease	ZEB1	increase	miR-200b —ZEB1	Participation of miR-200 in pulmonary fibrosis	miR-200 family were implicated in progression of fibrosis	25172416, 22189082

Table shows the miRNAs and corresponding target genes that are part of the network represented in Figure 2. These interactions of miRNAs and target genes that are relevant to CP have been validated in previous studies. Ref Nos column shows the pubmed IDs of the validated interaction that were published.

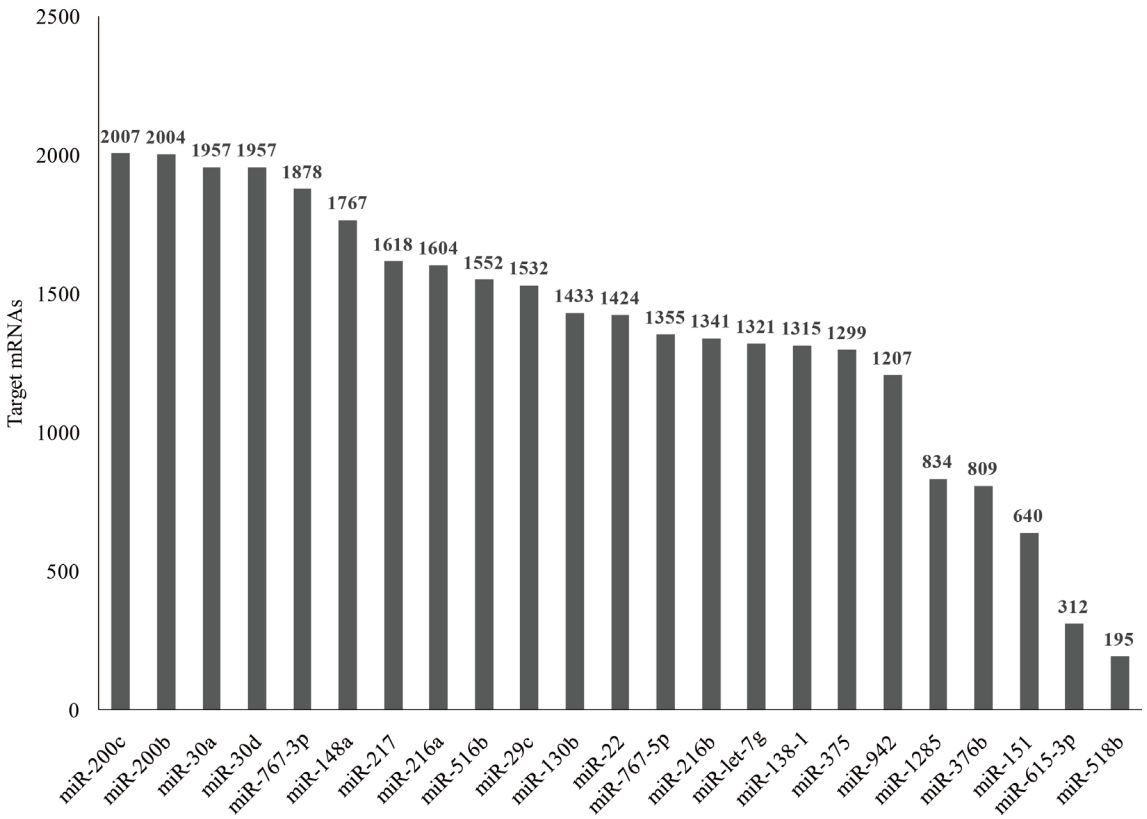

Figure S1. Number of mRNA transcripts identified by miRwalk for each differentially expressed miRNA.