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ORIGINAL ARTICLE

Pilot study identifying circulating miRNA signature specific to alcoholic chronic pancreatitis and its implication on alcohol-mediated pancreatic tissue injury

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Author contribution: Bishnupriya Chhatriya generated data, did formal analysis, and drafted the manuscript, reviewed and edited. Piyali Sarkar helped in data generation and data analysis. Srikanta Goswami conceptualized, designed the study, analyzed data, and wrote the manuscript. Sukanta Ray, Debashis Nath, and Kshaunish Das helped in finalizing the study design and drafting the manuscript apart from executing the clinical components of the study. Saroj K Mohapatra contributed to analytic tools, supervised data analysis and provided critical inputs to the manuscript. All authors read and approved the final manuscript, conception, and design or acquisition of data or analysis and interpretation of data. They have also been involved in drafting the manuscript or revising it critically for important intellectual content.

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Abstract

Background and Aim: Alcohol exerts its effects on organs in multiple ways. Alcoholic chronic pancreatitis (ACP) is a disease in which alcohol triggers the pathological changes in pancreas, leading to chronic inflammation and fibrosis. The molecular mechanism behind these changes is not clear. Identification of key circulating miRNA changes in ACP patients and determination of the fraction that is secreted from diseased pancreas not only could serve as potential biomarker for assessing disease severity, but also could help identifying the molecular alterations prevailing in the organ precipitating the disease, to some extent.
Methods: We performed microRNA microarray using the Affymetrix miRNA 4.0 platform to identify differentially expressed miRNAs in serum of ACP patients as compared to alcoholic control individuals and then found out how many of them could be pancreas-specific and exosomally secreted. We further analyzed a pancreatitis-specific gene expression data set to find out the differentially expressed genes in diseased pancreas and explored the possible role of those selected miRNAs in regulation of gene expression in ACP.
Results: We identified 14 miRNAs differentially expressed in both serum and pancreas and also identified their experimentally validated targets. Transcription factors modulating the miRNA expression in an alcohol-dependent manner were also identified and characterized to derive the miRNA–gene–TF interaction network responsible for progression of the disease.
Conclusions: Differentially expressed miRNA signature demonstrated significant changes in both pro- and anti-inflammatory pathways probably balancing the chronic inflammation in the pancreas. Our findings also suggested possible involvement of pancreatic stellate cells in disease progression.

Introduction

Alcohol alters biological function by direct interaction with cellular components, and also indirectly through the effect of alcohol metabolism by-products on the systemic oxidative and inflammatory state.[1] However, the causal cellular and molecular mechanisms for the harmful effects of alcohol remain ill-defined, and it is unclear how much is the contribution of common underlying mechanism as compared to unique cell or tissue-specific pathways mediating these effects. Alcohol abuse has frequently been found to be associated with the development and progression of both acute and chronic pancreatitis (CP).[2] Pancreas can

also metabolize alcohol either via oxidative pathways or via non-oxidative pathways.[3,4] Some studies have also attempted to explore the molecular changes inside pancreatic acinar cells.[5,6] However, regardless of decades of research, the actual mechanism of pathogenesis of alcoholic pancreatitis remains obscure and not much is known about the etiology of the disease and how the disease differs from nonalcoholic pancreatitis in terms of molecular changes.

One of the most effective means to understand the molecular etiology of the disease is to study the gene expression changes prevailing in the diseased tissue. However, comprehensive analysis of gene expression has multiple components. Alongside coding (mRNA) and noncoding (miRNA, long noncoding RNA) RNA expression changes in tissues, the changes in circulating miRNA is also very important because a very important fraction of circulating miRNA is exosomal and a portion of that must have been secreted from the diseased tissue, in this case inflamed and fibrotic pancreas. Moreover, very recent studies have also very specifically explored the role of pancreatic stellate cell (PSC)-specific exosomal miRNAs in the development of pancreatitis and pancreatic fibrosis.[7] Therefore, it is worth investigating the role of serum or plasma exosomal RNA in this process, assessing their overall contribution. Here, we have done profiling of circulating serum miRNAs deregulated in alcoholic chronic pancreatitis (ACP) patients by miRNA microarray and compared the altered miRNAs to the list of miRNAs already reported to be present as exosomal content in databases like ExoCarta and miRandola.[8,9] We further investigated how many of these circulating miRNAs could also be a result of similar change in expression pattern of that particular miRNA in diseased pancreatic tissue or activated PSC during fibrogenesis. Finally, we attempted to find out how these deregulated miRNAs contributed in the overall deregulation of gene expression in pancreas of the ACP patients by identification of their target genes or transcription factors (TFs) and prediction of pathways supposed to be perturbed in the process.

Methods

Patients and bio-specimen collection. The pilot study was conducted at Indira Gandhi Memorial Hospital, Agartala, India, and Institute of Post-Graduate Medical Education & Research, Kolkata, India. The diagnosis of ACP was made in the appropriate clinical setting and following standard guidelines (measurement of amylase, lipase, CA19-9, blood glucose level and imaging-based analysis such as ultrasonography, magnetic resonance imaging, and CT-scan wherever necessary). Identification of "alcoholic chronic pancreatitis" was based on the standard criteria to identify CP with alcohol consumption of 80 g/day for 5 days a week for >3 years. Hereby, four ACP patients and four alcoholic controls (individuals with similar alcohol consumption status but not having any pancreatic complications and who were "clinically normal") were selected from the recruits based on the criteria of matched age, sex, daily consumption of alcohol and duration of alcohol consumption. Only peripheral blood was obtained in clot vials and serum was isolated from them using standard methods. The study protocol was approved by the institutional ethics committee and written informed consent was obtained from all the participants. Relevant patient and control individual information is provided in Table S1, Supporting information.

Serum microRNA profiling. Total serum RNA enriched for small RNAs was isolated using the miRNAEasy kit from Qiagen using the glycogen method. Quality control and quantification of isolated RNA samples were performed, and the RNA integrity values for all the samples were between 8.0 and 9.0, while 260/280 ratios were also close to 2.0. Microarray experiment was performed using the Affymetrix miRNA 4.0 platform. Data files were submitted to NCBI Gene expression omnibus (GEO) (accession number: GSE128425). The raw data were analyzed using the R Bioconductor packages. Raw CEL files were read into an affybatch object in R. Robust multiarray average was used, which combines quantile normalization and median polish summarization methods. A large expression set object containing normalized data was thus created. Hierarchical clustering and principal component analysis were performed as part of initial quality control. Differential expression analysis was done using the Limma package of R Bioconductor.[10] Model matrix was designed and the data were fitted into the model. Contrast matrix was created and ebayes algorithm was used to determine differentially expressed miRNAs. An miRNA was considered differentially expressed if it passed the threshold of P-value <0.05 and fold change (FC) of 20% or more in either direction.

Tissue miRNA identification. A list of miRNAs expressed in CP tissue was manually curated from previously published literature.[11–15] The list was expanded using miRNAs in pancreatic tissue identified from miRmine,[16] which is a database containing a collection of expression profiles from different publicly available miRNA-seq data sets.

Identification of miRNAs secreted from tissue to serum. Differentially expressed miRNAs in serum were compared with the curated list of miRNAs in pancreatic tissue. miRNAs that were expressed in both serum and tissue in same direction were selected similar to other published reports.[17–20] We also looked into the databases ExoCarta and miRandola to find out whether these miRNAs are secreted in exosomes.

miRNA target identification. Experimentally validated targets of the selected miRNAs present in pancreatic tissue were then identified using the Web tool miRNet, resulting in experimentally validated targets in pancreatic tissue for a given list of input miRNAs.[21] miRNet gives targets validated by high-throughput experiments such as cross-linking, ligation, and sequencing of hybrids (CLASH), photo-activatable ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP), and Microarray apart from qPCR and other reporter assays.

Selection of gene expression data set. Extensive search was done in GEO as well as ArrayExpress for data sets containing gene expression data in CP and control individuals using the keywords "chronic pancreatitis" and "pancreatitis." Data sets were restricted to "*Homo sapiens* only," "array only," and "tissue only." Data sets were excluded if it contained methylation data, miRNA profiling data, or long noncoding RNA (lncRNA)

profiling data. Figures S1 and S2 show the exclusion and selection of data sets in a sequential manner.

Differential expression of genes in pancreatic tissue. Processed data obtained from the data set was log transformed and used for calculating differentially expressed genes (DEGs) in diseased pancreatic tissue of CP patients as compared to alcoholic controls. A model matrix was designed and then the data were fitted into the model. Subsequently, creating a contrast matrix and ebayes algorithm was used to find out the DEGs. The *P*-values were adjusted using the Benjamini–Hochberg correction method, which attempts to minimize false discover rate (FDR) and the genes that were below the cutoff value (P-adj < 0.05) were regarded as DEGs. The above analysis was done using "Limma" package of R Bioconductor.

Identification of miRNAs enriched with target DEGs in the desired direction. Combined analysis of miRNA and gene was done by using hypergeometric tests to identify miRNAs enriched with target genes with expression in reciprocal direction to the expression of miRNA. Here, all the DEGs were used as gene universe and one hypergeometric test was done for each miRNA. FDR correction was done on the *P*-value obtained after the tests on each miRNA and its targets, and miRNAs that were above the adjusted *P*-value cutoff of 0.05 were dropped from further study. The "Category" package of R bioconductor was used to do the above-mentioned analysis.[22]

The set of upregulated genes was then subjected to overrepresentation analysis by applying hypergeometric test, which is briefly described here. Let us consider two lists of genes: the first list being the set of upregulated genes, and the second, genes that are target of a given miRNA that is downregulated (here we assume that the miRNA reciprocally regulates expression of its target genes). The task is to find out whether genes that are target of this downregulated miRNA are also likely to be part of the list of upregulated genes. This is captured in a 2×2 contingency table as shown in Table S2. Overrepresentation analysis tests whether n_{11} and n_{22} are larger than expected relative to the other two cell numbers, and answers whether genes targeted by the miRNA also "tend to be upregulated." The test was implemented in the Category package, with a significant association detected at a threshold of $P < 0.05$.

Identification of TFs. Data corresponding to putative transcription factor-miRNA regulation information were downloaded from both PuTmiR and TransmiR, and the TFs having possible regulatory activity for the selected differentially regulated miRNAs (DEmiRs) were identified.[23,24] The list of identified TFs was compared with that of DEGs in pancreatic tissue of CP patients, and targets of differentially expressed transcription factor (DE-TFs) were identified using TRRUST and TF2DNA databases.[25,26]

Software. "R" codes for the data analysis are given in Appendix S1. Raw as well as processed data and other additional files required for analysis are available upon request.

The study has been approved by Institutional Ethics Committee of National Institute of Biomedical Genomics, Kalyani, West Bengal, India; Institute of Post Graduate Medical Education & Research, Kolkata, West Bengal, India; and Indira Gandhi Memorial Hospital, Agartala, Tripura, India. Appropriate prior written informed consent from the study participants have been taken following the guidelines of the Ethics Committees and in an approved format. We do not intend to publish individual patient data in the form of images, videos, voice recordings, and so forth. The study does not involve any animals.

Results

Description of overall plan. We have performed a microRNA microarray using total RNA from serum of four ACP patients as compared to four alcoholic control individuals and identified 72 upregulated and 78 downregulated miRNAs. The differentially expressed miRNAs were further investigated for their expression in pancreatic tissue and exosomal vesicles, and independent analysis of a gene expression data set identified 263 upregulated and 389 downregulated genes in pancreatic tissue of ACP patients compared with normal pancreas. This was followed by an integrated miRNA–mRNA analysis, resulting in 84 miRNA–gene interactions, which was finally used to build an interaction network having additional information of deregulated transcription factors totaling to 100 miRNA–gene–TF interactions. The schematic representation of the plan is shown in Figure 1.

Identification of differentially expressed miRNAs in serum. Serum miRNA profiling of four ACP samples and four alcoholic control samples revealed 150 differentially expressed miRNAs with *P*-value cutoff of less than 0.05 and FC of 20% or more in either direction. Among them, 72 miRNAs were found to be upregulated and 78 miRNAs were found to be downregulated. Expression results of all the deregulated miRNAs are represented in the volcano plot (Fig. 2a), while the heat map in Figure 2b shows clear differences in expression pattern of the miRNAs from those two groups.

Selection of pancreas-specific miRNAs. The characteristic pattern of differentially expressed miRNAs in ACP patients could partially originate from the disease-specific changes in the pancreas itself. A comparison of miRNAs altered in pancreatic tissue with that of deregulated in serum of ACP patients identified 32 miRNAs, which could be the pancreas-specific alteration of miRNAs reflected in serum. We also explored how many of these miRNAs were previously reported to be secreted in exosomes from available information in databases like ExoCarta and miRandola, yielding 28 out of 32 miRNAs also secreted through exosomes. Table S3 presents the list of 32 miRNAs having information about their availability in exosomes.

Identification of experimentally validated targets. Next, we aimed to explore the function of these altered miRNAs in the pancreas to have a better handle on understanding disease pathophysiology. In order to do so, we first wanted to identify their target mRNAs and wished to focus only on the experimentally validated targets as obtained from miRNet. Not all the miRNAs had experimentally validated targets, and we identified 3665 such targets for 19 of the 32 miRNAs (Figure S3). Thus, we derived a comprehensive target list supported by experimental

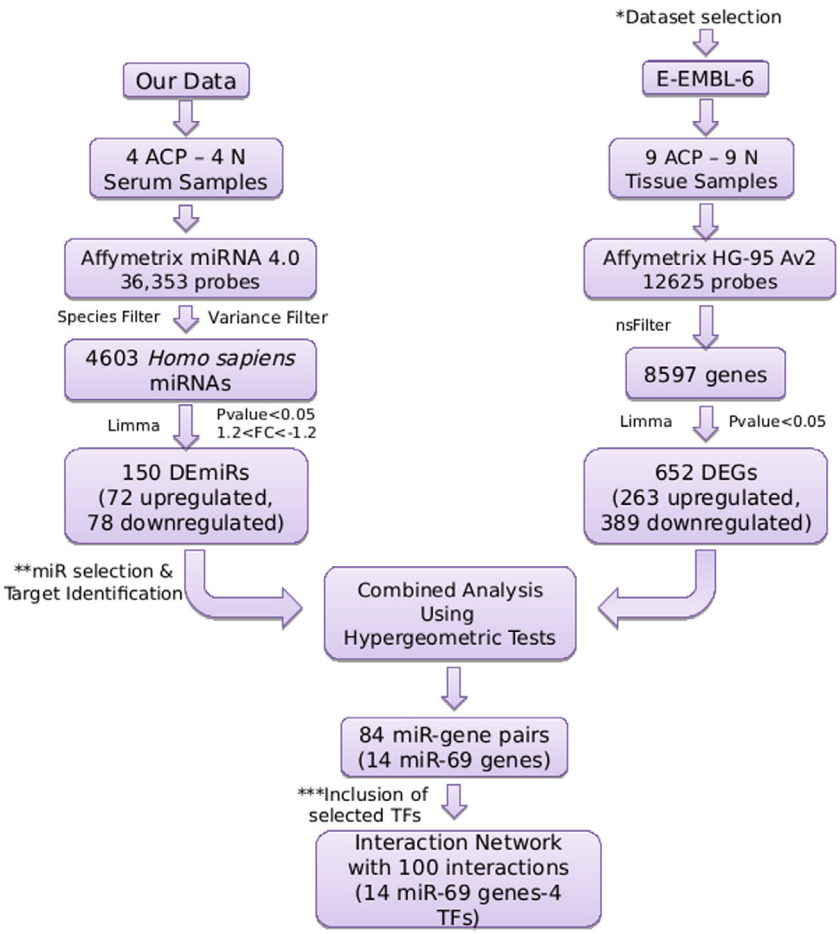

Figure 1 Schematic flowchart showing the overall workflow followed in this study. The portion indicated by * has been expanded in Figures S1 and S2, while the portion indicated by ** has been expanded in Figure S3 and the portion indicated by *** has been shown in Table S4. ACP, alcoholic chronic pancreatitis; DEG, differentially expressed gene; FC, fold change; TF, transcription factor.

evidence, some of which could be really responsible for development and progression of ACP.

Selection of data sets and identification of DEGs in tissues. An miRNA has multiple target mRNAs, and not all of these mRNAs are being targeted in every tissue. We planned to look for DEGs in the pancreatic tissue of CP patients. GEO and ArrayExpress were explored as described in the "Methods" section, and the data set E-EMBL-6 was selected. Subsequent quality check and analysis revealed 652 DEGs (263 upregulated and 389 downregulated genes) in pancreatic tissues from CP patients. Figure 2c shows the DEGs in a volcano plot, and Figure 2d shows upregulated and downregulated DEGs in a heat map.

miRNA–gene combined analysis. Finally, we tried to find out how our selected miRNAs are related to the DEGs obtained. The expression of target DEGs should be in the reciprocal direction to the expression of miRNAs if the miRNA is actually regulating its target DEG. We used hypergeometric tests to find out which miRNAs are enriched with targets in reciprocal direction of expression. Hypergeometric test is conventionally used in pathway enrichment analysis to identify which pathways are enriched in the selected genes. Here, we applied the same logic to identify which miRNAs are enriched with target genes with reciprocal direction of expression. The selection of miRNA (and its target DEGs) was made based on FDR-corrected *P*-value <0.05 as obtained from the hypergeometric tests. This test further strengthens that the 14 miRNAs out of 19 are enriched with DEG targets in reciprocal direction. The results are shown in Table 1.

Role of TFs. Another important aspect that required attention was how expression of these miRNAs is regulated and how alcohol might modulate their transcription. We wanted to see how many of the TFs identified from the databases to alter our selected miRNAs are also differentially expressed in pancreatic tissue and identified RUNX2, RUNX3, TGFB1, and ZEB1 as TFs which partially mediate effect of alcohol on pancreas by altering the expression of key miRNAs. We further obtained additional target information for these TFs from the databases like TF2DNA and TRRUST and compared the list with our DEG

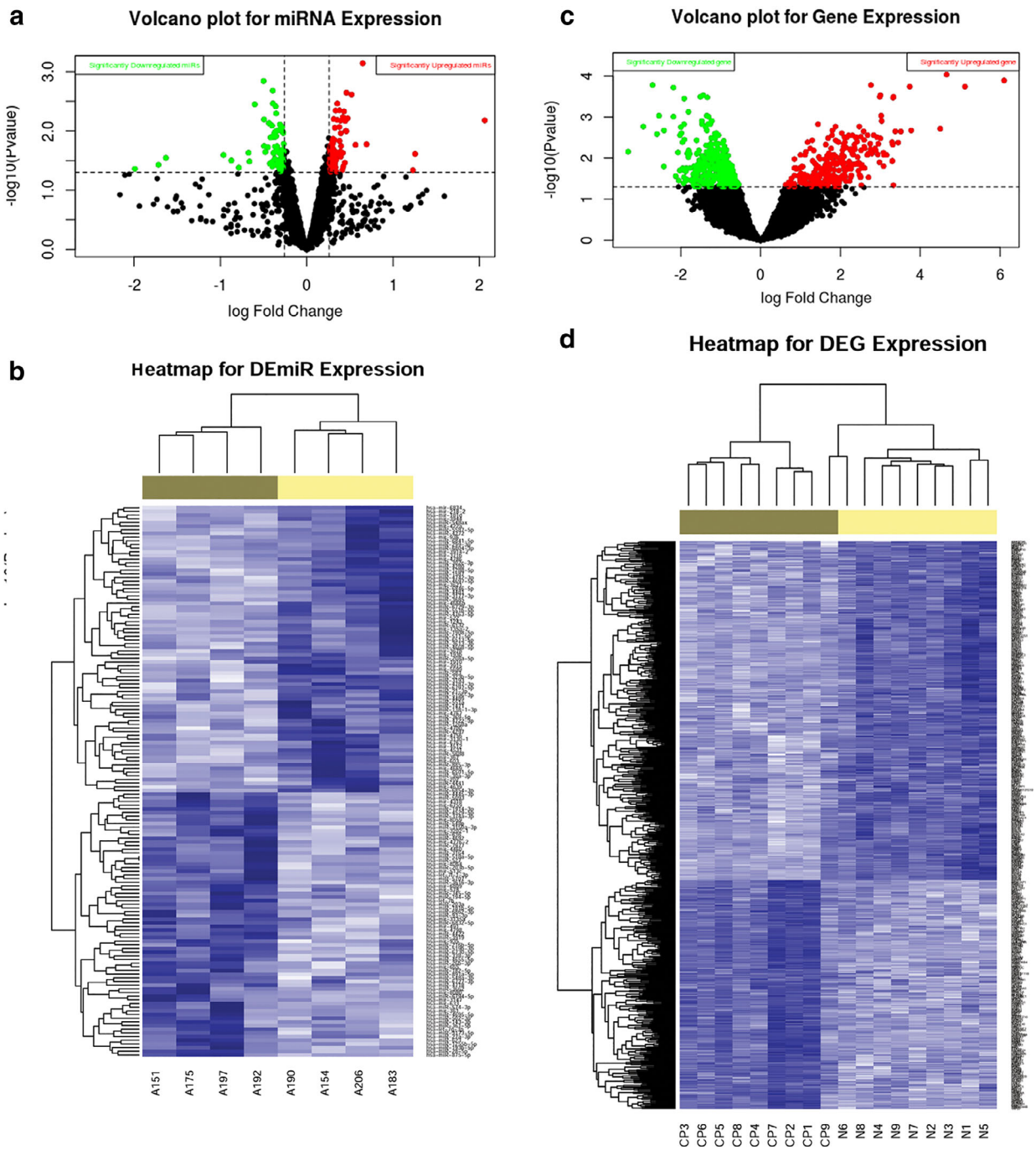

Figure 2 Identification of differentially regulated miRNAs and genes alcoholic chronic pancreatitis (ACP) patients as compared to alcoholic controls. Panels a and b show differentially regulated miRNAs (*P*-value < 0.05, 1.2 < fold change < −1.2) in serum of ACP patients as compared to alcoholic controls. While Panel a shows volcano plot, where red dots represent upregulated miRNAs and green dots represent downregulated miRNAs; Panel b shows the Heatmap, where brown colored bar represents cases and yellow colored bar represents controls. Panels c and d show differentially regulated genes (*P*-adj < 0.05) in pancreatic tissue of chronic pancreatitis patients as compared to controls in the data set E-EMBL-6. Panel c shows volcano plot, where red dots represent upregulated genes and green dots represent downregulated genes. Panel d shows the Heatmap, where brown colored bar represents cases and yellow colored bar represents controls. The color gradient is from white to dark blue based on increasing order of expression. All the images were constructed using "gplots" package in R. DEG, differentially expressed gene.

Table 1 Results of miRNA–gene combined analysis. Hypergeometric tests were conducted to identify miRNAs enriched with genes in reciprocal direction. Based on *P*-adj, 14 miRNAs were selected. Direction of expression (whether up- or downregulated) and fold change of expression in ACP patients as compared with normal individuals has been given

miRNA	Direction of expression	HyperG *P*-adj	Fold change
hsa-miR-27a-3p	Up	5.64E−19	3.972
hsa-miR-194-5p	Up	0.0000269	3.287
hsa-miR-361-5p	Up	3.19E−13	3.097
hsa-miR-143-3p	Up	1.27E−08	1.832
hsa-miR-502-3p	Up	2.07E−07	1.725
hsa-miR-181b-5p	Up	7.9E−14	1.518
hsa-miR-182-5p	Up	3.93E−12	1.395
hsa-miR-28-5p	Up	0.0000488	1.312
hsa-let-7b-5p	Up	3.89E−45	1.288
hsa-miR-216b-5p	Up	9.18E−07	1.250
hsa-miR-26b-3p	Up	0.00896649	1.241
hsa-let-7 g-3p	Up	0.00242843	1.226
hsa-miR-338-3p	Up	6.88E−11	1.210
hsa-miR-30a-3p	Down	0.01248905	−1.237

list. Table S4 shows the regulatory TFs and their target miRNAs. Combined interaction network of miRNA–DEGs and TFs is explored in Figure 3, and their analysis shows upregulation of both pro- and anti-inflammatory pathways toward development of persistent inflammation and fibrosis of pancreas in ACP. Table S5 shows miRNA–gene–TF interactions obtained from our analysis.

Discussion

The detailed molecular mechanism for the alcohol-induced CP is not clear. This fraction of miRNAs altered between ACP and normal alcoholic individuals not only contributes to the understanding of the biology of ACP but could also have the potential to function as putative biomarkers to detect severity of pancreatic injury in the disease. It will be worth mentioning that we have also compared total serum miRNA profile between normal nonalcoholic healthy individual and normal alcoholic healthy individual and found no significant difference (data not shown). This finding further justifies our miRNA–mRNA interaction analysis using the gene expression data set, where healthy nonalcoholic controls have been used. Now, integration of miRNA alteration results to the DEGs in pancreatic tissue from CP patients and also investigating how the TFs modulate the entire process being induced by alcohol yielded an miRNA–mRNA–TF interaction network. Exploration of that network showed upregulation of both the pro- and anti-inflammatory pathways contributing to the disease pathophysiology. let-7b-5p emerged as one of the major hubs of the network. This miRNA has been shown to be expressed in the pancreas of mice and humans and has been shown to play a potent anti-inflammatory effect. Anti-fibrotic effect of let-7b-5p is mediated mainly through inhibition of TGFB1 signaling, creating a feedback loop. Several studies also show the expression of let-7b-5p is induced by alcohol. However its involvement in pancreatic inflammation and fibrosis has not been directly shown earlier, and we are reporting for the first time its pivotal role in ACP.

The next important hub is miR-27a-3p, which is a pro-inflammatory miRNA that mediates its effects through inhibition of IL-10[27] and also induced by alcohol. miR-181b-5p and miR-182-5p are two other miRNAs reported to have anti-inflammatory and pro-inflammatory roles, respectively. miR-181b-5p has been shown to activate hepatic stellate cells and suppress autophagy. Inhibition of autophagy, when occurred in pancreas, leads to pancreatitis. Among the other miRNA candidates, miR-361-5p, also an antiautophagy miRNA, has been reported to be upregulated in acute pancreatitis patients.[28] miR-143-3p also found to be upregulated in rheumatoid arthritis and reported to be upregulated upon alcohol consumption.[29] miR-338-3p, miR-30a-3p, and miR-194-5p also act as anti-inflammatory and antifibrotic miRNAs, reported to be induced by alcohol, while miR-216b-5p and miR-28-5p are pro-inflammatory and pro-fibrotic miRNAs and have been shown earlier to be acting as markers of acute pancreatic injury and inflammation.

Similarly to the deregulated miRNAs, we also focused on deregulated TFs to find out how their alteration could explain the biology based on earlier evidence. *RUNX3*, which is overexpressed in our results, has been shown to be overexpressed in pancreatic ductal adenocarcinoma and promote inflammation. We have found downregulation of *RUNX2*. Although this transcription factor has been shown to be upregulated in pancreatic cancer,[30] its role in pancreatitis has not been demonstrated before. However, its pro-fibrotic role is well demonstrated in pulmonary fibrosis[31] and suppression of this could be linked to counterbalancing act against inflammatory signals. Transforming growth factor-β (*TGF-β*) is the master regulator of fibrosis and ethanol, and its metabolite acetaldehyde has been shown to increase *TGF-β1* expression. *TGFβ* family members are known to have important contribution in pancreatic development and pancreatic diseases.[32] Zinc finger E-box binding protein 1 (*ZEB1*) induces epithelial mesenchymal transition (EMT) in cancers and upregulates genes encoding inflammatory cytokines and known to promote fibrosis too.[33] Upregulation of *ZEB1* is also known to promote metastasis of pancreatic cancer.[34]

We observed that most of the miRNAs that came up in our study were having earlier evidence of being induced by alcohol in other system. Anti-inflammatory cytokines have already been implicated in etiology of ACP in experimental model of ACP, along with pro-inflammatory ones.[35,36] Furthermore, acute and moderate consumption of alcohol has also established anti-inflammatory role, as seen in both rat and humans.[37] Immune cells secreting anti-inflammatory cytokines have further proofs,[38] and several anti-inflammatory cytokines such as *IL-10* are high in the serum of pancreatitis patients.[39] Therefore, it is not surprising that we find a set of anti-inflammatory miRNAs that could explain, at least partially the balancing act of inflammatory and counterinflammatory pathways in disease pathophysiology.

It emerged from studies that chronic activation of PSCs plays a major driving role behind pancreatic fibrogenesis related to ACP. We also find that the identified miRNAs play a significant role in fibrogenesis, and their reported function could be extrapolated to PSC activation, supporting the emerging importance of PSCs in pathogenesis of ACP. It is possible that triggering of anti-inflammatory pathways acts as a "brake" to mediate

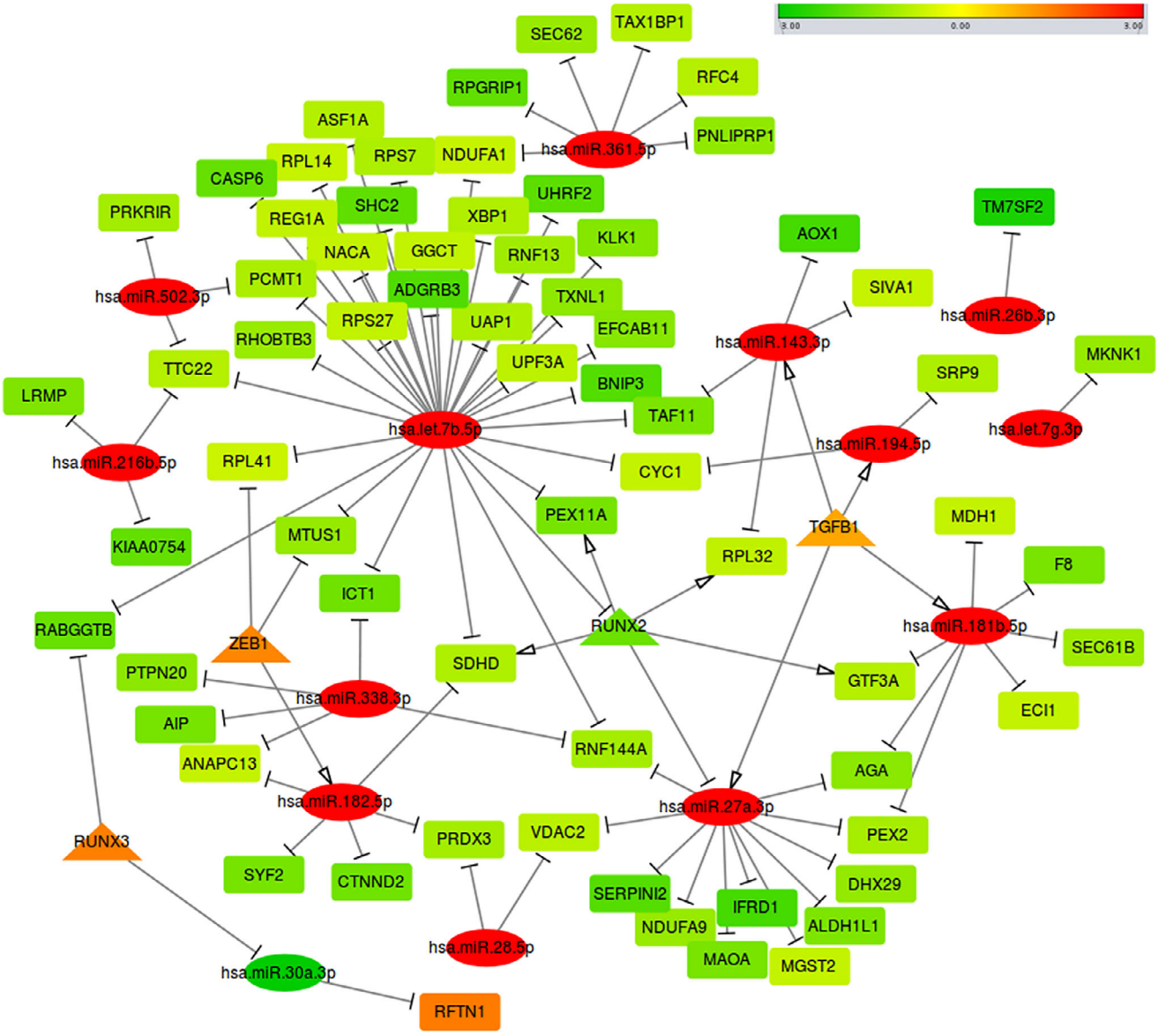

Figure 3 Interaction network involving miRNA–gene–transcription factor. It shows a network of DEmiRs targeting DEG, differentially expressed genes (DEGs). In addition to that, there are some DETFs that regulate both DEmiRs as well as DEGs. miRNAs are represented by elliptical shape, genes are represented by rounded rectangle shape, and transcription factors are represented by triangle shape. Color gradient from green to red shows the level of expression from low to high. "T" arrow represents inhibition or repression, "open delta" arrow represents activation. Network was constructed using Cytoscape.

controlled activation of PSCs only to carry out the repair of the damaged tissue, as required. However, the balance shifts toward progressive fibrogenesis and organ dysfunction if the insult continues. We have developed a model to summarize these findings, as described in Figure 4. We feel that the one drawback of our study is that the number of samples used for the initial serum miRNA microarray analysis was less. But, we present these results as pilot data and our findings from pilot data also corroborate with the published literature. Secondly, we ourselves have not performed any experiments to validate the role of the miRNAs. However, there are existing functional validation studies, which we have already discussed, supporting our finding and giving us overall confidence about our results. Still, it will be quite interesting to explore further how they could actually modulate alcohol-mediated pancreatic tissue damage.

Hence, we conclude that our study is first of its kind to focus on pancreatic tissue-specific circulating miRNAs in ACP to interrogate how they modulate the effect of alcohol in mediating pancreatic tissue damage. The importance lies in the fact that these miRNAs could further be investigated for their capability to function as biomarker to assess severity of the disease in terms of pancreatic injury.

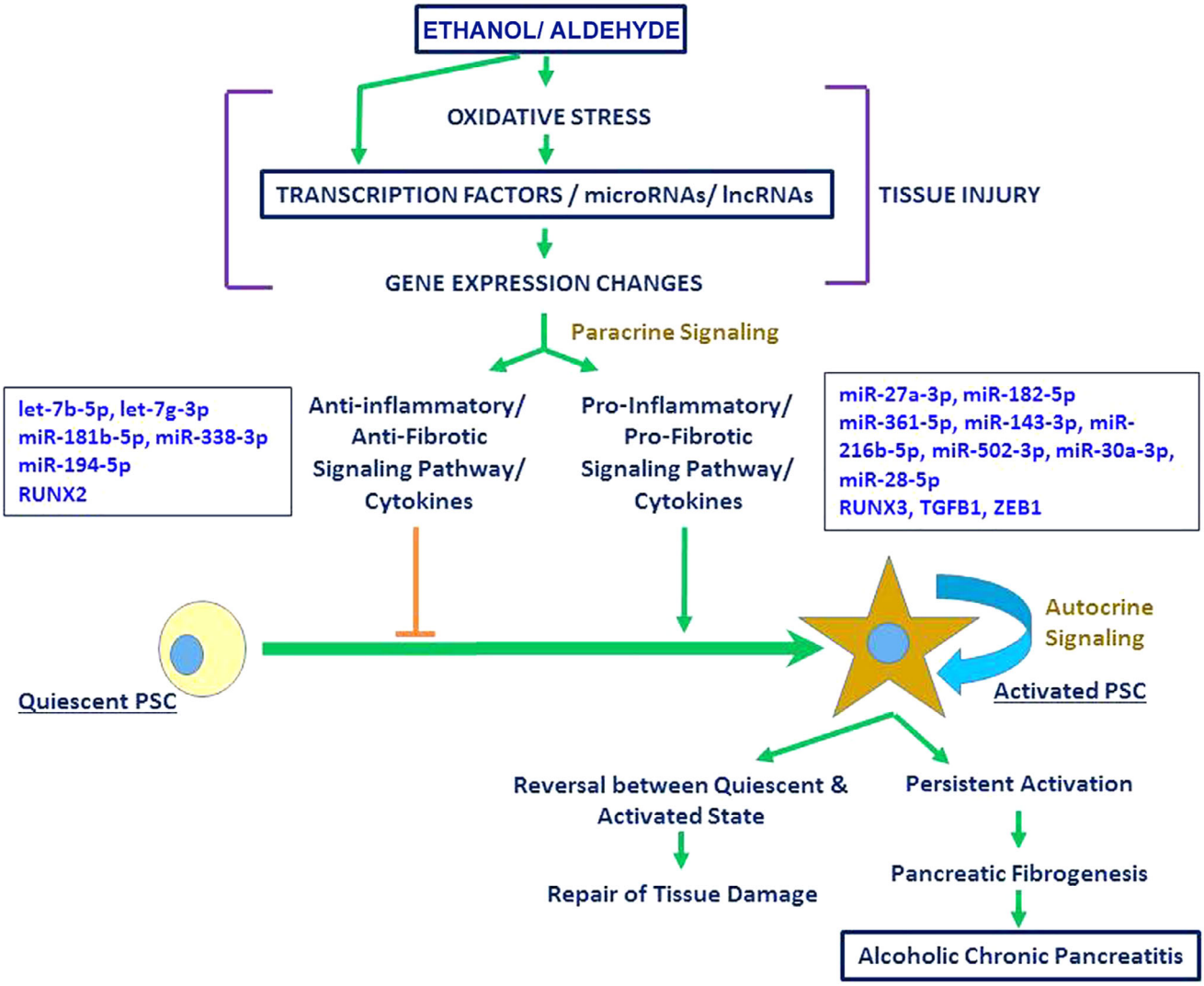

Figure 4 Proposed model highlighting possible mechanism of alcohol mediated tissue injury in chronic pancreatitis.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's website:

Appendix S1 Instructions for analyzing the data.

Figure S1 Schematic flowchart showing selection of tissue gene expression dataset from GEO.

Figure S2 Schematic flowchart showing selection of tissue gene expression dataset from Array express.

Figure S3 Schematic diagram to show how the miRNAs were filtered and selected for further study.

Table S1 Information about the ACP patients and alcoholic controls.

Table S2 Explanation of over representation analysis.

Table S3 List of miRNAs based on different selection criteria.

Table S4 miRNA-Transcription factor interactions.

Table S5 miR-Gene-TF interactions obtained from our study.