# Correlating tissue and plasma-specific piRNA changes to predict their possible role in pancreatic malignancy and chronic inflammation

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**Abstract.** The aggressiveness of pancreatic ductal adenocarcinoma is primarily due to lack of effective early detection biomarkers. Circulating non-coding RNAs serve as diagnostic or prognostic biomarkers in multiple types of cancer. Comparison of their expression between diseased tissue and relevant body fluids such as saliva, urine, bile, pancreatic juice, blood etc. may reveal mechanistic involvement of common non-coding RNAs. piwi-interacting RNAs (piRNAs) are a class of non-coding RNAs. The aim of the present study was to investigate plasma and tumour tissue piRNA changes in patients with pancreatic cancer (PC) and explore the possible role in tumorigenesis and pancreatic inflammation. Sequencing of circulating plasma small RNAs from patients with PC and chronic pancreatitis (CP) was performed and differentially expressed piRNAs were compared with those in tissues. Subsequent search for target genes for those piRNAs was performed followed by pathway and cluster analysis. A total of 36 piRNAs were shown to be deregulated in pancreatic tumour tissue and alteration of 11 piRNAs was detected in plasma of patients with PC. piRNAs hsa-piR-23246, hsa-piR-32858 and hsa-piR-9137 may serve a key role in PC development as their expression was correlated in both plasma and tumour tissue. Key piRNA-target interactions interfering with key biological pathways were also characterized. A total of 19 deregulated piRNAs in plasma samples of patients with CP was identified; these targeted genes responsible for chronic inflammation. Therefore, the present study provides a comprehensive description of piRNA alteration in pancreatic malignancy and inflammation; these may be explored for biomarker potential in future.

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*Abbreviations:* miRNA, microRNA; lnc, long non-coding; circRNA, circular RNA; piRNA, piwi-interacting RNA; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; PDAC, pancreatic ductal adenocarcinoma; CP, chronic pancreatitis; TE, transposable element

*Key words:* pancreatic ductal adenocarcinoma, chronic pancreatitis, piRNA, plasma, tissue

## Introduction

Pancreatic cancer (PC) is a disease with low incidence and high mortality rate. American Cancer Society estimates that ~66,440 people (31,910 female and 34,530 male patients) will be diagnosed with PC and ~51,750 people (24,480 female and 27,270 male patients) will die of the disease in the United States in 2024. Despite progress in research on PC and management of the disease, the 5-year survival rate remains ~10% (1). PC is the 4th leading cause of cancer death in the USA, after lung, colon and breast cancer and the 7th foremost cause of worldwide cancer-related death (1). Poor prognosis of PC is due to non-specific symptoms and silent growth until advanced progression of the disease; there are few diagnostic methods sufficiently sensitive and specific to detect the disease early (2). Furthermore, retroperitoneal position of the pancreas prevents accurate physical examination of the organ and there has not been consensus on the optimum usage of diagnostic imaging for early detection of PC. Even after using the most advanced imaging techniques, lesions <3 cm in size are not detected (2,3). Among non-invasive biomarkers, carbohydrate antigen 19-9 (CA 19-9) is the only molecule used in management of PC (4). However, there are reports of alteration of CA 19-9 in benign pancreatic diseases and gastrointestinal inflammation, thereby decreasing its specificity as a biomarker for pancreatic malignancy (4,5).

There has been some progress in molecular diagnosis. Technological advancements facilitate detection of circulating cancer cells, circulating microRNAs (miRNAs) and proteins for early diagnosis of PC and predict prognosis of the disease (6).

Non-coding (nc)RNAs such as long nc (lnc)RNAs, circular RNAs (circRNAs) and piwi-interacting RNAs (piRNAs) serve vital roles in the regulation of tumorigenesis, tumour progression and prognosis in multiple types of cancer including colon, breast, lung, gastric and liver cancer, PC, glioblastoma, leukemia (7). Multiple studies have established involvement of specific lncRNAs (Homeobox Transcript Antisense Intergenic RNA, Plasmacytoma Variant Translocation 1, H19-H19 Imprinted Maternally Expressed Transcript, myocardial Infarction Associated Transcript, GAS5-Growth Arrest Specific 5 etc.), circRNAs (circPDAC, circFOXK2-Circular RNA Forkhead Box K2, ciRS-7-Circular RNA Sponge For MiR-7, hsa_circ_0007534 etc.) and piRNAs (piR-162725, piR-017061) in the regulation of gene expression and control of several signal transduction pathways in PC (7,8). piRNAs are a type of short, single-stranded RNA 21-35 nucleotides in length. piRNAs interact with PIWI proteins to silence transposable elements (TEs) and maintain genome stability and integrity. piRNAs regulate endogenous genes mainly through RNA degradation (9,10). piRNAs-mouse PIWI (MIWI) protein interaction may target mRNAs with imperfect base pairing to promote their degradation by MIWI-dependent cleavage, thereby regulating gene expression and contributing to disease phenotype (9,10). piRNAs serve as non-invasive biomarkers since they are also found in body fluids such as blood, saliva, gastric juice and urine (11). To the best of our knowledge, however, there is little information on the role of piRNAs in pancreatic ductal adenocarcinoma (PDAC). Transcriptome analysis of pancreatic tumour tissues has identified lncRNAs, miRNAs and piRNAs that are altered in a tumour-specific manner (12). Another study reported candidate piRNAs isolated from plasma of patients with PC (13) and a separate study listed piRNAs that are differentially expressed (DE) in patients with PC (14). On the other hand, other studies have investigated the functional aspects of selected piRNAs and their interactions in PC (15,16).

The present study performed small RNA sequencing analysis of piRNAs from both tissues and plasma of patients with PC and controls. Target genes were subsequently identified and the pathways involved in disease development were predicted. A similar analysis using plasma samples from patients with chronic pancreatitis (CP) was performed to identify piRNAs that may contribute to chronic inflammation.

## Materials and methods

*Patients and bio-specimen collection*. A total of 16 healthy individuals and 15 pancreatic cancer patients were recruited between April 2015 to August 2019 with age range of 20 to 70 years for PC patients and 20 to 55 years for normal individuals. In both PC and normal individuals the female to male ratio was about 3:2. Surgical tissue and plasma samples of patients with confirmed PC (pancreatic ductal adenocarcinoma) and not undergoing any chemotherapy were obtained from the Institute of Postgraduate Medical Education & Research and the Chittaranjan National Cancer Institute (both Kolkata, India). The study was approved by the Institutional Ethics Committee (INST/IEC/2015/218 and IPGME&R/IEC/2022/318 for Institute of Postgraduate Medical Education & Research-Research Oversight Committee and CNCI-IEC-SG2-2023-69 for Chittaranjan National Cancer Institute-Institutional Ethics Committee). Written informed consent was procured from all participants prior to the study. A total of 5 ml peripheral venous blood was collected in vacutainer tubes (BD Biosciences) before routine surgery and plasma samples were processed as previously described (17). Normal plasma samples were collected from healthy individuals with no history of pancreatic disease and were processed in the same way. Tumour and adjacent normal pancreatic tissue (>5 cm from tumour margin) were collected from patients with PC. The samples were stored at -80˚C until use. Simultaneously, resected specimens were processed for histopathological assessment to confirm malignant or benign nature (Table SI).

*RNA isolation and quality control*. Total RNA enriched with small RNAs was isolated from the plasma samples using miRNAeasy Serum/Plasma advanced kit (Qiagen GmbH). Briefly, 200 $\mu$l plasma sample was centrifuged at high speed of 16,000 x g for 5 min at 4˚C to remove any cellular debris or particulate matter that may interfere with downstream RNA isolation. Next, QIAzol lysis reagent was followed by vortexing and phase separation after adding chloroform as per the manufacturer's instructions. The aqueous phase was then separated, and ethanol precipitation of RNA was performed followed by passage through the column provided with the kit. Column-bound RNA was washed and eluted using the buffer solution, according to the manufacturer's instructions. Quantification was performed using a multi-channel spectrophotometer (ND 8000; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Additionally, total RNA was isolated from tissue using QIAzol (Qiagen GmbH) and PureLink RNA mini kit (Ambion; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The quality of isolated total RNA was determined using Agilent RNA 6000 Nano chips in a 2100 Bioanalyzer (Agilent Technologies, Inc.) and NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). Quantification was performed using Qubit and the Quant-iT RNA assay kit broad range (Thermo Fisher Scientific, Inc.). Total RNA samples with RNA integrity number >7 were selected for small RNA library preparation and Illumina sequencing.

*Small RNA library preparation and sequencing*. The quality of isolated small RNA was checked using small RNA chips in a 2100 Bioanalyzer (Agilent Technologies, Inc.) and quantitation was performed using a Qubit Fluorometer. Small RNA sequencing library preparation was performed using Illumina® TruSeq® Small RNA Library Prep kit (Illumina, Inc.; cat. no. RS-200-0012) according to the manufacturer's instructions. A total of 10 ng isolated small RNA was used for library preparation. The first step was to ligate adapters to 3' and 5' ends of the RNA molecule. Subsequently reverse transcription and amplification were performed to generate a cDNA library, using the reagents provided in kit following manufacturer's instructions. Gel purification step that selects bands 145-160 bp long was performed to prepare the final small RNA sequencing library for clustering and sequencing. The quality of small RNA sequencing libraries

was checked using high sensitivity D1000 screen tape in a 2200 TapeStation (Agilent Technologies, Inc.) and final library quantification was performed using a Qubit Fluorometer (Thermo Fisher Scientific, Inc.). Single end 1X 50 bp sequencing of pooled libraries was performed in a Novaseq 6000 (Illumina, Inc.).

*Analysis of sequencing data*

*Preprocessing and quality control of piRNA sequencing data*. Initial quality control and visualization of small RNA sequencing data were performed using FastQC (version 0.12.0) (18) and MultiQC (v1.24) (19). Adapter trimming (Illumina TruSeq small RNA adapters) was performed using the TrimGalore tool (v0.6.10) (20). Sequence reads of a length of 24-35 nucleotides were retained in the analysis. Poor quality reads (Phred score <20) were filtered out.

*Alignment of reads to the reference genome and quantification of piRNA expression*. The filtered sequencing reads were aligned to the human genome reference (hg19) using Bowtie2 aligner (Version 2.5.1) (21). Quality-checking of the sequencing alignment data was performed using SAMtools (v1.21) (22), Sambamba (v0.5.0) (23) and Qualimap (v2.3) (24). Aligned sequencing reads were overlapped with other small RNA sequencing information from the DASHR (v2.0) database (in BED file format) (25) to filter out other small ncRNA. Raw piRNA expression counts were quantified using the Featurecounts tool (v1.6.0.3) (26), with a piRNAdb annotation file (version 1.7.5; reference genome, hg19) (27) in GTF format. For normalization of the count data, 'estimateSizeFactors' was used to calculate size factors for each sample, using the Median ratios method and normalized counts were obtained using 'counts' of DESeq2.

*Analysis of differential piRNA expression*. The R package DEseq2 (version 1.12.3) (28) was used to identify DE piRNAs. Wald test was used for assessing statistical significance with adjusted P-value <0.1 as the threshold and -log2FC >0.58 or <-0.58 for up- and downregulated piRNA, respectively. Visualization of DE piRNAs was performed through heatmap and volcano plots, using R packages pheatmap (10.32614/CRAN.package.pheatmap) and dplyr (10.32614/CRAN.package.dplyr). A detailed schematic of the data analysis pipeline and quality filtering is shown in Fig. S1.

*piRNA target identification*. Differentially expressed piRNAs and protein coding genes were used to predict the target genes for piRNAs using miRanda target prediction algorithm (29). Briefly, the FASTA sequences of DE piRNAs and gene coding transcripts were retrieved from piRNAdb (version 1_7_5; hg19 reference; pirnadb.org/) and Ensembl databases(Homo_sapiens.GRCh37.cdna.all.fa;hg19reference; ensembl.org/index.html), respectively, and miRanda was run using alignment score ≥170 and a binding energy ≤-20 kcal/mol. Next, the piRNA-target gene pairs were filtered based on degree of sequence complementarity in the primary (2-11 nucleotides) and secondary seed site (12-21 nucleotides) with no mismatch and wobble base pairing within the primary seed site and only one mismatch and no wobble base pairing within the secondary seed site, as reported previously with minor modifications (30). Additionally, targets for each piRNA were manually verified using piRNADb (27).

*Functional annotation of DE-piRNAs*. To understand the functional aspects of targets of DE piRNAs, gene set enrichment analysis (GSEA) was used. Gene Ontology (GO) (geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (genome.jp/kegg/) were used to perform the enrichment analysis. KEGG database integrates genomic information to explore metabolic pathways, genetic information processing and cellular processes, whereas GO analysis involves the computational examination of gene sets to identify and categorize biological processes (BPs), cellular components (CCs) and molecular functions (MFs). Enrichr was used for GO and KEGG analysis (31,32). P<0.05 was considered to indicate significant enrichment. The Cancer Genome Atlas-Pancreatic Adenocarcinoma (TCGA-PAAD) dataset was used through Gene Expression Profiling Interactive Analysis; gepia.cancer-pku.cn/).

*Predicting piRNA clusters from diseased and normal samples*. Most piRNAs in the genome originate from 25-35-bp-long discrete loci termed 'piRNA clusters', which serve a key role in the silencing of TEs (33). proTRAC command-line tool (34) was used with default parameters (sliding window size, 5,000 bp; sliding window increment, 1,000 bp; minimum fraction of hits with 1T(U) or 10A, 0.75; minimum size of piRNA cluster, 1,000 bp) to identify piRNA clusters in the tissue and plasma samples. Overlap of identified clusters with known repetitive elements in the genome was determined with Repeatmasker database (reference genome, hg19) annotation (35) and BEDtools package (36).

*Statistical analysis:* Wald test was used for assessing statistical significance with adjusted P-value <0.1 as the threshold and -log2FC >0.58 or <-0.58 in R package DEseq2 was used to identify DE-piRNAs. Pearson's correlation test. In GO enrichment analysis, hypergeometric distribution mathematic model was used to obtain the P-value of the Pathways. In KEGG pathways, multiple Benjamini and Hochberg testing was performed.

## Results

*Comparative analysis of DE piRNAs derived from small RNA sequencing of tissue and plasma*. Small RNA sequencing was performed to obtain a median of ~52.45 million reads (range, 28.32-65.68 million) in tumor tissues and adjacent normal samples. A median of 15.79 million reads (range, 3.29-92.69 million) was obtained in the case of the plasma samples (PC and CP cases and respective normal samples). Detailed metrics and quality control information of small RNA sequencing in tissue and plasma samples of patients with pancreatitis and PC are provided in Table SII.

*Landscape of DE piRNAs in pancreatic tumor tissues*. In pancreatic tumor tissues compared with normal tissue, 30 piRNAs were significantly upregulated and six piRNAs were significantly downregulated (Table I; Fig. 1A and C).

*Exploratory analysis of circulating piRNAs in plasma samples*. There were 16 normal samples and 15 samples from patients with PC. One piRNA was up- and 10 were downregulated (Table SIII; Fig. 1B and D).

Table I. Differentially expressed piRNAs in normal and pancreatic cancer tissue.

A. Upregulated piRNAs

| piRNA | Sequence | Log2 fold-change | Adjusted P-value |
|---|---|---|---|
| hsa-piR-33069 | 5'-AGACCTATGAAGAGATTGAAGAAGAAACTGAGGTCC-3' | 6.580811883 | 1.21x10-5 |
| hsa-piR-33150 | 5'-GGCGTGTGATGATTACCTGAGTATTTCTGACG-3' | 4.873765728 | 1.38206x10-4 |
| hsa-piR-33200 | 5'-TTTGCCATGATGAGAATTTATCTGAGG-3' | 4.58814715 | 6.842285x10-3 |
| hsa-piR-33072 | 5'-AGCCCTGAGGATGAAAGAACTATCCCTGAAGGGC-3' | 4.478109188 | 6.842285x10-3 |
| hsa-piR-28033 | 5'-GGCCAGCCTGGTCCACATGGGTCGGAA-3' | 4.200503537 | 6.842285x10-3 |
| hsa-piR-33124 | 5'-CTGTCCTTGATGTTACTGCTGTTCTGAGACAT-3' | 3.085192218 | 6.842285x10-3 |
| hsa-piR-28763 | 5'-GTTTAGACGGGCTCACATCACCCCATAAACA-3' | 2.409542772 | 6.842285x10-3 |
| hsa-piR-33110 | 5'-CGAGAATGATGAACGATGCTTCCAGATTCTGACAC-3' | 3.302763067 | 1.0722637x10-2 |
| hsa-piR-32876 | 5'-ATATCATGATGTTACTTTGATTCTCTGACC-3' | 5.176551309 | 1.4853741x10-2 |
| hsa-piR-33051 | 5'-AAACAATGATGGAGTTGCAAGGGTCTGAGC-3' | 2.954871635 | 2.0095922x10-2 |
| hsa-piR-33157 | 5'-GTGCTGGGATGAACGTTTTAACATCTGAGCAG-3' | 4.929144892 | 2.0802856x10-2 |
| hsa-piR-33052 | 5'-AAACTGATGATGCTTGAATTCCTGTTTACTCTGAAG-3' | 4.352093533 | 2.0979839x10-2 |
| hsa-piR-33061 | 5'-ACATGTGATGAGATCGTTGCTCTGATGG-3' | 2.816116428 | 2.1997222x10-2 |
| hsa-piR-7532 | 5'-TCTCATAATGAAGACATAGCCGATTCTCTGC-3' | 4.464241618 | 2.24885x10-2 |
| hsa-piR-7434 | 5'-TCTCAAAGTGAAAGGACCAGTTCGAAT-' | 2.161793669 | 2.24885x10-2 |
| hsa-piR-21852 | 5'-TGTGCTGACCATGGGCCCTGAGCGTCCT-3' | 4.420598295 | 2.6320863x10-2 |
| hsa-piR-13685 | 5'-TGCAGAGATCATACCCCAGAACCAAAAGGCC-3' | 3.789043899 | 3.0982636x10-2 |
| hsa-piR-33088 | 5'-CACCGTGATGAATAGATACTCTGAAGC-3' | 2.28739202 | 3.0982636x10-2 |
| hsa-piR-33159 | 5'-GTTCCAGGATGAAACCATGCGTATCTGAGC-3' | 2.222917393 | 3.0982636x10-2 |
| hsa-piR-20065 | 5'-TGGTCATTGACAATGGCTCCGGCATGTGC-3' | 2.846357394 | 3.3871133x10-2 |
| hsa-piR-16144 | 5'-TGCTGGGAAACGCAAAGCATCCGGAC-3' | 4.905862403 | 3.4132054x10-2 |
| hsa-piR-33152 | 5'-GGGCTGATGATGACCTCTGCAACTCTGAAGCAA-3' | 2.959235719 | 3.9836546x10-2 |
| hsa-piR-29906 | 5'-TACACCTAAGAAACAAGGAGGACTGGGA-3' | 2.521517367 | 3.9848324x10-2 |
| hsa-piR-7244 | 5'-TCGTTGCGGATGGCCAGCTGGAGGTGA-3' | 2.513102981 | 6.612213x10-2 |
| hsa-piR-9943 | 5'-TGACGGTTCCCTGTCTCTGAAAGACCTT-3' | 2.837158255 | 7.18151x10-2 |
| hsa-piR-10194 | 5'-TGAGAACCAATGGGAAGGAGCCTGAGC-3' | 2.094934269 | 7.6956152x10-2 |
| hsa-piR-25936 | 5'-TTTGAGGGTGATGATGGATTCTGTGT-3' | 3.248962354 | 8.9802476x10-2 |
| hsa-piR-30376 | 5'-TACCTCATGAAGATCCTCACCGAGCGCGGC-3' | 2.548138494 | 9.404138x10-2 |
| hsa-piR-10344 | 5'-TGAGACCAATGAAATCGCCAATGCCAAC-3' | 2.470225538 | 9.7043131x10-2 |
| hsa-piR-27076 | 5'-GCAAGGTGGGTCTCAGAGGTGATCGGCGA-3' | 2.088493166 | 9.7043131x10-2 |

B. Downregulated piRNAs

| | | | |
|---|---|---|---|
| hsa-piR-31535 | 5'-TAGGACATTATGACGTGCTTGGGTTC-3' | -3.492343956 | 3.3893191x10-2 |
| hsa-piR-32974 | 5'-GTCCTGCAATTCACATTAATTCTCACAGCT-3' | -2.903453446 | 1.21x10-5 |
| hsa-piR-23041 | 5'-CCCCTGGTGGTCTAGTGGTTAGGATTCGGC-3' | -2.806147201 | 6.265475x10-3 |
| hsa-piR-32870 | 5'-AGGGTGGTTCAGTGGTAGAATTCTCG-3' | -2.772172955 | 2.24885x10-2 |
| hsa-piR-32883 | 5'-CAAGAATTCTACCACTGAACAACCAATGC-3' | -2.218575542 | 5.546742x10-3 |
| hsa-piR-32938 | 5'-GCATTGGTGGTTCAGTAGTAGAATTCTCG-3' | -1.866421057 | 1.0722637x10-2 |

pi, piwi-interacting.

*Correlation between plasma and tissue samples of matched patients*. Matched plasma and tissue specimens were evaluated for piRNA expression profiles in four PDAC plasma samples. Normalized expression levels of 20 piRNAs were positively correlated in plasma and tissues of patients with PDAC (Pearson's correlation coefficient >0.3; Table SIV). This suggests a common trend of piRNA deregulation between biospecimens and increases the chances of tumour tissue piRNA alteration being reflected in plasma. A total of three piRNAs, hsa-piR-23246, hsa-piR-32858 and hsa-piR-9137, were highly correlated between tumour tissue and plasma of patients with PDAC and may have an important role in disease development.

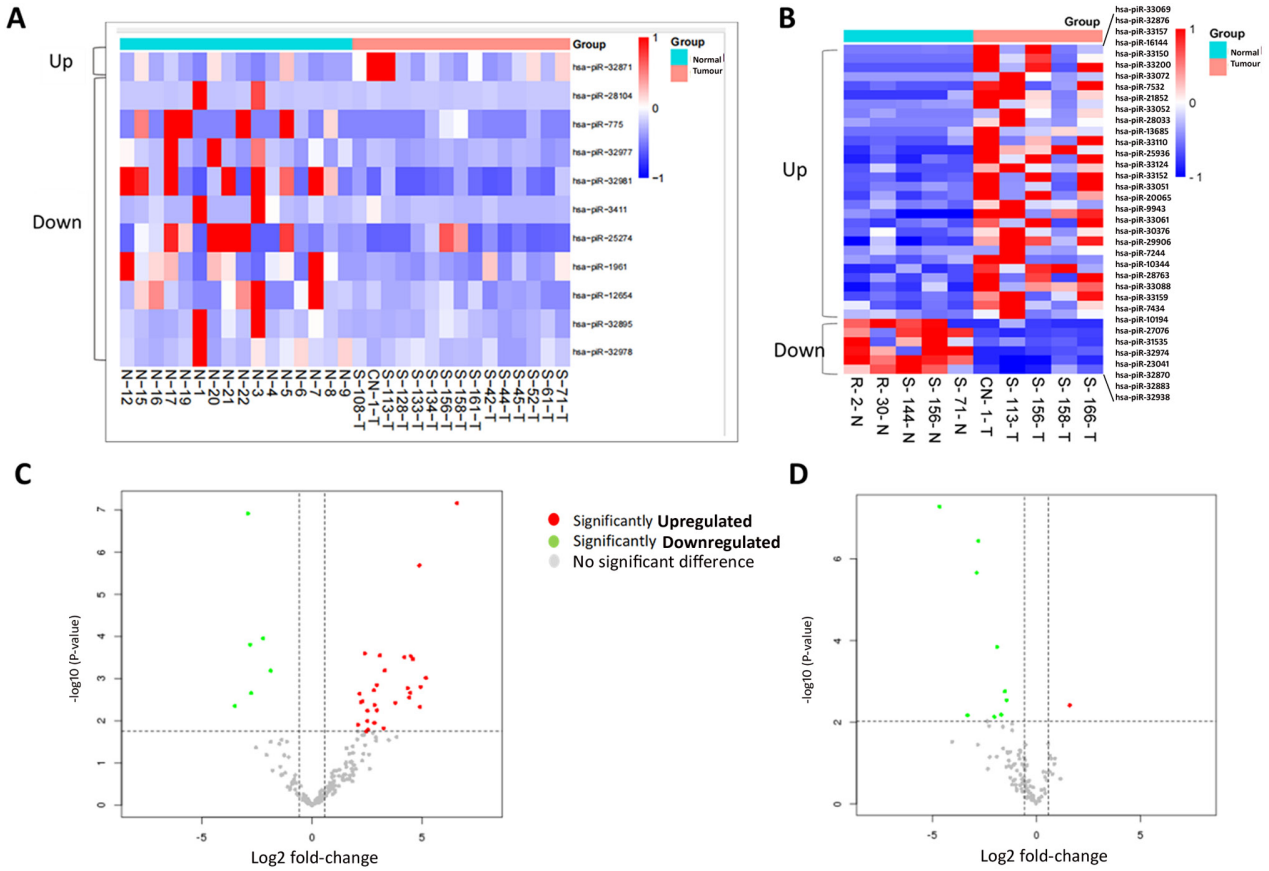


Figure 1. Expression of tissue- and plasma-specific piRNAs in patients with PDAC and healthy individuals. (A) Heatmap of expression of piRNAs across all adjacent normal (n=5) and tumour tissue (n=5). (B) Heatmap of the expression patterns of piRNAs across all plasma samples from healthy individuals (n=16) and patients with PDAC (n=15). Higher expression is shown in dark blue and the lower expression is shown in red colour. (C) Volcano plot shows DE piRNAs in tissue from tumour and adjacent normal samples. (D) Volcano plot showing the DE piRNAs in plasma of healthy individuals and PDAC samples. piRNA, piwi-interacting RNA; DE, differentially expressed; PDAC, pancreatic ductal adenocarcinoma.

*piRNA target prediction*. piRNAs have been found to operate in a manner similar to miRNA, as evident from studies, regulating gene expression through complementary base pairing and exhibiting an inverse correlation with target mRNA expression (9,10,37). By using Miranda algorithm and piRNAdb database to predict the targets of DE piRNAs, 413 mRNA targets for six downregulated DE piRNAs and 1,984 targets for 30 upregulated DE piRNAs were identified (Table SV).

*Pathway analysis of DE piRNA targets*. To determine the role of deregulated piRNAs in patients with PDAC, pathway analysis using predicted genes of DE piRNAs was performed. The top 10 GO classifications of BP, CC and MF were used (Fig. 2A and B). Using targets of upregulated piRNAs, top GO processes included 'monoatomic cation transport' and 'maturation of SSU-rRNA' in the BP subgroup, 'pre-ribosome, small subunit precursor' and '90S pre-ribosome' in the CC and 'mRNA 5' UTR binding' and 'histone demethylase activity' in the MF subgroup (Tables SVI-SXI). Similarly, using downregulated piRNA and targets, top GO processes were identified as cAMP-mediated signaling regulation, regulation of transcription and regulation of arginine-histamine methylation in BP group. Rough endoplasmic reticulum membrane and RISC complex were the top CC subgroup, while histone demethylase activity and promoter-specific chromatin binding were the top GO processes in the MF subgroup. Additionally, 154 KEGG pathways were identified among dysregulated piRNAs and their targets (Tables SXII and SXIII). Pathway enrichment analysis showed that several key pathways such as pathways of glycolysis and gluconeogenesis, pathways of bile secretion, pathways in cancer, glucagon signaling pathway, 'insulin signaling pathway' and MAPK signaling pathway were enriched (Fig. 3A and B) (Tables SXII and SXIII). These results indicated alteration of multiple malignancy-specific pathways in PC.

*Predicting piRNA clusters*. proTRAC was used to predict genomic location enriched with piRNA clusters in PC and normal samples. A total of 262 clusters were identified in plasma samples (Table SXIV) containing 40 piRNAs. Among these, 18 were exclusive to PC patients, 15 were common to both patient and normal samples and seven were unique to normal samples. A total of 34 clusters was identified in the patient genome while analyzing the tissue samples. Out of these, 17 clusters showed high normalized count reads. From these high-density clusters, 10 clusters demonstrated the presence of 25 enriched piRNAs (Table SXV). Similarly, within normal tissue samples, 24 clusters were identified alongside 12 highly concentrated

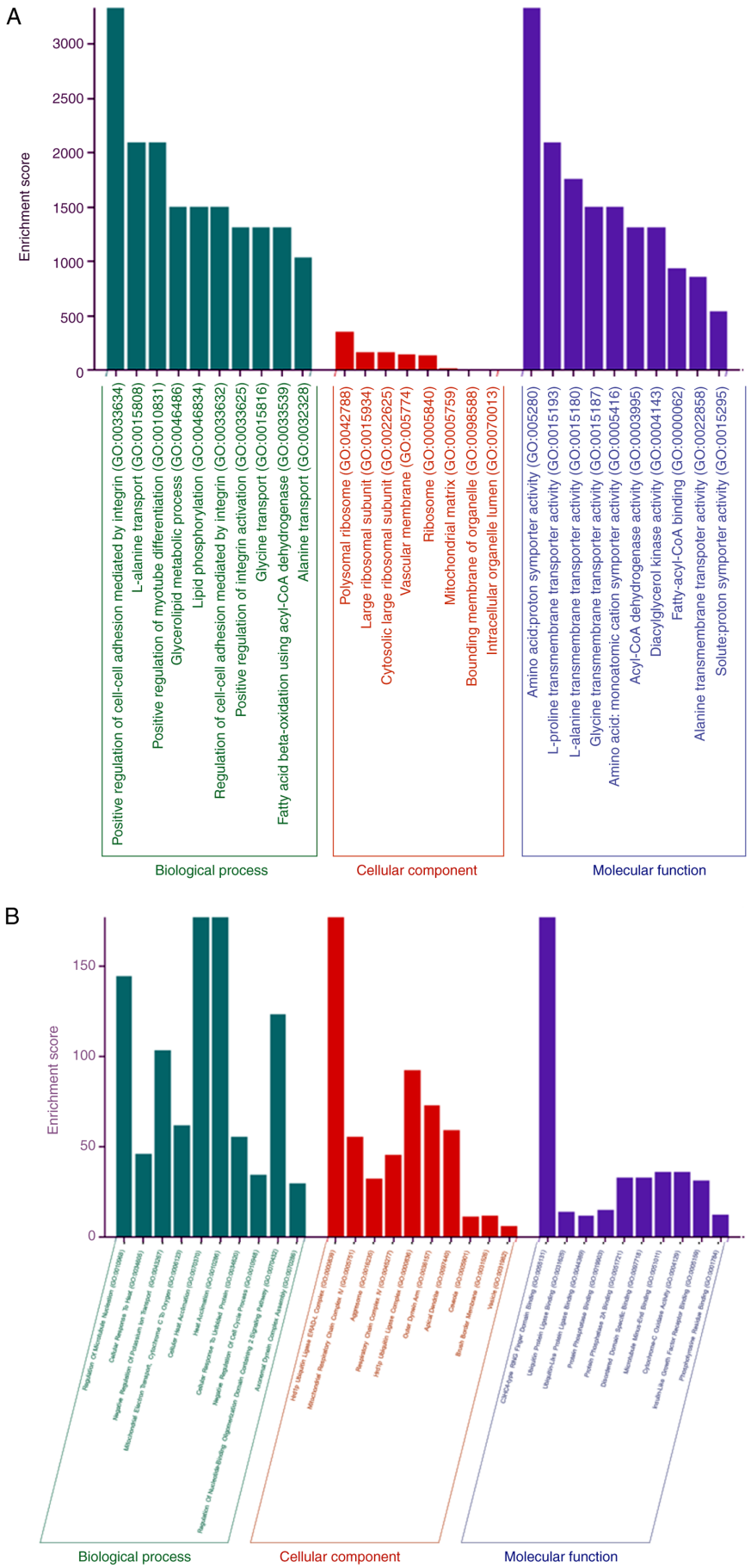


Figure 2. GO analysis with the targets of differentially expressed piRNAs in tissue samples. Top 10 pathways derived from GO analysis with the targets of (A) up- and (B) downregulated piRNAs. GO, Gene Ontology; pi, piwi-interacting.

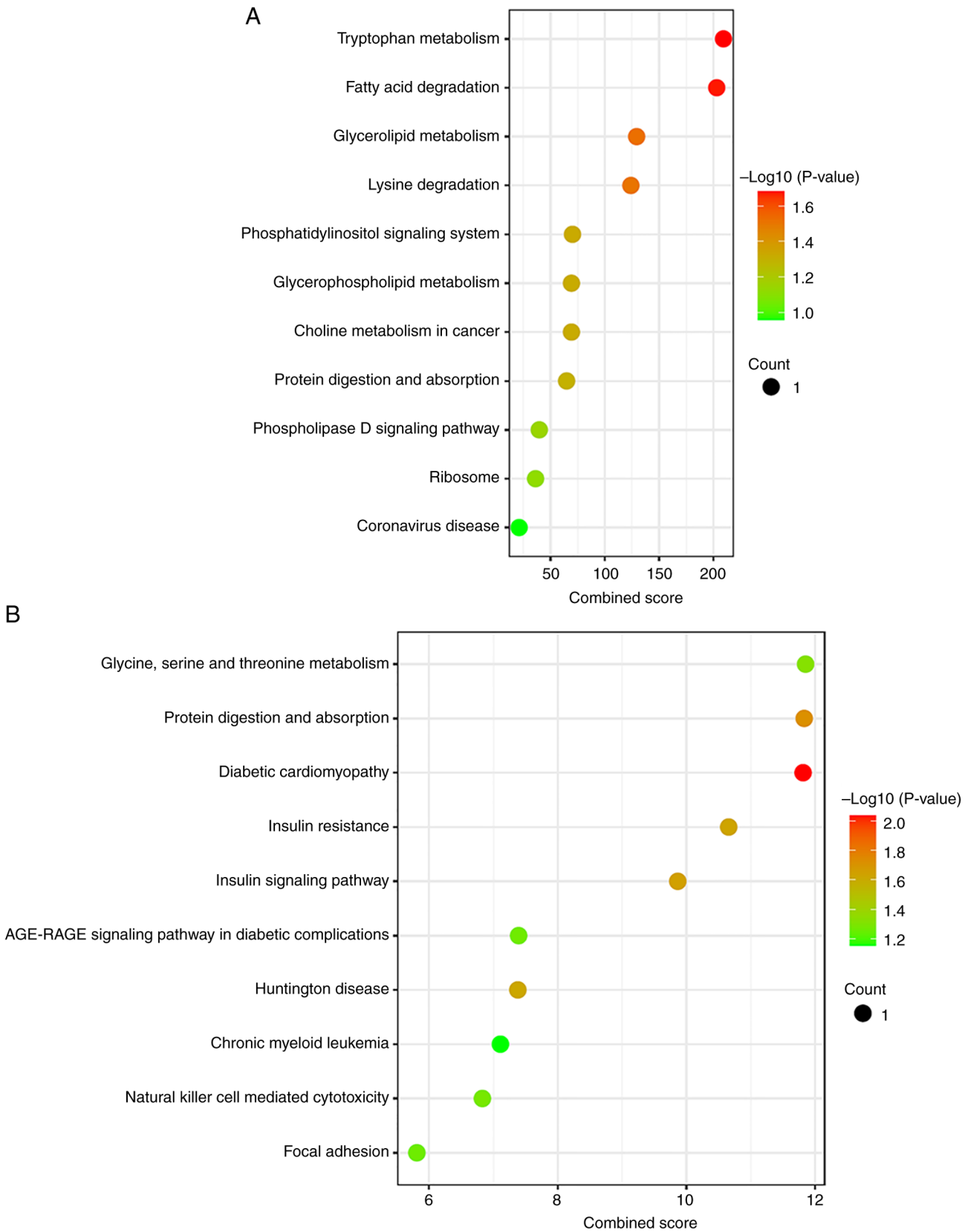


Figure 3. KEGG annotation and enrichment analysis with targets of differentially expressed piRNAs in tissue samples. Top 20 pathways derived from KEGG analysis with the targets of (A) up- and (B) downregulated piRNAs. pi, piwi-interacting; KEGG, Kyoto Encyclopedia of Genes and Genomes.

clusters. One cluster was the origin of six piRNAs, of which hsa-piR-32859, hsa-piR-22269, hsa-piR-20792, hsa-piR-32002 and hsa-piR-15181 were DE.

Although there was an asymmetric distribution throughout the chromosomes, clusters of piRNAs were more prevalent in chromosomes 9, 10, 18, 20 and Y (Table SXV; Fig. 4A). There

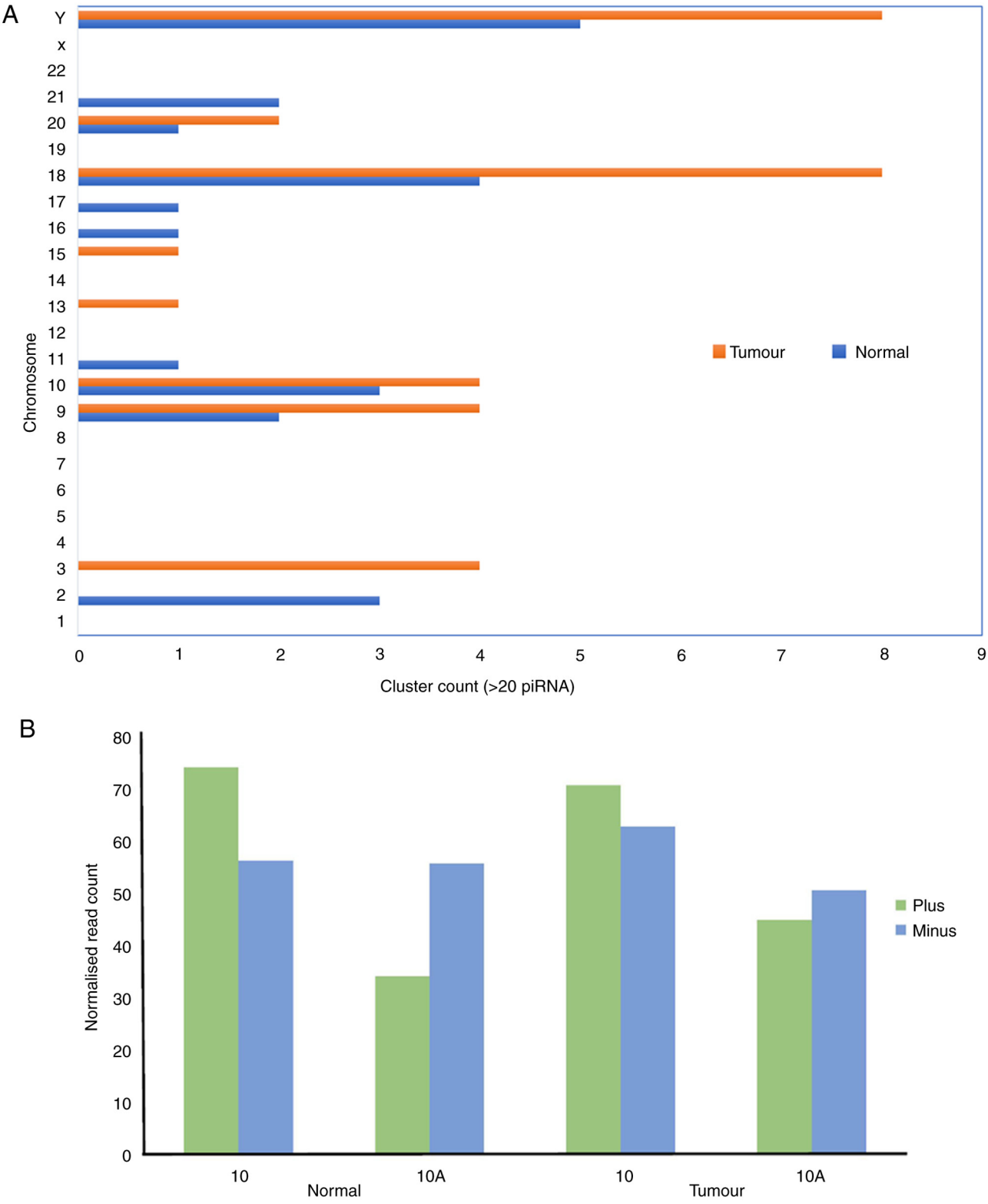


Figure 4. Distribution of piRNA clusters derived from tumour and adjacent normal samples. (A) piRNA clusters across the chromosome. (B) piRNA clusters from both groups show a bias of 1U and 10A. pi, piwi-interacting.

were no clusters detected on chromosomes 1, 4, 5, 6, 7, 8, 12, 14, 19, 22 and X. Furthermore, the nucleotide preference among the piRNAs was also investigated. Specifically, piRNAs encoded from the plus strand exhibited the strongest preference for 1U compared with those from the minus strand (9,10). However, a higher bias for 10A was observed in the minus compared with the plus strand (Fig. 4B). This indicated the importance of orientation in acquiring or determining the type of biogenesis pathway of piRNA production. Based on this genomic feature, orientation is key in determining the type of pathway by which piRNA is produced. Fig. S2 shows a representative image of piRNA cluster visualization.

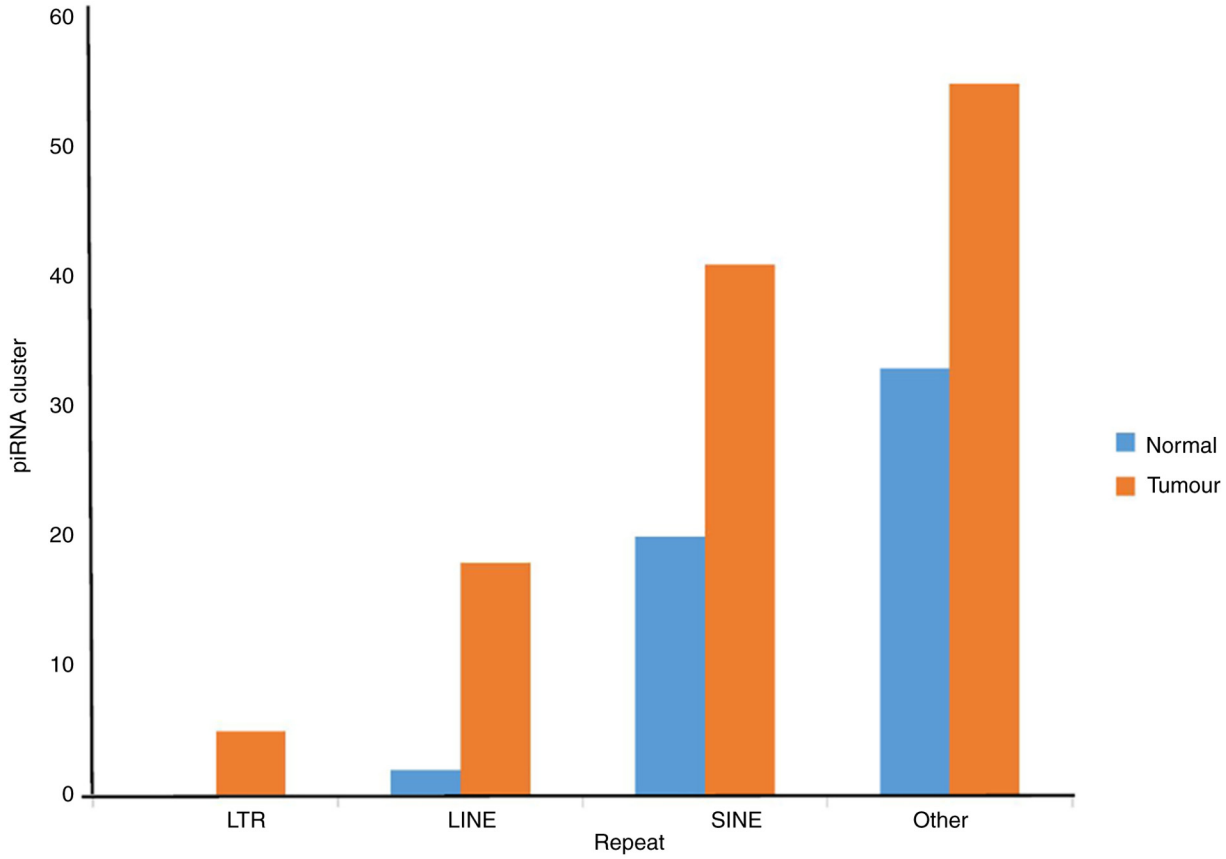


Figure 5. Distribution of the origin of the piRNA clusters among repeats and retro-repeats. pi, piwi-interacting; LTR, long terminal repeat; LINE, Long Interspersed Nuclear Elements; SINE, Short Interspersed Nuclear Elements).

*Functional annotation of the origin of piRNA clusters*. TEs or jumping genes occupy 45% of the entire human genome. TEs such as long Interspersed Nuclear Elements and SINE (Short Interspersed Nuclear Elements) are small repetitive sequences and easily enter or jump into any position of genome (38). This type of insertion leads to mutations and contributes to cancer development (38). To understand how piRNAs silence TEs, their origin was analyzed from the genome cluster and targeted coordinates in those regions were identified (Table SXV; Fig. 5) (39-41).

There was a higher density of piRNA origin found in tumour samples compared with normal samples. Additionally, density of piRNA clusters present in tumour samples was higher in SINE repeats. Long Terminal Repeats regions that harbour piRNAs were identified only in tumour samples. In normal samples, piRNAs were mapped to the DNA transposons like Tc1/mariner, DNA/TcMar-Tigger, DNA/hAT-Charlie and DNA/hAT-Tip100.

*DE piRNAs in plasma of patients with CP and healthy individuals*. Pre-operative plasma samples from eight patients with CP were used and circulating piRNA expression pattern was compared with that of healthy control individuals (n=16). A total of four upregulated piRNAs and 15 downregulated piRNAs were found in the plasma of patients with CP (Table SXVI; Fig. S3). piRNAdb was used to identify potential targets of those piRNA (Table SXVII). Most of the target genes contribute to chronic inflammation in multiple organs (Table II). The results of the present study suggested similar changes of piRNAs in pancreatic tissues of patients with CP that cause deregulation of target genes contributing to chronic inflammation. Additionally, the involvement of these target genes in PC was investigated using TCGA-PAAD. Almost all the genes were upregulated in pancreatic tumour tissue, indicating that the inflammatory condition in CP may also be present in pancreatic tumour tissue and promote carcinogenesis.

## Discussion

Circulating ncRNAs are being studied in greater detail for their role as potential disease biomarkers (6,7). It is hypothesized that the changes seen in various body fluids are the reflection of the changes in corresponding diseased tissue and may additionally indicate altered regulation of gene expression. Although cell-free and exosomal miRNAs have been studied, to the best of our knowledge, there are few studies on piRNAs (6,7). PC is very aggressive in nature and investigations on circulating miRNAs are quite a few (42,43). However, there is also not much information on circulating piRNAs in PC. Hence, it is necessary to identify non-invasive biomarkers for timely diagnosis. One study has reported identification of piR-168112 and piR-162725 in both PC cells and patient plasma. Expression level of piR-162725 was measured in patients along with CA19-9 level. Combined analysis of both the values of piR-162725 and CA19-9 in all the patients

Table II. Pro-inflammatory target genes for differentially expressed piwi-interacting RNAs in plasma of patients with chronic pancreatitis.

| Target gene | Role in modulating inflammatory pathways | GEPIA PAAD fold-change (TCGA data) | (Refs.) |
|---|---|---|---|
| HES7 | Significantly increased expression facilitates development of severe/very severe COPD | 1.8 | (66) |
| TRPM8 | Induces ophthalmological neuroinflammatory disease | 5.8 | (74,89) |
| INTS4 | Increases cell proliferation and inflammation signaling during development of glioma | 1.7 | (71,78) |
| SCAMP4 | Promotes systemic inflammation and contributes to development of SLE | 2.4 | (67,85) |
| API5 | Facilitates TLR4-dependent activation of antigen-presenting cells | 2.3 | (68,82) |
| IFT88 | Promotes inflammatory responses in non-ciliated macrophage | 2.0 | (72,73,81) |
| PDE3A | Promotes proinflammatory functions in platelets | 1.6 | (65,83) |
| TM9SF2 | Oncogene in colon cancer and promotes inflammation | 2.7 | (64,84) |
| EFCAB11 | Upregulated in inflammatory conditions resulting in asthma | 7.1 | (62,88) |
| SYNRG | Upregulated in sepsis associated lung inflammation | 2.3 | (61) |
| SLCO5A1 | Upregulated in oesophageal epithelial cells upon induction of inflammation by acidic bile salt | 2.9 | (71) |
| EED | Upregulated in neuroinflammation | 2.1 | (76,87) |
| G3BP2 | Promotes oscillatory shear stress-induced inflammation in endothelial cells | 4.3 | (70,77) |
| RYR2 | Promotes inflammation in spinal cord and diabetic cardiomyopathy; induces oxidative stress | 0.22 | (75,79) |
| WWP2 | E3 ubiquitin ligase that regulates pro-fibrogenic monocyte infiltration and activity in heart fibrosis | 1.9 | (63,80) |
| MACF1 | Alteration associated with metabolic syndrome and inflammation | 3.2 | (69,86) |

GEPIA, Gene Expression Profiling Interactive Analysis; PAAD, pancreatic adenocarcinoma; TCGA, The Cancer Genome Atlas; COPD, Chronic Obstructive Pulmonary Disorder; SLE, Systemic lupus Erythematosus; TLR, Toll Like Receptor.

increased the sensitivity to 89.7%, which is about 15% more than CA19-9 sensitivity alone (13).

piRNA expression patterns were investigated in tissue and plasma sample of the same patients. A positive correlation of expression of certain piRNAs was shown between tissue and plasma. Additional piRNAs were shown to be deregulated in plasma samples as well as in tissue samples (hsa-piR-23246, hsa-piR-32858 and hsa-piR-32858). hsa-piR-23041 is downregulated in PDAC tissue sample as per the piRDB database. To the best of our knowledge, there is little information on disease association of piRNAs compared with other ncRNAs. piRNAs have evolved as a countermeasure to suppress TEs. piRNA clusters are sites throughout the genome from where most piRNAs are synthesized. These clusters generally overlap with a large number of TEs. Hence, piRNA sequences derived from each cluster are homologous to TEs in the same cluster and to similar TEs residing in other parts of the genome (44,45). Therefore, it is key to determine expression data of piRNAs from the clusters, while considering suppression of TEs by piRNAs in both cis- and trans- context. From the present expression and cluster analysis, hsa-piR-15181, hsa-piR-22269, hsa-piR-32859, hsa-piR-32002 and hsa-piR-20792 were overexpressed in tumour samples and were identified in relevant piRNA clusters through proTRAC analysis. There are two types of piRNA biogenesis pathways. The primary maturation pathway produces piRNAs and the secondary maturation pathway amplifies those piRNAs. The primary maturation pathway shows a bias for U at position 5' (46) and the secondary amplification pathway shows a bias for A at position 10. Here, it was shown that bias differed between groups. The number of piRNAs with a bias for A at position 10 was comparatively lower than the bias for 'U'. This bias for 'A' is indicative of the fact that there is more piRNA formation through primary maturation pathway. It is necessary to conduct additional research to determine biogenesis of piRNAs. The presence of TEs in tumor samples suggests piRNAs are generated more frequently to silencing TEs. piRNAs are hypothesized to modulate other cellular functions by targeting specific mRNAs and hence, identification of their targets may identify the pathways they modulate to mediate disease development or progression. Metabolic reprogramming has been proposed as a key hallmark of malignancy. The uptake and catabolism of amino acids are aberrantly altered and in general, amino acids promote the survival and proliferation of cancer cells under cell stress and

provide growth advantage to the tumour (47,48). Significant downregulation of multiple amino acid catabolism pathways was revealed in the present study, as well as fatty acid degradation pathways, suggesting potential modulation of metabolic reprogramming by piRNAs. Glutaryl-CoA dehydrogenase (GCDH) is a key enzyme involved in the degradative pathway of L-lysine, L-hydroxylysine and L-tryptophan metabolism (49). This gene was a direct target of upregulated piR-7244. GCDH gene has been previously reported as a tumour suppressor gene in hepatocellular carcinoma (50) and may function in the same manner in PDAC. Similarly, diacylglycerol Kinase Gamma (DGKG) gene is a member of the type I subfamily of diacylglycerol kinases, which are involved in lipid metabolism (51). DGKG is a target of upregulated piR-10194 identified in our results. The present study found alteration of lipid catabolizing pathways from our pathway analysis. Therefore, DGKG gene expression might contribute to observed suppression of lipid catabolizing pathways.

Nucleotide-binding oligomerization domain receptor-2 (NOD2) exerts oncogenic effects via activation of the NF-κB and ERK signaling pathways. Activation of NOD2 signaling through upregulation of either NF-kB or ERK signaling pathways revealed that gasdermin D is involved in this pathway (52). To the best of our knowledge, there are no studies of gasdermin D in PC, however other gasdermin family proteins (gasdermin E) have been shown to promote chemo-resistance in PC (53). Ras-related nuclear protein-guanine nucleotide release factor) has also been observed as an upregulated target of the present downregulated piRNAs and is an important component of the microtubule nucleation process. Microtubule dynamics is an important player in cancer (54) and nucleation is the most important regulatory step. Unfolded protein response (UPR) is constitutively active in PDAC, likely contributing to disease progression and acquisition of therapeutic resistance (55). Disabled Homolog 2 Interacting Protein) and DAXX (Death Domain Associated Protein), which serve as regulators of UPR (unfolded protein response) (56), were targets of downregulated piRNAs hsa-piR-28096 and hsa-piR-23041, respectively, indicating the potential role of these altered piRNAs in modulating UPR-driven signaling in pancreatic cancer. Among upregulated pathways, the insulin and the AGE-RAGE signaling pathways are implicated in PC (17). Notable genes such as SMAD3 (Sma- And Mad-Related Protein 3), TGFBR2 (Transforming Growth Factor beta Receptor 2) and PPP1R3B (Protein Phosphatase 1 Regulatory Subunit 3B) were upregulated (targets of downregulated piRNAs) may play an important role in piRNA-mediated development of PC. Another upregulated pathway, focal adhesion and associated focal adhesion kinase, has also been reported in the metastasis of PC and the integrin signaling pathway is instrumental in this process (57). Caveolins have also been found to modulate integrin function (58) and 3D collagen architecture is also reported to regulate cell adhesion (59). The identification of upregulated target genes regulating the focal adhesion pathway (integrin Subunit Beta 6, CAV3-Caveolin 3, COL4A6-Collagen Type IV Alpha 6 Chain) provides insight to the possible mechanism. All the aforementioned findings indicate the potential roles of altered piRNAs as well as their altered targets in carcinogenesis. According to the results of the present study, hsa-piR-23246, hsa-piR-32858 and hsa-piR-9137 may serve as plasma biomarkers.

DE piRNAs were also identified in the plasma of patients with CP. To the best of our knowledge, the present study is the first on the alteration of piRNAs in patients with CP. CP is a progressive fibro-inflammatory disorder and is considered a pre-malignant condition for PC (60). Therefore, it is key to identify characteristic changes in the serum or tissue piRNAs in these patients to identify inflammation and malignancy specific alterations. After identification of the target genes, their biological functions were investigated; >50% of the target genes were proinflammatory and were reported to promote inflammation in other organs (61-76). Among these genes, transient Receptor Potential Cation Channel Subfamily M Member 8), SCAMP4 (Secretory Carrier Membrane Protein 4), TM9SF2 (Transmembrane 9 Superfamily Member 2) and G3BP2 [GTPase Activating Protein (SH3 Domain) Binding Protein 2] expression is also increased in patients with PDAC (77-89). CP increases the risk of PC, and overexpression of these genes not only promotes CP, but also maintains the inflammatory milieu in pancreatic tumour tissue.

The present results suggested that piRNAs hsa-piR-23246, hsa-piR-32858 and hsa-piR-9137 may be used as potential biomarkers to distinguish pancreatic malignancy. Additionally, alteration of specific piRNAs in pancreatic tumour tissues could drive the process of tumorigenesis. However, the present study did not assess the expression status of these three piRNAs in other gastrointestinal disease, which would determine the specificity and sensitivity of the signature. Validation of the piRNAs in a different cohort of patient samples and healthy individuals was not performed. Functional validation of the altered piRNAs and their target genes should be performed in future.

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**Availability of data and materials**

The data generated in the present study may be found in the Indian Biological Data Centre under accession number INCARP000298 or at the following URL: (inda.rcb.ac.in/indasecure/userstudydetails).

**Authors' contributions**

BS performed experiments, analyzed data and wrote the manuscript. SC and BM performed sequence analysis and wrote the manuscript. SR, HS, IG and KD designed the study and wrote the manuscript. NKB designed and supervised the study. SG conceptualized, designed and supervised the study. All authors have read and approved the final manuscript. SG and BS confirm the authenticity of the raw data.

**Ethics approval and consent to participate**

The present study was approved by Institutional Ethics Committee of National Institute of Biomedical Genomics (Kalyani, India; approval no. CERTIFICATE-SG1-MARCH 05 2014), Institute of Post Graduate Medical Education & Research (Kolkata, India; approval nos. INST/IEC/2015/218 and IPGME&R/IEC/2022/318) and Chittaranjan National Cancer Institute (Kolkata, India; approval no. CNCI-IEC-SG2-2023-69). Written informed consent was obtained from the study participants.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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