

Identification of candidate miRNA biomarkers for pancreatic ductal adenocarcinoma by weighted gene co-expression network analysis

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Accepted: 25 January 2017 / Published online: 15 February 2017
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Abstract

Purpose Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive malignancy with a dismal prognosis which is, among others, due to a lack of suitable biomarkers and therapeutic targets. Previously, basic gene expression analysis methods have been used for their identification, but recently new algorithms have been developed allowing more comprehensive data analyses. Among them, weighted gene co-expression network analysis (WGCNA) has already been applied to several cancer types with promising results.

Methods We applied WGCNA to miRNA expression data from PDAC patients. Specifically, we processed microarray-based expression data of 2555 miRNAs in serum from 100 PDAC patients and 150 healthy subjects. We identified network modules of co-expressed miRNAs in the healthy subject dataset and verified their preservation in the PDAC dataset. In the non-preserved modules, we selected key miRNAs and carried out functional enrichment analyses of their experimentally known target genes. Finally, we tested their prognostic significance using overall survival analyses.

Results Through WGCNA we identified several miRNAs that discriminate healthy subjects from PDAC patients and that, therefore, may play critical roles in PDAC development. At a functional level, we found that they regulate p53, FoxO and

ErbB associated cellular signalling pathways, as well as cell cycle progression and various genes known to be involved in PDAC development. Some miRNAs were also found to serve as novel prognostic biomarkers, whereas others have previously already been proposed as such, thereby validating the WGCNA approach. In addition, we found that these novel data may explain at least some of our previous PDAC gene expression analysis results.

Conclusions We identified several miRNAs critical for PDAC development using WGCNA. These miRNAs may serve as biomarkers for PDAC diagnosis/prognosis and patient stratification, and as putative novel therapeutic targets.

Keywords Pancreatic cancer · PDAC · miRNA expression · WGCNA · Systems biology · Biomarkers

1 Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy and the fourth most common cause of cancer death worldwide, with a five-year survival rate of approximately 3–6% and a median survival rate of 2–8 months [1]. These poor survival rates are mainly due to the asymptomatic and invasive nature of the disease at advanced stages, often with metastasis at the time of diagnosis, and to a chance of only 10% of being operable [2]. Therefore, suitable diagnostic and prognostic biomarkers and therapeutic targets are considered crucial, but they are currently scarce. Although several gene and protein biomarkers have been proposed [3], none of them is sensitive and/or specific enough. For example, the biomarkers carbohydrate antigen 19–9 (CA 19–9), carcinoembryonic antigen (CEA) [4] and LGR5 [5] have so far not met their initial expectations. Other biomarkers, such as KRAS, CDKN2A, TP53 and SMAD4, have been proposed

Electronic supplementary material The online version of this article (doi:10.1007/s13402-017-0315-y) contains supplementary material, which is available to authorized users.

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based on their high mutational status and their involvement in important cellular pathways, such as those regulating apoptosis and cell cycle progression, DNA damage control, and Hedgehog, TGF- β , Wnt/Notch and KRAS signalling [2]. Also, microarray-based expression profiling studies have revealed hundreds of genes that are differentially expressed in PDAC tissues compared to normal pancreatic tissues that, as such, may serve as biomarkers [6, 7].

While the identification and validation of biomarkers are ongoing we should, however, reflect on their failures in order to guide future research. One major drawback of the interpretation of microarray-based expression data is that it addresses individual genes and does not consider correlations among genes. Certainly, the identification of differentially expressed genes may provide biologically and pathologically relevant information, but thanks to recent bioinformatic advancements, new and effective algorithms to interpret gene expression data may allow a more comprehensive identification of new biomarkers. As has extensively been reported [8–11], genes with similar expression patterns may code for proteins with similar biochemical and functional properties, that may form complexes or may act in similar pathways. Therefore, differential co-expression network analysis algorithms have been developed and applied to investigate gene and microRNA (miRNA) expression data [12–15]. As such, the weighted gene co-expression network analysis (WGCNA) algorithm [16] represents a novel systems biology approach that detects key genes related to a sample trait in a network of co-expressed genes. WGCNA is based on similarities in expression profiles among samples and detects, in the network, highly co-expressed genes that are closely connected. These sub-network regions are called modules that have been proven to be conserved across phylogenies, to be enriched in protein-protein interactions and often to consist of functionally related genes [8, 11, 17–19]. By constructing two gene networks, based on normal and tumour tissue expression data, it is possible to identify modules and genes that exhibit different network properties. These distinguishing elements may be involved in pathological processes and could serve as diagnostic and prognostic biomarkers or as potential therapeutic targets. Recently, researchers have been using WGCNA to obtain new and promising candidate biomarkers [20–23]. Previously, we also applied WGCNA to PDAC gene expression data [24] and identified new candidate biomarkers, besides others that were previously reported as potential PDAC biomarkers, thus confirming the reliability of this method.

In addition to genes and proteins, also miRNAs may serve as biomarkers since they can act as tumour promoters (“oncomiRs”) or suppressors, by silencing or promoting known cancer-related genes and pathways, respectively [25]. As for other cancers, aberrant expression of several miRNAs has been linked to PDAC development, and they have been proposed as diagnostic and prognostic biomarkers, and also as indicators of tumour aggression, metastasis, therapy resistance

and response [25–27]. Furthermore, miRNAs have been found to play a role in epithelial-mesenchymal transition (EMT) [28], which has previously been suggested to be an essential process related to the progression of several cancers, including PDAC [29, 30]. Moreover, miRNA profiles can distinguish cancers from disorders with similar clinical presentations such as chronic pancreatitis and pancreatic intraepithelial neoplasia (PanIN) [31]. In recent years, miRNA expression profiles have extensively been generated from PDAC patient-derived blood samples, which has resulted in the identification of miRNAs for the diagnosis of pancreatic cancer [32, 33]. Although there are still some methodological challenges to be addressed for the correct quantification of these circulating miRNAs [34], they represent attractive alternatives to tissue-derived miRNAs, including abundance, non-invasive isolation and inexpensive amplification.

Here, we analysed microarray-based miRNA expression profiles from PDAC patient- and healthy individual-derived serum samples. In particular, we applied WGCNA to identify key miRNAs potentially involved in the pathogenesis of PDAC and validated them as diagnostic/prognostic biomarkers. These miRNAs may shed further light on the biological mechanisms underlying PDAC development. They may also serve as potential therapeutic targets.

2 Materials and methods

2.1 miRNA expression data and pre-processing

The recent microarray-based expression dataset GSE59856 [35] was downloaded from the NCBI Gene Expression Omnibus (GEO). It contains quantile normalized miRNA expression data of serum samples obtained from 150 healthy subjects and 100 PDAC patients. The dataset was produced using microarray platform 3D-Gene Human miRNA V20_1.0.0 and was based on miRNA sequences listed in a recent release (v20) of the miRBase database (www.mirbase.org) for probe design, which allows the expression assessment of 2555 miRNAs. As reported before [24], data analyses were performed using the R programming language (v3.3.0), Bioconductor packages (v3.0) and R functions in the WGCNA package [36]. The R codes for reproducing our analyses are provided in the Supplementary Materials.

In order to detect outlier samples, we followed the standardized connectivity (Z_K) method as proposed before [37]. The Z_K score represents the overall strength of connections between a given node and all of the other nodes in a network. Samples with a Z_K score < -2 have to be considered as outliers [37]. To assess comparability between normal and PDAC samples, necessary for subsequent analyses, we used the *softConnectivity* function from package WGCNA. This function measures, across the two datasets, the correlation of the expression level of each miRNA

and the correlation of the connectivity of each miRNA, i.e., its co-expression levels with other miRNAs, in the dataset. Datasets are comparable if these two correlations are positive and the p -values are significant. Moreover, higher correlation values (ranging from 0 to 1) indicate higher comparability between the normal and PDAC datasets. Generally, correlation values are better for expression than for connectivity parameters. We also applied the *pickSoftThreshold* function of the *WGCNA* package to assess whether the topology of the networks was scale-free, as is required for WGCNA analysis. This function provides a *Scale-free Topology Fit Index* that, for scale-free topology networks, reaches values above 0.9 for low powers (< 30) as defined in [16].

2.2 Weighted gene co-expression networks and their modules

We created two weighted gene co-expression networks based on the normal and PDAC miRNA expression data, using standard *WGCNA* procedures [16], including the creation of a matrix of adjacencies and its transformation into a topological overlap matrix (TOM) that was used as input for the hierarchical clustering analysis. In the resulting dendrograms, the function *cutreeHybrid* from the R package *dynamicTreeCut* was applied to identify network modules present in the normal dataset (used here as reference dataset). The preservation levels of normal network modules in the PDAC network were assessed by the function *modulePreservation* from the *WGCNA* package, that provides a preservation Z-score for each module. Modules with high Z-scores (> 10) are well preserved between normal and PDAC networks, whereas values < 10 are indicative for a moderate to low preservation [38]. The *grey* and *gold* modules are special *WGCNA* modules that were not considered (for details see [24]).

2.3 Detection of hub miRNAs and their functional annotations

The *WGCNA* functions *moduleEigengenes* and *signedKME* were used to identify highly connected intra-modular miRNAs, also called hub miRNAs. In particular, the *moduleEigengenes* function calculates the eigengene of a module, that is a virtual gene representing the gene expression profile of the entire module. The *signedKME* function measures the distance of the expression profile of a gene to that of the module eigengene and, thus, quantifies how close a gene is to a module, i.e., it measures the module membership of a gene. Within a module, the genes with the highest module membership scores are considered hub genes of that module. In analogy to previous studies [24, 39], we here selected the 20 most connected hub miRNAs for each non-preserved module.

Next, we carried out functional enrichment analyses of known miRNA targets in order to facilitate the interpretation of the biological mechanisms related to these miRNAs. The

most interesting miRNAs were used as input for the miRNet web tool [40] to identify the biological pathways, processes, molecular functions and cellular components statistically enriched for the corresponding miRNA target genes. In particular, this tool identifies the enriched KEGG and REACTOME pathways and Gene Ontology (GO) terms based on experimentally confirmed miRNA targets. Since this tool exploits validated miRNA targets, it ensures a higher reliability than tools based on predicted miRNA targets.

2.4 Survival analyses

We used the SurvMicro web tool [41] to carry out survival analyses in order to validate the identified hub miRNAs as prognostic PDAC biomarkers. Survival analyses were performed on an independent PDAC dataset present in The Cancer Genome Atlas (TCGA) (<http://cancergenome.nih.gov/>) containing miRNA expression and survival data derived from 54 PDAC patients (see Table 1 for the description of the GEO and TCGA cohorts). The SurvMicro tool uses the 2013 version of the TCGA collection and, therefore, allows the analysis of only 54 of 154 patients currently collected in TCGA. The SurvMicro tool divides samples into high-risk and low-risk groups through the median of the prognostic index obtained via a Cox regression model. After this, it generates the hazard ratios (HR) with relative confidence intervals (CI) and p -values.

3 Results

3.1 WGCNA prerequisites for normal and PDAC datasets: outlier samples, batch effects, comparability and scale-free topology

The GEO microarray expression dataset GSE59856 [35] contains miRNA expression data of serum samples obtained from 150 and 100 healthy subjects and PDAC patients, respectively. Since weighted gene co-expression network analysis (WGCNA)

Table 1 NCBI GEO and TCGA cohorts used for the survival analysis

		GSE59856	TCGA
Number of patients	-	100	54
Gender	Male	64	26
	Female	36	28
Age	Median	68	66
	(range)	(33–81)	(41–88)
Tumour stage	I	1	4
	II	18	46
	III	27	2
	IV	54	2

Note that TCGA data were obtained at level 3 from the May 2013 run

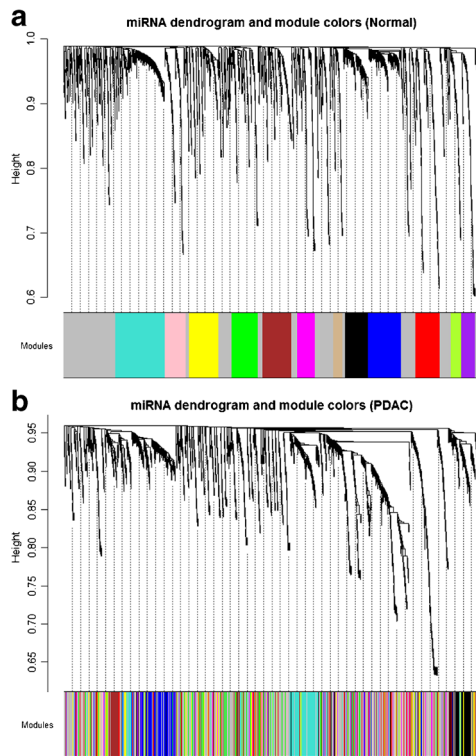


Fig. 1 Clustering dendrograms and modules identified by weighted gene co-expression network analysis (WGCNA). In (a) the clustering diagram and 12 modules for the normal dataset are shown. In (b) the clustering diagram of the PDAC dataset and the normal modules imposed on the PDAC network are shown

requires more than 15 samples to construct a network and to generate robust and refined results, our sample size was largely sufficient. The WGCNA approach is sensitive to the presence of outlier samples and batch effects [17], generally due to systematic and technical differences between different platforms and laboratories. The dataset we chose avoids batch effect issues since it is based on the same type of microarray platform and since the assays were carried out by the same operators. Based on the presence of outliers, we removed 6 and 5 samples from the normal and PDAC datasets, respectively. As required for WGCNA, we also verified whether the normal and PDAC datasets were comparable, i.e., sufficiently similar to highlight even slight differences. This assessment was carried out by the *softConnectivity* function, which provides positive correlation values when there is high comparability between two datasets. Our datasets were found to be comparable since the overall miRNA expression correlation was 0.85 ($p < 1e-200$) and the overall miRNA connectivity was 0.3 ($p < 3.3e-51$) (Supplementary Fig. 1). The connectivity parameter indicates how similar the co-expression level of a miRNA is compared to all other miRNAs in the network. An additional network requirement for WGCNA analysis is scale-free topology, a fundamental property of metabolic and signalling networks in which some nodes (here miRNAs) are more connected (“hub nodes” or “central nodes”) than others (“peripheral nodes”). We found that

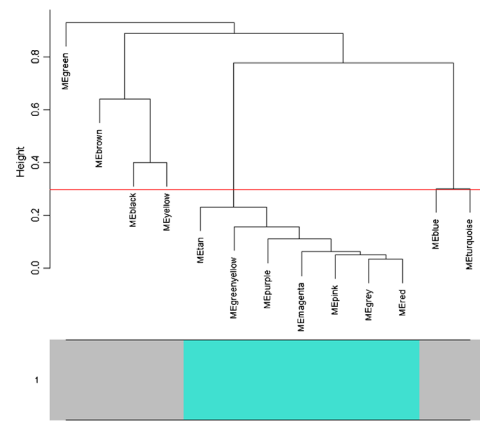


Fig. 2 Meta-module identification. The module network dendrogram was constructed by clustering module eigengene distances. The horizontal line represents the threshold (0.3) used for defining the meta-modules. The *Tan*, *Greenyellow*, *Purple*, *Magenta*, *Pink* and *Red* modules clearly represent a meta-module (hereafter referred to as “M6” meta-module) in the PDAC network, while no meta-module was identified in the normal network (not shown)

the normal and PDAC networks exhibited a scale-free topology since the *Scale-free Topology Fit Index* reached values above 0.9 for low powers (< 30), i.e., the powers were 7 and 5 for the normal and PDAC networks, respectively (Supplementary Fig. 2). This result also indicates that no batch-effects were present in the original microarray datasets.

3.2 Identification of modules able to distinguish PDAC from normal miRNA datasets

After constructing weighted gene co-expression networks derived from the normal and PDAC miRNA expression datasets,

Table 2 Modules identified in the normal network by WGCNA, relative size and preservation (Z-score)

Module	Module size (genes)	Z-score
turquoise	284	16.102
black	133	15.303
blue	186	12.367
brown	169	11.512
greenyellow	58	6.504
purple	80	4.682
yellow	169	4.288
red	138	3.694
pink	122	3.475
green	151	1.977
magenta	101	1.190
tan	56	0.473

Modules with Z-scores > 10 well maintain their characteristics in the PDAC network, so they are defined as preserved modules. Modules with Z-scores < 10 are low preserved, so they can distinguish normal and pathological conditions [38]. There could be an undesirable correlation between module size and Z-score [38], but in this table the R^2 is only 0.4262

Table 3 Hub miRNAs identified in the PDAC network

Module	Hub miRNAs
Green	miR-302d-3p, miR-382-5p, miR-513c-5p, miR-519c-3p, miR-545-5p, miR-548y, miR-641, miR-873-5p, miR-924, miR-942-3p, miR-1289, miR-2115-3p, miR-3115, miR-3149, miR-3678-5p, miR-4520-2-3p, miR-4527, miR-5007-5p, miR-6866-5p, miR-6882-5p
Yellow	miR-135a-3p, miR-204-3p, miR-423-5p, miR-575, miR-1343-5p, miR-3918, miR-4419a, miR-4450, miR-4459, miR-4476, miR-4497, miR-4530, miR-4638-5p, miR-4665-5p, miR-4673, miR-6076, miR-6768-5p, miR-6889-5p, miR-6893-5p, miR-6895-5p
M6 meta-module	miR-196a-3p, miR-548aq-3p, miR-552-5p, miR-890, miR-1269a, miR-1298-3p, miR-2355-3p, miR-4502, miR-4647, miR-4682, miR-4704-5p, miR-4778-5p, miR-4780, miR-6509-5p, miR-6509-3p, miR-6715b-3p, miR-6740-5p, miR-6764-3p, miR-7154-5p, miR-8070

we set out to identify network modules. According to WGCNA, a module is a highly connected sub-graph, that is a group of strongly co-expressed genes. Generally, these modules are composed of genes with similar biochemical and functional properties or belong to similar pathways [8, 11, 17–19]. Through hierarchical clustering, we identified in the normal network 12 modules of different sizes in terms of miRNA number. Each module was labelled by a different colour, according to WGCNA package functions (Table 2). Considering normal as the reference network and PDAC as the test network, we assessed the preservation level across the two networks by evaluating whether the network properties of each reference module were maintained in the test network. This evaluation has previously been performed to e.g. identify sex- and species-associated differences in brain networks or to determine whether certain cellular pathways are perturbed in particular situations, including cancer [11, 20, 21, 38]. We found that, as expected, some modules exhibited similar characteristics in both networks while others were not preserved and, therefore, potentially related to the development of PDAC. By imposing modules belonging to the normal network onto the PDAC network, we found that many module-associated colour labels still clustered together (i.e., the *turquoise*, *blue* and *black* modules) indicating a good preservation between the two networks (Fig. 1). Instead, other modules (i.e., *green* and *yellow*) were found to be scattered throughout the PDAC network, indicating a limited preservation. In order to exactly quantify the module preservation levels, we used the *modulePreservation* function from the WGCNA package. For each module, a Z-score was calculated representing the preservation level (Table 2). The higher the

Z-scores, the more the modules are preserved, whereas modules with Z-scores <10 are lowly preserved and, thus, may distinguish normal from pathological conditions. In particular, the *greenyellow*, *purple*, *yellow*, *red*, *pink*, *green*, *magenta* and *tan* modules were found to be not preserved.

We also ascertained whether there were relationships between modules within each network by verifying the presence of meta-modules [42]. By performing hierarchical clustering on the eigengenes of each module it is possible to determine how close, i.e., co-expressed, the modules are to each other. Therefore, a meta-module represents a cluster of co-expressed modules that can be considered as a higher order of organization of the miRNA profiles [42]. Within the PDAC network we identified a meta-module composed of the *greenyellow*, *purple*, *red*, *pink*, *magenta* and *tan* modules, hereafter referred to as the *M6* meta-module (Fig. 2), that may be indicative of a common regulatory process encompassing the six modules.

3.3 Identification of hub miRNAs and functional enrichment analysis of their targets

Since the *green* and *yellow* modules and the *M6* meta-module were found to be not preserved between the normal and PDAC networks, we set out to identify the central nodes (hub miRNAs) that represent them by selecting miRNAs with the highest module membership scores. These miRNAs may play important roles in the pathogenesis of PDAC. The top 20 hub miRNAs identified for each module in the PDAC network are listed in Table 3. In order to subsequently allow a biological and functional

Table 4 Functional annotation of hub miRNAs: enriched KEGG pathways

Module	Functional annotations
Green ($p < 0.01$)	FoxO signaling, p53 signaling, Prostate cancer, Cell cycle, Pancreatic cancer, Chronic myeloid leukemia, Viral carcinogenesis, colorectal cancer, Proteoglycans in cancer, Glioma, Bladder cancer, Hepatitis B, Pathways in cancer.
Yellow ($p < 0.05$)	Viral carcinogenesis, cell cycle, p53 signaling, bladder cancer, chronic myeloid leukemia, glioma, pancreatic cancer
M6 ($p < 0.05$)	Glioma, chronic myeloid leukemia, ErbB signaling, FoxO signaling, non-small cell lung cancer, Wnt signaling, Hippo signaling, pathways in cancer, colorectal cancer

Table 5 Functional annotation of hub miRNAs: enriched REACTOME pathways

Module	Functional annotations
Green ($p < 0.001$)	Gene expression, generic transcription pathway, cellular responses to stress, oxidative stress induced senescence, signaling by ERBB4, VEGFR2 mediated vascular permeability, translocation of GLUT4 to the plasma membrane, cellular senescence
Yellow ($p < 0.05$)	Pre-NOTCH expression and processing, oncogene induced senescence, cell cycle, mitosis, pre-notch transcription and translation
M6 ($p < 0.05$)	Gene expression, activation of BH3-only proteins, intrinsic pathway for apoptosis, membrane trafficking, translocation of GLUT4 to the plasma membrane, vesicle-mediated transport, generic transcription pathway.

interpretation of these hub miRNAs, we performed a functional enrichment analysis limited to experimentally confirmed miRNA target genes. This choice was made to avoid false positive target genes due to prediction algorithms. The hub miRNA list of each module (or meta-module) was processed using the miRNet web tool to identify enriched KEGG and REACTOME pathways and Gene Ontology (GO) terms (Tables 4, 5 and 6). Genes targeted by the *green* module miRNAs were found to be significantly enriched in cancer pathways, including PDAC, and mainly related to gene expression regulation at both the transcription and translation level. Also the *yellow* module was found to be related to cancer pathways, including PDAC, and to be enriched in genes involved in cell cycle regulation. According to KEGG and REACTOME resources, the *M6* meta-module was mainly related to cancer-associated pathways, apoptosis and transcription regulation.

Using the miRNet tool, miRNA-target interaction networks were created for each module to graphically highlight which miRNAs had a large number of interactions and to calculate the node degree and betweenness, both of which are indicators for node centrality (Fig. 3). We found that miR-302d-3p exhibited the highest node degree and betweenness in the *green* module, miR-6893-5p in the *yellow* module and miR-548aq-3p in the *M6* meta-module. The interaction graph also allowed us to identify the genes (mRNAs) targeted by different miRNAs. By doing so, we found that the *CREBRF*, *GATA6*, *NUFIP2* and *YOD1* (*green* module), *AGO2*, *CCNF*, *KREMEN1*, *LINC00598*, *RAB15*, *SAMD4B*, *SUMO1* and

TPM3 (*yellow* module) and *NUFIP2* (*M6* meta-module) genes were regulated by at least five miRNAs.

3.4 miRNA candidate biomarkers allow the stratification of PDAC patients into high- and low-risk groups

In order to validate our candidate miRNAs as prognostic biomarkers, we assessed whether hub miRNAs in the *green* and *yellow* modules and the *M6* meta-module were associated with the overall survival (OS) of PDAC patients [21, 24]. To this end, we performed both single-miRNA and multi-miRNA (top 20 hub miRNAs) survival analyses in each module by applying the SurvMicro tool to an independent miRNA expression dataset of PDAC patients. By using the top 20 hub miRNAs as input for the SurvMicro tool, we found that the miRNA profiles in all modules successfully stratified PDAC patients into high- and low-risk groups as revealed by Kaplan-Meier survival plots (Fig. 4). Specifically, we found that the OS times of the high-risk group patients were at least two-fold shorter than those of patients in the low-risk group (HR 2.79 [95% CI 1.46–5.33] $p = 0.001939$ for the *green* module, HR 2.05 [95% CI 1.08–3.89] $p = 0.02841$ for the *yellow* module and HR 2.44 [95% CI 1.28–4.65] $p = 0.00645$ for the *M6* meta-module). In addition, we set out to identify miRNA expression signatures that were most significantly associated with PDAC survival outcomes by carrying out single-miRNA survival analyses. We found that an increased expression of each miRNA listed in Table 7, except miR-552-5p, was associated with a better OS.

Table 6 Functional annotation of hub miRNAs: enriched ($p < 0.01$) Gene Ontology (GO) terms

	GO biological process	GO molecular function	GO cellular component
Green	Regulation of translation	(none)	(none)
Yellow	(none)	Purine nucleotide binding, purine ribonucleotide binding, nucleotide binding, ATP binding, adenylyl nucleotide binding, adenylyl ribonucleotide binding.	nucleolus
M6	(none)	Zinc ion binding, chromatin binding, transition metal ion binding.	(none)

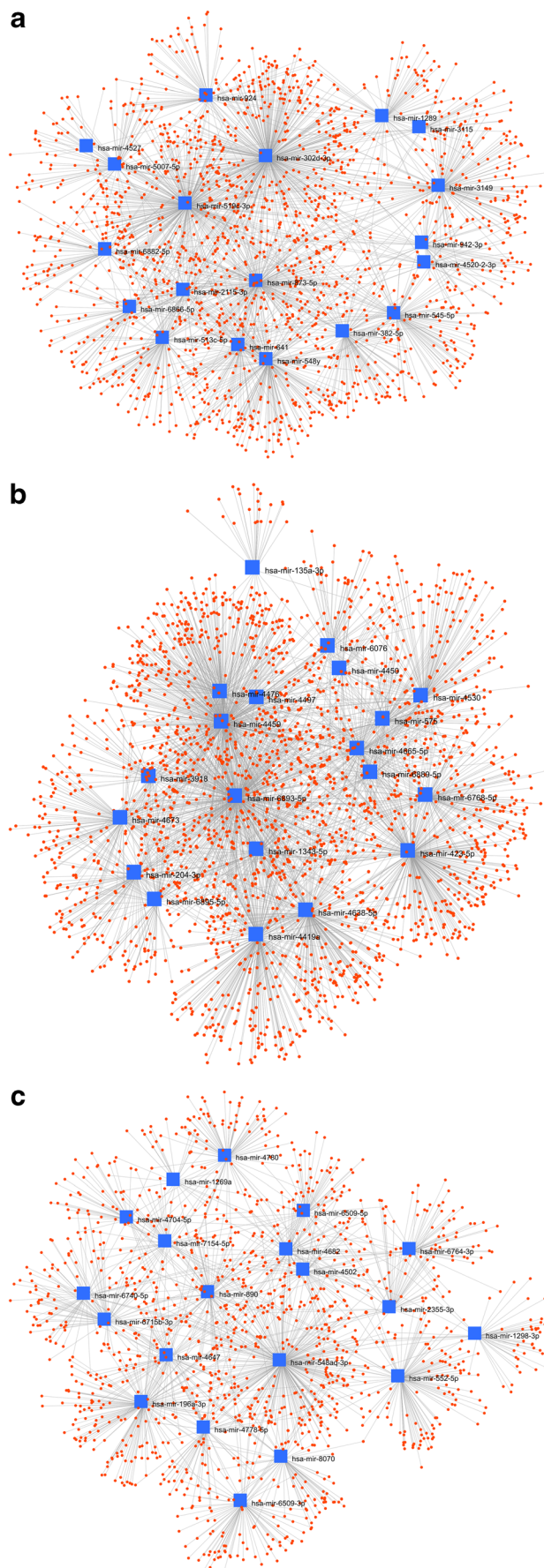


Fig. 3 Interaction network of hub miRNAs in the *green* (a) and *yellow* (b) modules and the *M6* meta-module (c) with their experimentally validated target genes using the miRNet tool. This network graphically highlights which miRNAs exhibit a large number of interactions

4 Discussion

Pancreatic ductal adenocarcinoma (PDAC) is a deadly tumour for which the identification of new diagnostic/prognostic biomarkers and therapeutic targets is of critical importance. Since circulating miRNAs may serve as such, we initiated the identification of miRNA biomarkers and targets through the analysis of microarray-based miRNA expression data obtained from serum of PDAC patients and healthy subjects. Through weighted gene co-expression network analysis (WGCNA) we compared networks in normal and PDAC samples and found that eight out of twelve network modules, i.e., network regions enriched in co-expressed miRNAs, in the normal network were not preserved in the PDAC network. These miRNA modules may play important roles in PDAC development and, therefore, the hub miRNAs within these modules may serve as new diagnostic/prognostic biomarkers or as therapeutic targets. We assessed the potential prognostic value of these miRNAs by validating them in an independent miRNA expression dataset. Through PDAC overall survival (OS) analyses we found that the miRNA expression profiles in all modules, and at least two hub miRNAs in each module, were able to effectively discriminate between two distinct prognosis groups.

Among the *green* module hub miRNAs, miR-302d-3p (previously known as miR-302d) has previously been found to be over-expressed in serum of both PDAC and chronic pancreatitis patients compared to healthy controls [43], whereas miR-942 has been found to be over-expressed in serum of PDAC patients compared to both patients with chronic pancreatitis [33] and healthy subjects [43]. In comparison with serum from healthy subjects, miR-513c-5p (also known as miR-513) has been found to be upregulated in serum of both PDAC and chronic pancreatitis patients [43]. Among the *yellow* module miRNAs, miR-135a-3p (previously known as miR-135a*) has been found to be 17-fold and 21-fold upregulated in serum of patients with PDAC and pancreatitis, respectively, compared to serum of healthy subjects [43]. Also miR-575 was found to be higher expressed in serum of patients diagnosed with PDAC than in serum of healthy subjects, i.e., 2-fold [44] or 4-fold [45]. Conversely, miR-4497 was found to be down-regulated in serum of PDAC patients compared to healthy subjects [46]. Moreover, it has been reported that the serum levels of eight hub miRNAs in this module (miR-204-3p, miR-423-5p, miR-575, miR-4450, miR-4476, miR-4497, miR-4530 and miR-6893-5p) are individually able to discriminate PDAC patients from healthy individuals [35], as revealed by LIMMA R analysis. Among the hub miRNAs

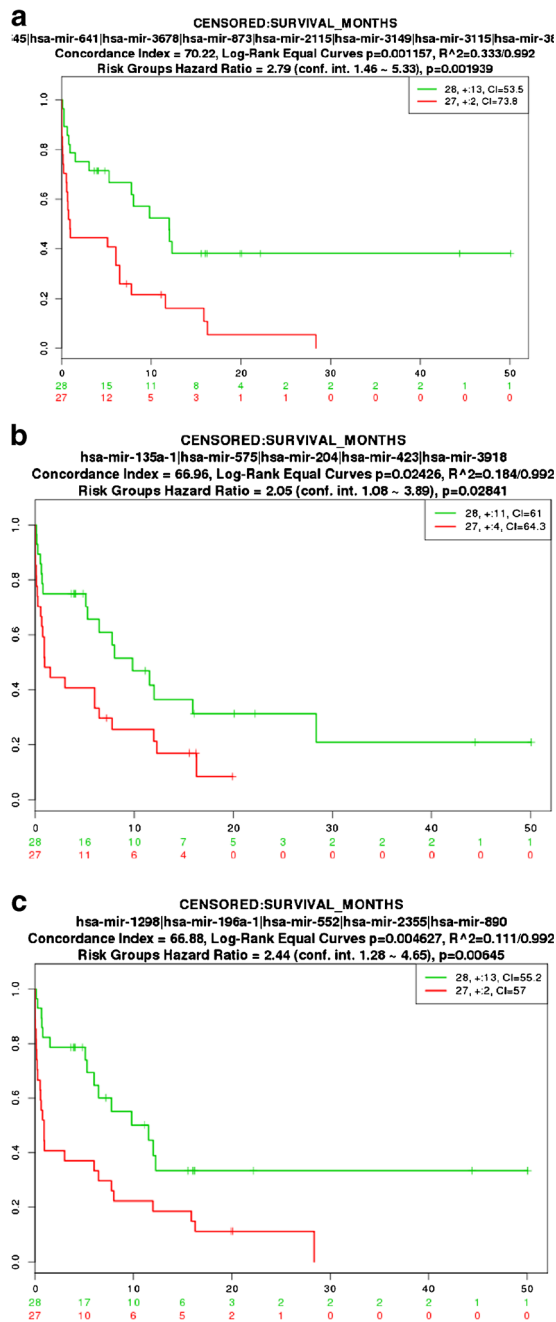


Fig. 4 Kaplan-Meier survival plots for overall survival related to the *green* (a) and *yellow* (b) modules and the *M6* meta-module (c) using the top 20 hub miRNA signatures. The X and Y axes represent survival time (months) and percent of survival, respectively. Lower curves represent high-risk groups and upper curves represent low-risk groups

in the *M6* meta-module, miR-196a-3p (also known as miR-196a*) has not been investigated in PDAC before, whereas the role of miR-196a-5p (also known as miR-196a), which originates from the same pre-miRNA precursor, has extensively been studied. miR-196a has repeatedly been found to be significantly over-expressed in serum of PDAC patients compared to both pancreatitis patients and healthy subjects [43, 47–49]. Moreover, it has been found that miR-196a levels

Table 7 Single-miRNA survival analysis on an independent PDAC miRNA expression dataset

Module	Hub miRNA	Hazard ratio (HR) [Confidence interval 95%]	p-value (< 0.05)
Green	miR-3115	2.58 [1.36–4.92]	0,0038
	miR-3149	2.54 [1.33–4.84]	0,0045
	miR-513c-5p	2.27 [1.20–4.29]	0,0114
	miR-519c-3p	2.12 [1.12–4.00]	0,0209
	miR-924	2.02 [1.08–3.79]	0,0285
	miR-548y	2.00 [1.06–3.78]	0,0320
	miR-302d-5p	1.95 [1.04–3.69]	0,0387
Yellow	miR-3918	2.31 [1.22–4.36]	0,0100
	miR-575	2.02 [1.08–3.79]	0,0285
M6	miR-1298-3p	2.55 [1.34–4.87]	0,0043
	miR-552-5p	2.34 [1.22–4.50]	0,0105
	miR-890	1.99 [1.05–3.75]	0,0341

may inversely correlate with patient survival [50]. At the functional level, miR-196a has been found to inhibit apoptosis and to promote the proliferation and invasion of PDAC cells by down-regulating the tumour suppressor gene *ING5* (inhibitor of growth family member 5) [51] and the metastasis-related gene *NFKBIA* (NF-kappa-B inhibitor alpha) [52]. MiR-196a has also been identified in serum-derived exosomes of PDAC patients, although at very low levels [53]. Since the miR-196a levels are usually high in both PDAC cells and in serum of PDAC patients, its low level in exosomes is likely due to selective miRNA loading/sorting during exosome biogenesis. Specific sequence motifs are known to be involved in miRNA sorting into exosomes [54] following rules resembling those of the RNA export language [55].

Furthermore, we identified enriched KEGG and REACTOME pathways and GO terms through functional enrichment analysis of hub miRNA target genes. By doing so, we found that the obtained modules were enriched in PDAC-related pathways. Interestingly, also prostate and colon cancer-specific pathways emerged, and the miRNA profiles in prostate, colon and pancreatic cancers turned out to be more similar than those in lung and breast cancers [56]. In addition, we found that according to KEGG, REACTOME and GO, all modules were highly related to cell cycle and gene expression regulation.

We also identified genes targeted by the highest number of hub miRNAs, using only experimentally validated miRNA-target interactions. In the *green* module, the GATA6 transcription factor was found to be highly targeted. In the past, its role in PDAC has extensively been investigated. It has e.g. been reported that genomic rearrangements in PDACs frequently include amplification of the *GATA6* gene which, accordingly, has been found to be over-expressed [57, 58]. Conversely, silencing of this gene has resulted in inhibition of pancreatic cancer cell growth [59]. A possible underlying mechanism

Table 8 Previously identified hub genes [24] targeted by hub miRNAs identified here. Only experimentally validated miRNA target genes collected by the miRNet tool were used for this comparison

Module	Hub miRNAs	Hub genes
Green	miR-302d-3p	CYCS, MANEAL, SEC23B
	miR-513c-5p	CYCS
	miR-519c-3p	SEC23B
	miR-873-5p	POLDIP2
	miR-924	FLVCR1
	miR-1289	BPNT1
	miR-3149	MANEAL
	miR-6882-5p	MFSD6
Yellow	miR-3918	CYCS
	miR-4419a	ZDHHC3
	miR-4459	TBC1D24
	miR-4497	BPNT1
	miR-6076	CYCS
	miR-6768-5p	TMEM51
	miR-6893-5p	BPNT1, CYCS
M6 meta-module	miR-196a-3p	ELOVL6
	miR-548aq-3p	C15ORF52
	miR-4780	MCU

may be a GATA6-mediated induction of the Wnt signalling pathway [60]. More recently, GATA6 has also been suggested to act as a tumour suppressor, since it can inhibit epithelial-mesenchymal transition (EMT), and has been associated with a better patient outcome [61]. Within the *yellow* module, we found that the tropomyosin 3 gene (*TPM3*), the small ubiquitin-like modifier 1 gene (*SUMO1*), the RISC catalytic component argonaute 2 gene (*AGO2*) and the cyclin F gene (*CCNF*) were highly targeted by hub miRNAs. Interestingly, by comparing the gene expression profiles of early-recurrent PDAC cases (within 12 months after surgery) with those of late-recurrent cases (over 12 months), it has been found that *TPM3* can discriminate between these two groups [62]. The *SUMO1*, *AGO2* and *CCNF* genes have respectively been found to be 42, 6 and 9-fold higher expressed in primary pancreatic tumours than in normal pancreatic tissues or normal duct epithelial cells [63–65]. Moreover, PDAC-associated missense mutations have been identified in the *AGO2* (K709 M) [66] and *SAMD4B* (I668L) [67] genes, although their functional significance is still unclear [68–71]. Therefore, in addition to the suggested miRNAs, these genes deserve further investigation in relation to PDAC initiation and/or progression.

By comparing the experimentally validated targets of the key miRNAs identified in this work with the hub genes that we previously identified using the same method [24], a relationship emerges. In particular, we found that many key miRNAs target many hub genes (Table 8), which may explain

the altered expression profiles of the hub genes that we previously highlighted. The *BPNT1*, *CYCS*, *MANEAL* and *SEC23B* hub genes are, for example, targeted by at least 2 key miRNAs. Possibly, also other relationships between our two studies may exist but, as yet, the experimentally validated miRNA targets are only partially known.

Taken together, we conclude that our work confirms the validity of some already suggested PDAC biomarkers and has revealed new candidate miRNAs that may be exploited as diagnostic or prognostic biomarkers or as therapeutic targets. Although there is overlap between known candidate biomarkers and the ones that we identified here, further studies are needed to evaluate their practical performance. In this respect, some factors should be carefully considered, including confounding factors such as age, gender and ethnicity of the patients [72–74], differences in tumour stages and treatment regimens, as well as technical factors, such as blood storage conditions and processing [75, 76]. Moreover, since miRNAs can be found in serum in a free form, associated to HDL or enclosed in exosomes or microvesicles, researchers have to pay special attention to the isolation of miRNAs from the desired fraction in order to avoid contamination [77]. Exosome sampling can e.g. also be performed using buccal swabs, thereby taking advantage of the non-invasiveness and higher yield than from blood. Moreover, it is possible to analyse PDAC-specific exosomal sub-populations based on the presence of the Glypican-1 antigen [78] and, by doing so, to validate candidate miRNA biomarkers for patient stratification or therapy response only in exosomes released from PDAC cells.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

This article does not contain any studies with human participants or animals performed by any of the authors.

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