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Transcriptomic analysis of the Aquaporin (AQP) gene family interactome identifies a molecular panel of four prognostic markers in patients with pancreatic ductal adenocarcinoma

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ABSTRACT

Background: This study aimed to assess the differential gene expression of aquaporin (AQP) gene family interactome in pancreatic ductal adenocarcinoma (PDAC) using data mining techniques to identify novel candidate genes intervening in the pathogenicity of PDAC.
Method: Transcriptome data mining techniques were used in order to construct the interactome of the AQP gene family and to determine which genes members are differentially expressed in PDAC as compared to controls. The same techniques were used in order to evaluate the potential prognostic role of the differentially expressed genes.
Results: Transcriptome microarray data of four GEO datasets were incorporated, including 142 primary tumor samples and 104 normal pancreatic tissue samples. Twenty differentially expressed genes were identified, of which nineteen were downregulated and one up-regulated. A molecular panel of four genes (Aquaporin 7 – AQP7; Archain 1 – ARCN1; Exocyst Complex Component 3 – EXOC3; Coatomer Protein Complex Subunit Epsilon – COPE) were identified as potential prognostic markers associated with overall survival.
Conclusion: These outcomes should be further assessed *in vitro* in order to fully understand the role of these genes in the pathophysiological mechanism of PDAC.

Introduction

Pancreatic cancer (PC) is one of the leading cancer-related causes of death worldwide and the fourth cause of cancer mortality in the US [1,2]. More than 85% of the cases presenting with pancreatic cancer are ductal adenocarcinomas (PDAC). In most cases, the tumor is located in the head of the pancreas [3,4]. Due to the retroperitoneal location of the pancreas, the early stages of this type of cancer are usually asymptomatic. The disease is generally advanced when it is diagnosed and is associated with poor prognosis [5]. In fact, the 5-year survival rate of PC is approximately 6% [5]. Depending on the degree of differentiation along with the effect of tumor microenvironment, the malignancy may show poorly to well-formed glands or infiltrating cells forming sheets [3,4]. In spite of the continuous research efforts, the PC-related mortality rates are increasing and it is projected that in 2030 pancreatic cancer will be the second cancer-related cause of mortality [6].

It is well known that the tumor development, invasion, migration and metastasis depend on the tumor microenvironment and metabolism [7]. In addition, many studies have shown that water balance and glycerol metabolism play a crucial role in maintaining cell

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function [8,9]. Aquaporins (AQPs) constitute a family of 13 (AQP0-12) transmembrane channel proteins that facilitate the transcellular water movement. In fact, they have been implicated in tumor growth and metastasis, by enhancing cell migration, cell-matrix adhesion, glycerol uptake, and by interacting with oncogenes [8,9]. For instance, AQP1, AQP3, AQP5, and AQP9 have been associated with the pathogenesis of colorectal cancer [10,11]. Further evidence has also highlighted the role of AQP1 in tumor angiogenesis and cell migration [7]. In the same context, the expression level of AQP3 was increased in gastric cancer compared to normal gastric mucosa [10,11], thus having been implicated in the epithelial to mesenchymal transition (EMT) and PI3K/AKT/SNAIL signaling pathways [12].

Despite the recognized role of the expression of certain members of the AQP gene family in normal and cancer conditions [13–16], their expression in PDAC has not been fully investigated. This information prompted us to study the role of AQP gene family members and their interactome in PDAC. The aim of our study was to assess the differential expression of AQP0-12 mRNA in PDAC compared with healthy tissue using transcriptome microarray data of three independent pancreatic adenocarcinoma datasets. Furthermore, we investigated the AQP protein interactors, structured their gene network and performed Kaplan-Meier analysis in order to identify novel candidate genes that in conjunction with AQP genes may be used as biomarkers.

Materials and methods

ConsensusPathDB construction of the AQP interactome

A bioinformatics analysis of the matrix of interactors associated with the AQP gene family was performed in order to identify possible partners of the AQP genes involved in cancer. The AQP gene network was analyzed using the ConsensusPathDB [17] during March 2018 (http://cpdb.molgen.mpg.de/). The ConsensusPathDB is a database that integrates networks of interactions regarding binary and complex protein-protein, genetic, metabolic, signaling, gene regulatory and drug-target interactions. The choice of the ConsensusPathDB platform was based on its integration of 32 different public databases for interactions that have been curated, thus establishing a high level of data extraction quality.

AQP interactome gene expression profile in pancreatic cancer

We used the PubMed Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/gds) to investigate the expression profile of the AQP gene family interactome in patients with pancreatic cancer compared with healthy controls. PubMed GEO is a repository of publicly available curated gene expression datasets, as well as original series and platform records. All microarray data used in this study were downloaded in November 2018. The differentially expressed genes (DEGs) were identified using three independent pancreatic adenocarcinoma microarray datasets including GSE15471, GSE16515, GSE28735 and GSE32676, with 246 samples in total ($36 + 45+36 + 25 = 142$ primary tumor samples and $16 + 45+36 + 7 = 104$ normal control samples). These datasets were generated from the following detecting microarray platforms: [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array regarding GSE15471, GSE16515, GSE32676 and [HuGene-1_0-st] Affymetrix Human Gene 1.0 ST Array regarding GSE28735. GSE15471, GSE18670 and GSE32676 datasets were composed of matched tumor samples and adjacent non-tumor samples. The gene expression data were log transformed and median centered per array. Genes were considered to be significantly overexpressed or underexpressed when the p value derived from the comparison of the PDAC group vs. the control group corresponded to $p < 0.05$.

GeneMANIA analysis of the significantly differentially expressed components of the AQP interactome in pancreatic cancer

GeneMANIA (http://genemania.org/) [18] is a tool using gene ontology algorithms in order to predict the function of a gene or a set of genes to generate a connectivity map. Functional and interaction enrichments were performed during November 2018. The functional annotation enrichment analysis of Gene Ontologies (GO) was performed with using the GeneMania software mainly due to its attribute to produce both the standard GO enrichment analysis along with predictions of functional relationships between genes by mapping known relationships from other organisms via orthology. The significant enrichments were further assessed by calculating their false discovery rates (FDR). The functions of the resulting proteins of genes within the AQP gene network were extracted from GeneCards - The Human Gene Compendium (http://www.genecards.org/), which is a database of human genes providing systematic information on all genomic and functional aspects.

Investigation of the potential prognostic value of the significantly differentially expressed members of the AQP interactome

To evaluate the potential prognostic significance of the significantly differentially expressed AQP interactome members, we constructed survival curves using the publicly available survival data of The Cancer Genome Atlas (TCGA). In order to perform the analysis, we used the PROGgeneV2 Prognostic Database (Indiana University) [19] and we calculated the median value of gene expression to group the patients in either high expressing (above median) or low expressing (below median) each significantly differentially expressed gene.

Gene set enrichment analysis (GSEA) of the four prognostic markers

We performed GSEA using the David Bioinformatics Resources 6.7 [20,21] and GeneMania in order to obtain further information regarding the four prognostic markers mediated biological pathways involved in the pathogenesis of PDAC.

Statistical analysis

The results were analyzed using GraphPad Prism 8.0 for Mac (GraphPad Software, San Diego, CA). Normal distribution of the data was performed by application of the D'Agostino and Pearson Omnibus normality test. Comparisons of gene expressions were performed with two-tailed unpaired t-test for parametric data and Mann-Whitney U test for nonparametric data. P values were corrected for multiple comparisons by the calculation of Q statistic (Benjamini-Hochberg). Differences that had a $Q < 0.05$ were deemed significant. Correlations were performed by the calculation of the Pearson (r) or the Spearman's rank (ρ) correlation coefficients for parametric or non-parametric data, respectively. Deming regression analysis was performed in order to assess cause-and-effect relationships among significant genes. Kaplan-Meier survival curves were generated using the PROGgeneV2 Prognostic Database (Indiana University) software. Differences were deemed significant with a $P \leq 0.05$.

Results

Trial flow and ConsensusPathDB analysis of the AQP interactome in humans

A trial flow of the present study is presented in Fig. S1. The gene components of the AQP interactome that were derived from the

Table 1
Summary of gene symbols and description of the AQP interactome members.

Gene Symbol	Gene Description
AQP1	Aquaporin 1
AQP2	Aquaporin 2
AQP3	Aquaporin 3
AQP4	Aquaporin 4
AQP5	Aquaporin 5
AQP6	Aquaporin 6
AQP7	Aquaporin 7
AQP8	Aquaporin 8
AQP9	Aquaporin 9
AQP10	Aquaporin 10
AQP11	Aquaporin 11
AQP12A	Aquaporin 12A
AQP12B	Aquaporin 12B
RRM2B	Ribonucleotide Reductase Regulatory TP53 Inducible Subunit M2B
SLC5A1	Solute Carrier Family 5 Member 1
ARCN1	Archain 1
COPA	Coatomer Protein Complex Subunit Alpha
COPB1	Coatomer Protein Complex Subunit Beta 1
COPB2	Coatomer Protein Complex Subunit Beta 2
COPE	Coatomer Protein Complex Subunit Epsilon
COPG1	Coatomer Protein Complex Subunit Gamma 1
COPG2	Coatomer Protein Complex Subunit Gamma 2
RHAG	Rh Associated Glycoprotein

ConsensusPathDB platform are demonstrated in Table 1. A total of 24 interacting proteins were revealed through the construction of the AQP interactome in humans. The network representing the AQP interactome was named network 1.

PubMed GEO analysis of the differential gene expression of AQP interactome components in pancreatic cancer

Out of the 24 proteins of network 1, sufficient PubMed GEO data

Table 2
Summary of the differential gene expression of the AQP interactome in PDAC as compared to healthy tissue.

Gene Symbol	Fold Changes (Actual)	Fold Changes (Hodges-Lehmann)	*P* values	*Q* values
Upregulated				
ARCN1	0.3066	0.1780	0.0024	0.0026
Downregulated				
AQP1	−0.4921	−0.2992	0.0029	0.0026
AQP2	−3.125	−0,4418	<0.0001	0.0002
AQP3	−0.2692	−0.2209	0.0003	0.0005
AQP4	−0.6839	−0.1053	0.0001	0.0002
AQP6	−2.531e+014	−0.3844	<0.0001	0.0002
AQP7	−0.1454	−0.2429	<0.0001	0.0002
AQP10	−0.1194	−0.2083	0.0030	0.0026
AQP11	−0.2923	−0.4005	0.0035	0.0029
AQP12B	−0.1307	−0.3651	<0.0001	0.0002
COPE	−0.0448	−0.0749	0.0465	0.0302
COPG2	−3.0250	−0.5018	0.0004	0.0006
RHAG	−0.1744	−0.3741	<0.0001	0.0002
RHCE	−0.0618	−0.0779	0.0029	0.0026
RHD	−0.0617	−0.0857	0.0252	0.0170
RHBG	−0.1306	−0.3010	<0.0001	0.0002
GYPB	−1.083	−0.2160	<0.0001	0.0002
MIP	−2.945	−0.5095	<0.0001	0.0002
COPZ2	−0.2350	−0.1918	0.0040	0.0032
EXOC3	−0.6242	−0.1700	0.0014	0.0017
Not significantly different				
AQP5	−0.9547	−0.1499	0.8178	0.3717
AQP8	−0.4774	−0.1957	0.1807	0.0995
AQP9	0.1226	0.4840	0.6306	0.3098
RRM2B	−0.0950	−0.0593	0.2684	0.3717
SLC5A1	−0.1073	0.005936	0.9368	0.4154
COPA	−0.08528	0,008088	0,9035	0.2484
COPB1	−0.1669	−0.06236	0.3251	0.1689
COPB2	1.064	0.1058	0.1370	0.0781
COPG1	−0.01181	0.0296	0.2741	0.1466
RHCG	−0.02837	−0.1438	0.0742	0.0465
COPZ1	−0.03610	−0.04561	0.1374	0.0781

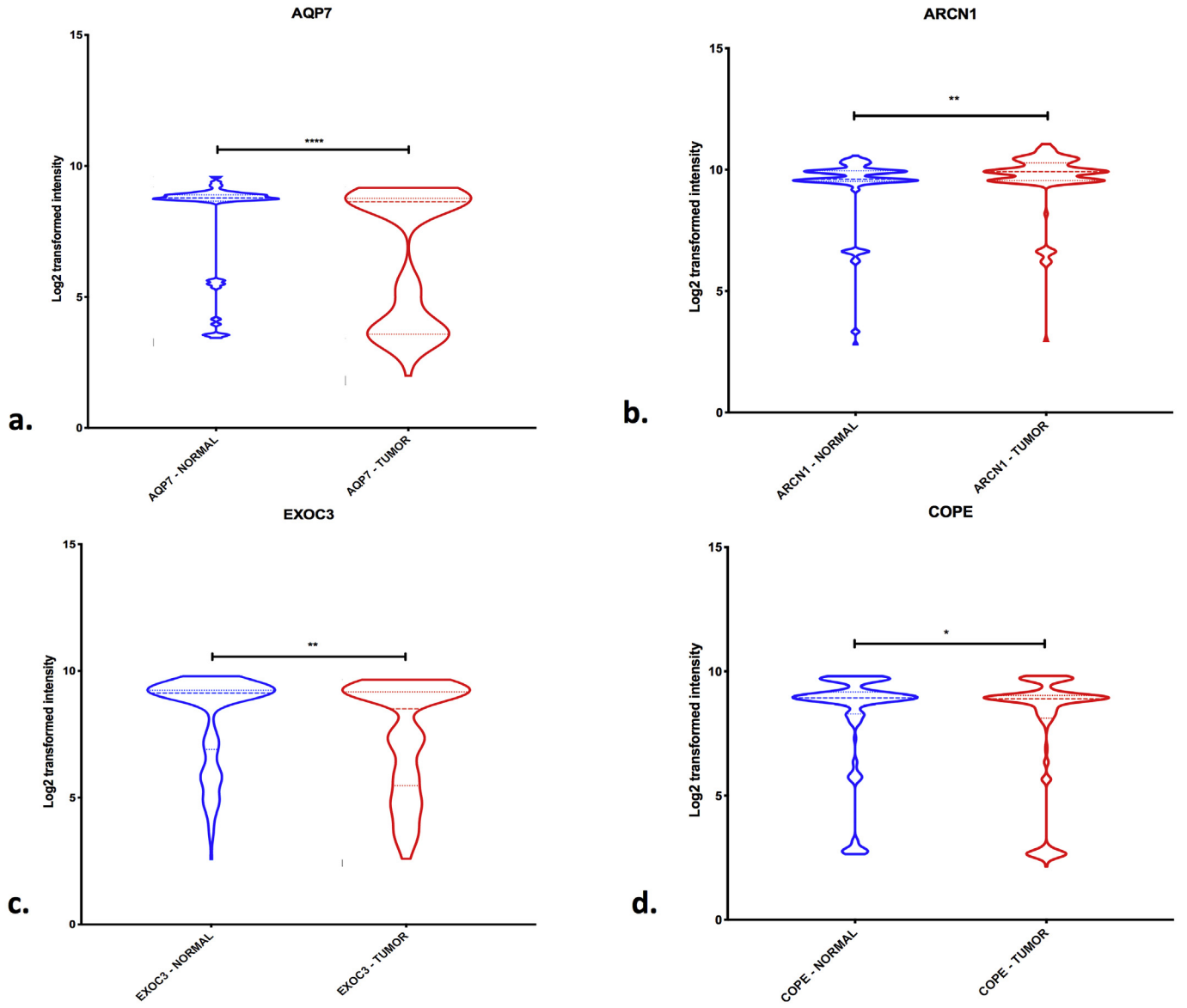

Fig. 1. Violin plots with bars demonstrating the differential gene expression profile in normal and PDAC tissue samples. **a.** AQP7 is significantly under-expressed in PDAC group. **b.** ARCN1 is significantly over-expressed in PDAC group. **c.** EXOC3 is significantly under-expressed in PDAC group. **d.** COPE is significantly under-expressed in PDAC group.

regarding their gene expression level was available regarding 22 genes (91.7%) and 13 DEGs were identified. No data was available regarding the AQP12A and C5A1 genes. Subsequently, we interrogated PubMed GEO regarding the gene expression profile of the above genes. The clinical and pathological characteristics regarding the included samples and patients are presented in Table S1. Thirteen DEGs were identified from the network 1 and were further assessed regarding potential interacting genes in the GeneMania platform (Fig. S2). The additional genes were also assessed regarding their expression level. The cumulative outcomes are presented in Table 2 (network 2). There was one up-regulated gene and nineteen down-regulated genes (Table 2; Fig. 1). No significant difference was reported regarding the expression level of eleven genes (Table 2).

Correlations among DEGs

The expression of AQP2 was correlated with AQP1, AQP3 and AQP6. AQP1 expression was found to be negatively correlated with AQP2 expression ($p = 0.0064$ and Spearman's $r = -0.1454$; Fig. 2a) and positively correlated with AQP3 ($p < 0.0001$ and Spearman's $r = 0.4907$; Fig. 2b) and AQP6 ($p < 0.0001$ and Spearman's $r = 0.3376$; Fig. 2c).

Deming regression analysis revealed the equations describing the significant correlations between AQP1 and AQP2, AQP2 and AQP3, along with AQP2 and AQP6 gene expressions. The negative correlation between AQP1 and AQP2 is described by the equation: $AQP2 = -4.715 \times AQP1 + 46.27$. Furthermore, the positive correlation between AQP2 and AQP3 is described by the equation: $AQP3 = 2.446e+014 \times AQP2 - 9.224e+014$. In addition, the positive correlation between AQP2 and AQP6 is described by the following equation: $AQP6 = 1.007e+015 \times AQP2 - 5.662e+015$.

Functional enrichment analysis of the DEGs

All the DEGs of the AQP interactome (Table 2) underwent GO

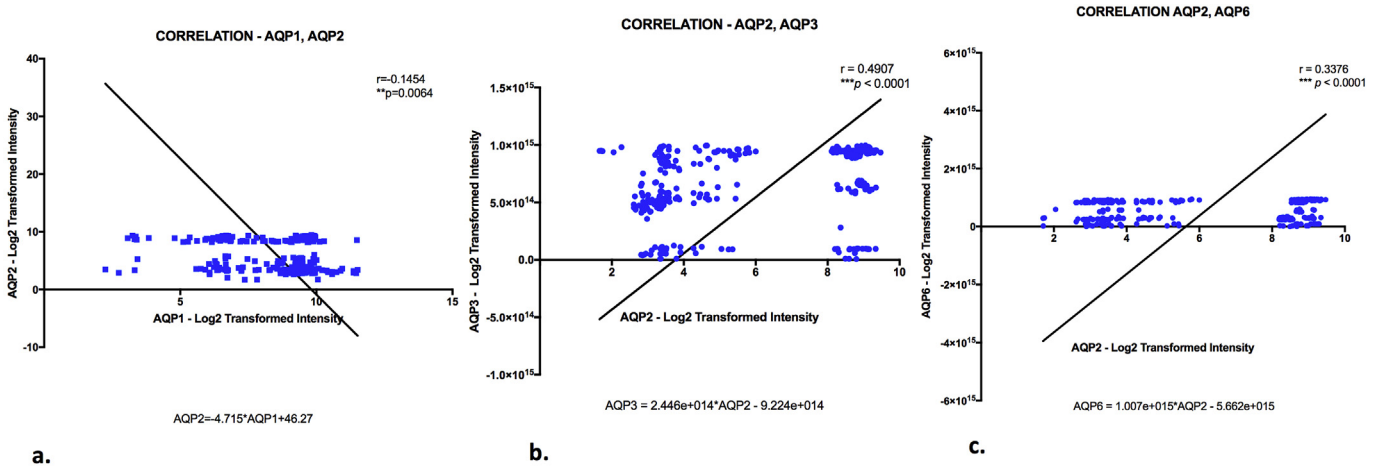

Fig. 2. Deming regression analysis revealed significant associations of the gene expression levels of AQP1 and AQP2, AQP2 and AQP3, AQP2 and AQP6. **a.** Negative correlation between AQP1 and AQP2 is described by the equation: AQP2 = −4.715 × AQP1+46.27. **b.** Positive correlation between AQP2 and AQP3 is described by the equation: AQP3 = 2.446e+014 × AQP2-9.224e+014. **c.** Positive correlation between AQP2 and AQP6 is described by the following equation: AQP6 = 1.007e+015 × AQP2-5.662e+015.

Table 3
Functional annotation enrichment analysis of GO for the differentially expressed AQP interactome genes: top 3 GOs.

Function	FDR	Genes in network	Genes in genome
Water transport	9,25e-07	12	38
Golgi-associated vesicle	7,98e+01	9	49
Establishment of vesicle localisation	9,57e+05	7	94

Abbreviations: GO = Gene Ontologies; FDR=False Discovery Rate.

enrichment analysis. In fact, DEGs were introduced in the GeneMANIA software and functional enrichment was performed (Table 3). The FDRs were also calculated regarding each DEG.

AQP7, ARCN1, COPE and EXOC3 expression were indicators for prognosis of patients with pancreatic adenocarcinoma

We further investigated whether the up-regulation or down-regulation of DEGs in pancreatic cancer could affect patient survival. Pancreatic adenocarcinoma data with gene expression and clinical information from The Cancer Genome Atlas (TCGA) was used to investigate their prognostic significance. One hundred and seventy pancreatic cancer patients were included in this analysis. Kaplan-Meier survival curves were constructed for all DEGs of the AQP interactome. The median survival period was 661 days for AQP7 high expression group, and it dropped to 596 days in AQP7 low expression group (HR: 0.86 [95% CI: 0.76–0.97]; $p = 0.0135$; Fig. 3a). ARCN1 low expression group had an increased median survival period, 695 days, as compared with the median survival of the high expression group which was 545 days (HR: 3.1 [95% CI: 1.5–6.39]; $p = 0.00215$; Fig. 3b). Furthermore, the median survival for the COPE high expression group was 652 days and for the COPE low expression group was 596 days (HR: 0.58 [95% CI: 0.39–0.86]; $p = 0.00667$; Fig. 3c). In addition, the median survival was 691 days for the EXOC3 high expression group and 545 days for the EXOC3 low expression group (HR: 0.45 [95% CI: 0.21–0.97]; $p = 0.0428$; Fig. 3d). These results indicated that ARCN1 was an adverse factor while AQP7, COPE, EXOC3 were beneficial factors regarding the median survival of pancreatic adenocarcinoma patients.

Gene set enrichment analysis of the four prognostic markers

Following the construction of the Kaplan-Meier survival curves, the four prognostic markers underwent gene set enrichment analysis. Specifically, the four markers were introduced as a gene set in DAVID and GeneMANIA algorithms. Gene Enrichment analysis provided similar results, as displayed in Fig. S3 (DAVID) and Fig. S4 (GENEmania). Regulation of cellular water and glycerol homeostasis, intracellular protein transport, along with Golgi and COPI vesicle coat represented the most significant GO annotations associated with the four markers.

Discussion

The present study explored alterations regarding the gene expression profile of the AQP interactome in pancreatic adenocarcinoma at the genome-wide scale by integrating three pancreatic cancer transcriptome microarray datasets. In this way, the gene expression modifications were identified with enhanced accuracy at the molecular level, as compared with studies based on a single dataset. In fact, a total of 246 samples were included and analyzed in the present study. In this study, we have constructed the gene network of AQPs through which we report 20 DEGs related to pancreatic cancer. These genes should be further assessed regarding their role in the development and progression of PDAC as well as their potential use as drug targets. These novel gene candidates are AQP1, AQP2, AQP3, AQP4, AQP6, AQP7, AQP10, AQP11, AQP12B, COPE, COPG2, RHAG, RHCE, RHD, RHBG, GYPB, MIP, COPZ2, EXOC3 and ARCN1.

Our analysis indicated that PDAC was marked by dysfunctions of water and alcohol transport, transport to the Golgi and subsequent modification. The four identified biomarkers were all implicated in these processes, along with COPI vesicle coat. AQP7 is involved in the movement of water, glycerol and urea across cell membranes. It has also been implicated in the pathogenesis in human urothelial and hepatocellular carcinoma [22,23]. In fact, AQP7 was down-regulated in human hepatocellular carcinoma compared to normal tissue according to one study [23]. The present study provided the

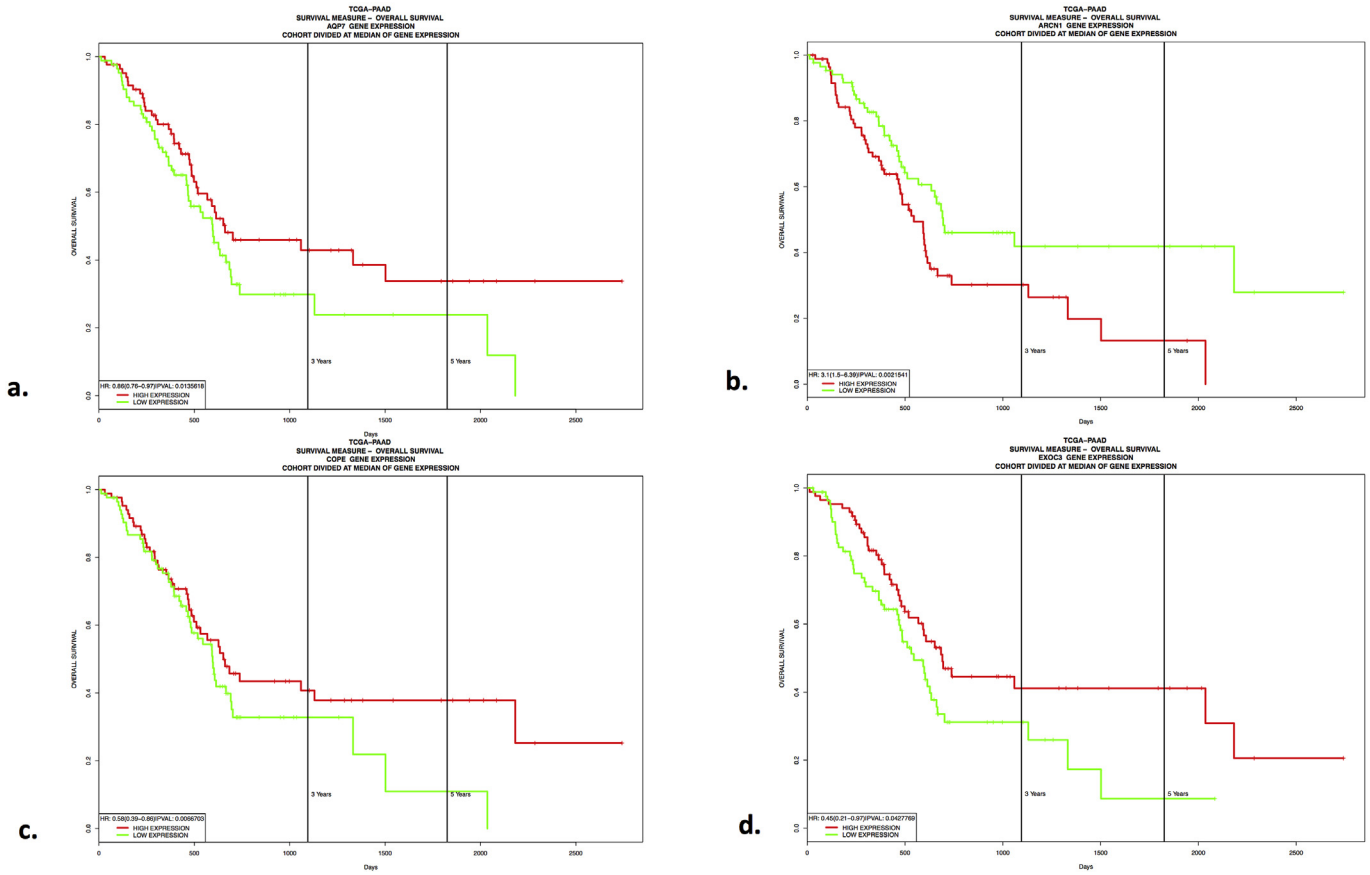

Fig. 3. Kaplan-Meier curves demonstrating the differences in survival between PDAC groups with different expression levels of AQP7, ARCN1, COPE and EXOC3. **a.** The median survival period was greater for the AQP7 high expression group, as compared with the low expression group (HR: 0.86 [95% CI: 0.76–0.97]; $p = 0.0135$). **c.** ARCN1 low expression group had an increased median survival period, as compared with the median survival of the high expression group (HR: 3.1 [95% CI: 1.5–6.39]; $p = 0.00215$). **c.** The median survival for the COPE high expression group was greater, as compared with the COPE low expression group. **d.** The median survival was increased for the EXOC3 high expression group (HR: 0.45 [95% CI: 0.21–0.97]; $p = 0.0428$).

first evidence, to the best of our knowledge, supporting the downregulation of AQP7 in PDAC compared with normal tissue. This downregulation may be attributed to transcriptional inhibition, given the consistently decreased mRNA and protein expression levels of AQP7. However, the exact mechanisms underlying this dysregulation require further assessment.

ARCN1 is an intracellular protein that maps in a genetic region where multiple disease-associated chromosome translocations occur in a genetic region [24–26]. ARCN1 sequence is well conserved and this protein may play a fundamental role in the eukaryotic cell biology [24–26]. In addition, it presents similarities to heat shock proteins and clathrin-associated proteins, while it may be involved in vesicle structure or trafficking. According to our outcomes, ARCN1 was upregulated in PDAC tissue samples. Moreover, the increased expression level of ARCN1 was associated with decreased survival.

COPE is an epsilon subunit of a coatomer protein complex [27,28]. It is required for budding from Golgi membranes, and is essential for the retrograde Golgi-to-Endoplasmatic reticulum transport of dilysine-tagged proteins [27,28]. The coatomer complex consists of at least the alpha, beta, beta', gamma, delta, epsilon and zeta subunits. According to our outcomes, COPE was underexpressed in PDAC tissue compared to normal tissue. Furthermore, patients with low expression level of COPE presented lower survival compared to the high expression group.

EXOC3 is a component of the exocyst complex essential for targeting exocytic vesicles to specific docking sites on the plasma membrane [29,30]. At least eight components of the exocyst complex, including this protein, are found to interact with the actin cytoskeletal remodeling and vesicle transport machinery [29,30]. The complex is also essential for the biogenesis of epithelial cell surface polarity [29,30]. In the present study, the expression level of EXOC3 was decreased in PDAC tissue compared to normal. Patients presenting with lower expression level of EXOC3 were associated with decreased survival, thus proposing its potential as a prognostic marker.

The strength of these four biomarkers is based on their potential for easy adaption and reproducibility, using immunohistochemistry auto-stainers in a standardized manner. Given the rapid development of artificial intelligence, along with big data processing techniques in interpreting test results, future immunohistochemistry interpretation may be characterized by greater uniformity and subjectivity.

The present study demonstrates a four gene molecular prognostic panel that can provide prognostic information regarding patients with PDAC. This panel can be used as an adjunct to current staging systems to provide enhanced prognostic information. Moreover, the present study identifies the differences between patients with PDAC and normal controls regarding the expression profile of the AQP interactome. This valuable resource should be further assessed in order to improve knowledge of tumor biology and improve the treatment options.

The limitations of the present study are (i) the preparation of tissue samples in four different laboratories and (ii) the employment of two different Affymetrix chips. Nonetheless, the strengths of the current study are (i) the clear protocol as demonstrated in the flow chart, (ii) the inclusion of four datasets, (iii) the large number of tissue samples analyzed, thus making the evidence stronger and (iv) the analysis of survival data and the construction of Kaplan-Meier curves related to gene expression profiles.

Conclusion

In this this study we identified for the first time 20 novel genes differentially expressed in PDAC, along with the significant correlations between DEGs. We also identified the predicted biological processes in which APQ interactome is involved. These processes should be further evaluated. Finally, we have identified a molecular panel of four genes that may be used as prognostic biomarkers associated with survival in PDAC for the first time, along with the signaling pathways that they mediate. Further translational as well as clinical research should elucidate the potential benefit in the pathophysiological context of PDAC.

Conflicts of interest

The participating authors declare no conflicts of interest.

Ethical approval

Does not apply.

Informed consent

Does not apply.

Acknowledgements

Does not apply.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pan.2019.02.006.

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