Identification of potential hub genes associated with the pathogenesis and prognosis of pancreatic duct adenocarcinoma using bioinformatics meta-analysis of multi-platform datasets

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Abstract. Pancreatic duct adenocarcinoma (PDAC) is a highly malignant type of cancer with a low five-year survival rate. Gene alterations are crucial to the molecular pathogenesis of PDAC. Therefore, the present study analyzed gene expression profiles to reveal genes involved in the tumorigenesis of PDAC. A total of eight gene expression profiles (GSE15471, GSE16515, GSE41368, GSE62165, GSE62452, GSE71729, GSE71989 and GSE91035) and a PDAC dataset were acquired from the Gene Expression Omnibus and The Cancer Genome Atlas (TCGA) database, respectively. Differentially expressed genes (DEGs) were screened using functional annotation, Gene Ontology (GO) enrichment analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and protein-protein interaction (PPI) network construction. A Cox proportional hazards model was then constructed and used to analyze the data. A total of 136 DEGs (67 up- and 69 downregulated genes) were identified between PDAC tissues and normal tissues. The ‘extracellular matrix-related’ genes were the most enriched in the GO term analysis. ‘Pancreatic secretion’, ‘phosphoinositide-3-kinase–protein kinase B/Akt (PI3K-Akt) signaling pathway’, ‘protein digestion and absorption’ and ‘ECM-receptor interaction’ were the most enriched categories in KEGG pathway analysis. Following PPI network construction, the 10 most significant genes [albumin, epidermal growth factor, matrix metalloproteinase (MMP) 9, epidermal growth factor receptor, fibronectin 1, MMP1, plasminogen activator inhibitor-1, tissue inhibitor of metalloproteinase 1, plasminogen activator urokinase (PLAU) and PLAU receptor] exhibiting a high degree of connectivity, were identified as the hub genes likely to be associated with the pathogenesis of PDAC. In addition, a prognostic predictive system for PDAC, composed of five genes (laminin subunit γ 2, laminin subunit β 3, serpin family B member 5, amphiregulin and secreted frizzled related protein 4), was constructed. This was validated in the GSE62452 dataset (using 66 PDAC samples with outcome data) and TCGA PDAC dataset (using 146 PDAC samples with outcome data). In conclusion, the present study revealed potential hub genes involved in PDAC progression, providing directive significance for individualized clinical decision-making and molecular-targeting therapy in patients with PDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly complex and malignant type of cancer in humans. It is the seventh leading cause of cancer-associated mortality, and is expected to rise to the third due to its increasing incidence and poor prognosis (1). Despite considerable improvements in surgical, radiation and chemotherapeutic treatments, 80% of patients with PDAC miss the optimal period for effective systemic therapy due to a lack of symptoms, anesis or disease regression at the time of diagnosis (2). Hence, the five-year overall survival rate for PDAC remains at 3-5%. There is thus an urgent need to identify new biomarkers to improve the understanding of the molecular mechanisms involved in PDAC pathogenesis (3). Potential prognostic biomarkers and novel therapeutic targets may help to improve current poor treatment outcomes.

Microarrays have been widely used to identify more sensitive and effective biomarkers for PDAC. Shen et al (4) reported that ribosomal protein genes nucleoporin 170, nucleoporin 160 and heterogeneous nuclear ribonucleoprotein U may be useful as molecular markers for early diagnosis of the disease. Ger et al (5) reported that the fms related tyrosine kinase kinase 3 and poly(rC) binding protein 3 could potentially be used as prognostic biomarkers for pancreatic cancer. Another analysis considered dickkopf WNT signaling pathway inhibitor 1 and high mobility group AT-hook 2 to be hub genes that are strongly associated with Wnt family member 3A and tumor protein...
p53, respectively (6). In addition, KRAS, TP53, CDKN2A, SMAD4, RNF43, ARID1A, TGFβr2, GNAS, RREB1 and PBRM1 were identified as driver genes in PDAC (7). Variation in the significantly expressed genes associated with PDAC pathogenesis between different studies may be due to small sample sizes, the use of different microarray platforms and different statistical methods. To overcome these limitations, integrative meta-analysis using different microarray platforms with larger sample sizes may prove to be a powerful bioinformatics tool, improving the accuracy and reliability of data analysis.

In the present study, multiple Gene Expression Omnibus (GEO) datasets containing a large number of samples were acquired from two different microarray platforms (Affymetrix and Agilent). The RobustRankAggreg (RRA) 1.1 package (8) in R is based on a statistical model that allows for the evaluation of the significance of results. It can be used to identify differentially expressed genes (DEGs) across multiple datasets from different microarray platforms. By defining the rank vector for each gene, based only on the datasets where it is present, the results include DEGs that are not present in every dataset (9). Hence, the gene expression module was investigated to reveal the genes influencing PDAC tumorigenesis.

Materials and methods

Selection and retrieval of microarray datasets. A total of 8 gene expression profiles [GSE15471 (10), GSE16515 (11), GSE41368 (12), GSE62165 (13), GSE62452 (14), GSE71729 (15), GSE71989 (16) and GSE91035 (17)] from two platforms (Affymetrix and Agilent) were retrieved from the GEO (http://www.ncbi.nlm.nih.gov/geo/) database, using the keywords ‘pancreatic ductal adenocarcinoma’, ‘Homo sapiens’ and ‘microarray’. The selected microarray datasets met the following inclusion criteria: i) Expression profiling by array; ii) samples included human PDAC and corresponding adjacent or normal pancreatic tissue; iii) n>10; and iv) the gene expression profile is complete. A total of 8 microarray datasets were retrieved from two different microarray platforms. The data included 452 PDAC samples and 204 normal pancreatic tissue samples (Table I). For further validation, normalized datasets (fragments per kilobase of transcript per million mapped reads upper quartile) of 146 PDAC samples (with complete expression profiles and clinical prognoses) were retrieved from The Cancer Genome Atlas (TCGA) database (7).

Data pre-processing and DEG analysis. Initially, log₂ conversion and quantile normalization was performed on each individual GEO dataset. DEGs of each dataset were then screened using the limma package (https://bioinf.wehi.edu.au/limma) with R/Bioconductor software for functional enrichment analysis. This included the following gene ontology (GO) categories: Molecular function (MF), biological process (BP) and cellular component (CC), as well as enrichment analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. P<0.05 was set as the threshold value for MF, BP and CC; and P<0.01 was set as the threshold value for KEGG analysis.

Functional enrichment analysis. The functions of common DEGs were further analyzed using the clusterProfiler package with R/Bioconductor software for functional enrichment analysis. This included the following gene ontology (GO) categories: Molecular function (MF), biological process (BP) and cellular component (CC), as well as enrichment analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. P<0.05 was set as the threshold value for MF, BP and CC; and P<0.01 was set as the threshold value for KEGG analysis.

Results

Identification of DEGs. A total of 136 DEGs (67 up- and 69 downregulated genes) were identified between PDAC tissues and normal tissues by analyzing eight gene expression profiles retrieved from the GEO database. Volcano plots of the gene expression profile data, and a heat map and histogram of DEGs across the datasets are displayed in Figs. 1-3.

Functional enrichment analysis. To elucidate the functions of common DEGs, GO and KEGG pathway enrichment analyses were performed. The GO results determined that, in the MF
category, the upregulated genes were predominantly enriched in ‘extracellular matrix constituent’ and ‘glycosaminoglycan binding’, whilst the downregulated genes were mainly enriched in ‘serine-type endopeptidase activity’, ‘serine-type peptidase activity’ and ‘serine hydrolase activity’. In the BP category, the upregulated genes were mainly enriched in ‘ECM organization’

| Author, Year       | Dataset     | Count | Tumor | Normal | Platform     | Region     | (Refs.) |
|--------------------|-------------|-------|-------|--------|--------------|------------|---------|
| Badea et al, 2009  | GSE15471    | 78    | 39    | 39     | GPL570       | Romania    | 10      |
| Pei et al, 2009    | GSE16515    | 52    | 36    | 16     | GPL570       | USA        | 11      |
| Frampton et al, 2012| GSE41368    | 12    | 6     | 6      | GPL6244      | Italy      | 12      |
| Janky et al, 2014  | GSE62165    | 131   | 118   | 13     | GPL13667     | Belgium    | 13      |
| Yang et al, 2014   | GSE62452    | 130   | 69    | 61     | GPL6244      | USA        | 14      |
| Moffitt et al, 2015| GSE71729    | 191   | 145   | 46     | GPL20769     | USA        | 15      |
| Schmittgen, 2015   | GSE71989    | 22    | 14    | 8      | GPL570       | USA        | 16      |
| Schmittgen, 2016   | GSE91035    | 40    | 25    | 15     | GPL22763     | USA        | 17      |

Figure 1. Volcano plots of genes that are significantly different between pancreatic tumor and adjacent non-tumor tissues. (A) GSE15471; (B) GSE16515; (C) GSE41368; (D) GSE62165; (E) GSE62452; (F) GSE71729; (G) GSE71898; (H) GSE91035. The x-axis indicates the fold-change (log-scaled); the y-axis indicates the P-values (log-scaled). Each symbol represents a different gene, and the red and blue symbols indicate upregulated and downregulated genes, respectively. P<0.05 was considered to be statistically significant and a fold change (log-scaled) of mean + 2SD was set as the threshold value.
and 'extracellular structure organization', whilst the downregulated genes were enriched in 'antimicrobial humoral response' and 'digestion'. In the CC category, the upregulated genes were mainly enriched in 'ECM' and 'collagen-containing ECM', whilst the downregulated genes were not enriched. 'Pancreatic secretion', 'phosphoinositide-3-kinase-protein kinase B/Akt (PI3K-Akt) signaling pathway', 'protein digestion and absorption' and 'ECM-receptor interaction' were the most enriched pathways in the KEGG pathway analysis. The results of the functional enrichment analysis are exhibited in Figs. 4-6 and support the results of previous studies (20,21).

Hub gene identification using PPI network construction and modular analysis. A PPI network based on the DEGs was constructed using Cytoscape software and the STRING database (Fig. 7). The ten genes with the highest degree of connectivity [albumin (ALB), epidermal growth factor (EGF), MMP9, epidermal growth factor receptor (EGFR), fibronectin 1 (FN1), matrix metalloproteinase (MMP) 1, plasminogen activator inhibitor-1 (SERPINE1), tissue inhibitors of metalloproteinases (TIMP1), plasminogen activator urokinase (PLAU) and PLAU receptor (PLAUR)] were selected as the hub genes; two modules with MCODE scores>5 were selected from the PPI network (Fig. 7B and C). Coincidentally, Module 1 was composed of the 10 hub genes previously selected (Table II).

Construction of the prediction system. The prognostic prediction system, composed of five signature genes (LAMC2,
Figure 3. Histogram of the sources of the 136 DEGs. Bar height represents the number of DEGs across multiple datasets linked by dots.

Figure 4. Gene ontology enrichment analysis of upregulated genes in pancreatic tumor compared with adjacent non-tumor tissues. (A), Molecular function, (B), Biological process, (C), Cellular component.
LAMB3, SERPINB5, AREG and SFRP4), was constructed using the survival information of 66 patients in the training set (GSE62452). The risk score formula for each patient was calculated as shown below: Risk score=(1.0656) x LAMC2 + (-0.5804) x LAMB3 + (-0.4488) x SERPINB5 + (0.3060) x AREG + (-0.5294) x SFRP4. The area under the ROC curve was found to be 0.862, and consequently, specificity and sensitivity were both determined to be highest when the risk score was 0.960 (Fig. 8). The PDAC patients of the GSE62452 dataset were divided into a high-risk group (risk score, ≥0.960; n=33) and a low-risk group (risk score, <0.960; n=33). The patients in the low-risk group (45.5%; 95% CI, 30.1‑68.7%) had a significantly higher survival rate than those in the high-risk group (48.0%; 95% CI, 33.5‑68.7%; P=4x10^{-6}; Fig. 9A).

Discussion

The pathogenesis of PDAC is extremely complex. Whilst there have been many studies on the biological mechanisms underpinning PDAC, the results are inconsistent and various aspects remain unclear. This could be attributable to the three following aspects: i) Small study sample sizes; ii) datasets retrieved from different platforms; and iii) different statistical analysis methods. However, using a combination of multiple datasets from different platforms, the present study aimed to improve the accuracy and reliability of these results. In the present study, eight PDAC datasets (GSE15471, GSE16515, GSE41368, GSE62165, GSE62452, GSE71729, GSE71989 and GSE91035) were used to construct the prediction system. A dataset retrieved from TCGA, consisting of the clinical prognostic information of 146 patients with PDAC, was used as an independent validation dataset for the prognostic prediction system. The individual risk score of each patient was calculated using the aforementioned formula. A risk score of 0.960 was used as the threshold and the patient samples were divided into high- and low-risk groups (n=73 each). KM survival analysis showed that the high-risk group (48.7%; 95% CI, 37.8‑62.7%) had significantly poorer OS scores than the low-risk group (48.1%; 95% CI, 36.0‑64.4%; P=0.008; Fig. 9B).
from two platforms (Affymetrix and Agilent) were analyzed, and the RRA package was used to account for the differences between the platforms. Finally, a dataset from TCGA was used to verify the data and to further improve reliability.

A total of 136 DEGs were identified from the GEO datasets. These included 67 up- and 69 downregulated genes, which were differentially expressed in PDAC samples, compared with the normal controls.

Figure 6. Pathway analysis for differentially expressed genes between pancreatic tumor and adjacent non-tumor tissues.

Figure 7. PPI network of DEGs. The MCODE algorithm was applied to this network to identify neighborhoods where proteins were densely connected, the gradient from green to red represents the change from down to upregulation; two significant modules with a score>5.0 were selected. (A) PPI network of DEGs; (B) Module 1, MCODE score=8.182; and (C) Module 2, MCODE score=5.2. PPI, protein-protein interaction; DEGs, differentially expressed genes.
Table II. Hub genes with high degree of connectivity.

| Name    | MCODE cluster | MCODE score | Degree |
|---------|---------------|-------------|--------|
| FN1     | Cluster 1     | 7.363636    | 20     |
| ALB     | Cluster 1     | 7.363636    | 33     |
| MMP1    | Cluster 1     | 7.363636    | 16     |
| TIMP1   | Cluster 1     | 7.363636    | 14     |
| MMP9    | Cluster 1     | 7.363636    | 23     |
| SERPINE1| Cluster 1     | 7.363636    | 13     |
| PLAUR   | Cluster 1     | 7.363636    | 12     |
| PLAU    | Cluster 1     | 7.363636    | 11     |
| EGF     | Cluster 1     | 7.363636    | 26     |
| EGFR    | Cluster 1     | 7.363636    | 23     |

FN1, fibronectin 1; ALB, albumin; MMP, matrix metallopeptidase; TIMP1, tissue inhibitor of metalloproteinases 1; SERPINE1, serpin family E member 1; PLAUR, plasminogen activator urokinase receptor; PLAU, plasminogen activator urokinase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor.

GO enrichment analysis determined that the most significant enrichments in MF, BP and CC were related to the extracellular matrix (ECM), especially for the upregulated genes. For KEGG pathways analysis, ‘pancreatic secretion’, ‘PI3K-Akt signaling pathway’ and ‘ECM-receptor interaction’ were highly enriched. High enrichment of ECM related genes and pathways suggests that ECM regulation is closely associated with PDAC progression. The ECM is a complex, three-dimensional structure composed of both structural and non-structural proteins (22,23). It plays a fundamental role in facilitating cell differentiation, apoptosis, proliferation and migration (24). The ECM is also the most abundant component in the tumor microenvironment (TME), which profoundly influences the behavior of cancer cells (25). Furthermore, certain studies have suggested that alterations in the ECM can induce cell transformation and metastasis, promoting the development and progression of tumors (26,27). In the TME of PDAC, some ECM proteins, such as collagen, fibronectin and laminin, are significantly upregulated (28). Each of these proteins promotes the growth and invasion of PDAC cells (29-32). Moreover, the results of the present study indicated LAMC2 and LAMB2 are significantly associated with PDAC prognosis.

Based on PPI network analysis of the common DEGs in the selected studies, the 10 most highly connected genes (ALB, EGF, MMP9, EGFR, FN1, MMP1, SERPINE1, TIMP1, PLAU and PLAR) were screened and Module 1 is, coincidentally, composed of the 10 hub genes. Furthermore, FN1 and PLAU have previously been identified as hub genes in a similar study (10). Multiple studies have determined that these genes are associated with tumorigenesis and progression. For example, the FN1 gene encodes fibronectin, which is a major constituent of the ECM within the TME. The binding of FN1 to its receptor activates the FN1 signaling pathway in pancreatic cancer cells, and promotes tumor cell survival, invasion, metastasis and angiogenesis (33). The expression of fibronectin in pancreatic cancer cells is also associated with a low survival rate (34,35). In addition, FN1 participates in the progression of ovarian cancer by increasing the expression level of matrix metalloproteinase MMP-9 (36). MMPs and TIMPs are important enzymes in the process of ECM degradation. Expression of the MMP-9 protein is upregulated during the progression of various cancer types, including pancreatic cancer, and is directly involved in tumor cell migration, invasion, metastasis, tumor-related inflammation and angiogenesis (37,38). Therefore, its expression is associated with malignant progression (39).

The MMP-1 protein is one of the most widely expressed MMPs. It activates the G protein-coupled receptor protease-activated receptor-1 (PAR1) and induces secretion of bioactive proteins (most prominently interleukin-8, growth-regulated oncogene-α and C-C motif chemokine ligand 2) to regulate tumor migration (40). TIMP-1 enzyme is a natural inhibitor of MMPs, but it can also stimulate cell proliferation and prevent apoptosis, promoting cancer progression (41). The binding of TIMP1 to receptors on the cell surface activates the PI3K/Akt signaling pathway via Ras, and the EGF signal then induces TIMP1 expression (42,43). High plasma TIMP1 levels may interact with EGFR signaling, and thereby reduce the anti-tumor effects of EGFR inhibitors (44). The EGFR and EGFR proteins are widely recognized for their role in numerous cancer types, including PDAC (45,46). Upon binding to EGF, EGFR forms homologous dimers, auto-phosphorylates and interacts with downstream factors to activate genes involved in cell proliferation, differentiation, survival and migration (47,48). As such, inhibitors of the EGFR signaling pathway have received attention as potential therapeutic agents (49). Moreover, EGF-induced activation of EGFR increases MMP-9 expression levels through the activation of the PI3K/Akt pathway in patients with glioblastoma (50).

In the present study, the ALB gene showed the highest connectivity to other genes in Module 1A, and this gene encodes the most abundant protein found in human blood (51). Cachexia is expressed in <80% of patients with PDAC, and plasma albumin is often reduced (52,53). In the current study, it was speculated that besides digestive and absorption disor-
ders, cachexia may also be related to the low expression of ALB. Nevertheless, investigations into the regulatory genetic mechanism of ALB in PDAC have rarely been reported. PLAU, PLAUR and SERPINE1 represent the main components of the system. This system not only participates in cell signaling pathways (such as angiogenesis, cell growth, cell adhesion and migration) to influence cancer-related processes, but also in the disruption of the ECM through various pathways, such as the downregulation of the tumor suppressor gene p53, interference of Hoxa5 function, inhibition of the p38 pathway and activation of mitogen-activated protein kinase 1 (54-57).

An accurate and credible prognostic prediction system may help to better evaluate patient prognosis, provide a basis for clinical decision-making and indicate new therapeutic targets. Of the five genes associated with prognosis in the present study, LAMC2 and SER5 have also been reported to be prognosis-associated genes in PDAC (6,10). The LAMC2 and laminin subunit α3 proteins are important components of laminin 5. Through interaction with cell surface receptors, they participate in a variety of biological processes, including cell adhesion, differentiation, tumor angiogenesis and metastasis. The upregulation of LAMC2 is considered an indicator of adverse prognosis and the high metastatic potential of multiple cancer types, including colorectal cancer and lung adenocarcinoma (58). LAMC2 gene-silencing has been shown to significantly inhibit cell migration and invasion in head and neck squamous cell carcinoma cells (59). The LAMB3 protein regulates epithelial mesenchymal transition-related proteins and MMP-9 via the activation of the Akt signaling pathway, to promote the invasion and metastasis of tumors in papillary thyroid cancer (60). The important role of LAMA3 in the metastasis of lung adenocarcinoma has also been reported (61).

SERPINB5 is regarded as a tumor suppressor and an important senescence-associated biomarker. Notably, it is expressed in PDAC, but not in healthy pancreatic tissue (62). There is abundant evidence that SERPINB5 plays an onco-genic and pro-metastatic role (63,64), and is associated with the prognosis of patients with PDAC (62).

AREG is a ligand of EGFR and is overexpressed in various cancer tissues. It has been demonstrated that high AREG expression can be used as an independent prognostic indicator of poor overall survival in patients with PDAC, and there is a significant association between AREG/EGFR co-expression and poor tumor differentiation (65). The AREG protein has been clinically indicated as a prognostic and predictive biomarker, and numerous novel strategies have been developed to disrupt AREG-mediated oncogenic pathways (66).

SFRP4 is a Wnt-signaling antagonist. Due to its pro-apoptotic properties, SFRP4 provides axon-guidance information and functions as a tumor suppressor in multiple tissue types. Highly methylated SFRP4 induces transcriptional silencing, which activates abnormal Wnt signaling, leading to tumorigenesis and tumor progression (67). Moreover, SFRP4 monotherapy (or in combination with chemotherapy) can inhibit proliferation, reduce cell survival and initiate apoptosis in cancer stem cells in breast, prostate and ovarian cancer cell lines. This increases cell sensitivity to chemotherapy, amplifying the treatment effect (68). However, the mechanism of SFRP4 in PDAC remains poorly characterized.

Certain limitations of the present study should be noted. Firstly, the effects of certain patient and disease characteristics (tumor grade/stage, sex, age and race) on gene expression were not accounted for. Secondly, the GEO and TCGA data were obtained from public databases, and consequently, evaluation of data quality was challenging. Thirdly, variation in the accuracy of the prediction system for different molecular subtypes of PDAC has not been investigated due to a lack of consensus on the clinical classification of different molecular subtypes. Fourthly, TCGA dataset was used to validate 136 DEGs identified in the meta-analysis of the GEO datasets, but the results are not as similar as anticipated. This could be due to the small sample size of normal tissues in TCGA dataset. Finally, analysis was performed on gene expression databases; further molecular experimentation is required to validate the results.

In conclusion, the present study confirmed certain DEGs that were previously indicated in similar studies, and has also...
identified a set of genes that were not discovered in individual analyses. Therefore, this technique should be considered useful for the further analysis of heterogeneous expression datasets, improvement of DEG recognition techniques and the discovery of new biomarkers for PDAC diagnosis or therapeutic targeting. Furthermore, the present study identified 10 hub genes that may be involved in the pathogenesis of PDAC using multi-platform, multi-gene expression profile datasets and bioinformatics meta-analysis. In addition, a reliable predictive system, composed of five genes, for determining the prognosis of patients with PDAC has been constructed. The results of the current study may help to guide individualized clinical decision-making and future molecular-targeted therapies.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the GEO and TCGA databases.

Authors' contributions

XT and YP conceived and designed the study. YQP and YM drafted the manuscript. YM and YP collected and analyzed the data. LP critically revised the manuscript. XL, JX and LP were responsible for interpretation of the data. The manuscript was revised by YP XT, with final approval from YP.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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