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EXPERT REVIEWS

Gene-expression profiling in pancreatic cancer

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Pancreatic cancer has one of the worst prognoses, owing principally to a late diagnosis and the absence of good treatments. In the last 5 years, up to 12 molecular pathways involved in pancreatic cancer have been described. Global gene-expression profiling and the use of microarray databases have allowed the identification of hundreds of genes that are differentially expressed in pancreatic cancer. However, validation of these genes as biomarkers for early diagnosis, prognosis or treatment efficacy is still incomplete. Additionally, microRNAs have emerged as a potential source of variation between cancer and normal samples, and several of them have been identified as being deregulated in pancreatic tumors. An integrative point of view in the study of pancreatic cancer that makes use of all the whole-genome technologies has revealed several molecular mechanisms that affect pancreatic cancer development. These results should encourage the use of more personalized medicine in this pathology. Recent developments and future perspectives are discussed.

Keywords: gene expression • microarray • miRs • pancreatic cancer

Pancreatic cancer is a disease with a high rate of mortality that is generally diagnosed at advanced stages, when no successful therapies are available. A typical feature of pancreatic cancer is the local invasive potential and the distance it can spread in early developmental stages of the disease. The molecular mechanisms involved in the high tumorigenicity of pancreatic cancer are not well-known. However, a great gathering of molecular lesions, which occur in pancreatic cancer cells but not in normal cells of the pancreas, has been widely described in recent years. Many authors have demonstrated the role of these lesions in maintaining the transformed phenotype of the tumor cells, in spite of the remaining doubts regarding the cellular origin of pancreatic cancer and the sequence of molecular events that drive the progression from premalignant lesions to invasive cancer. Excellent analyses of such molecular characterizations have been summarized in recent reviews [1,2].

Among all tumor pathologies, pancreatic cancer has one of the worst prognoses. Recently published cancer statistics show that survival rate for pancreatic cancer patients has not improved substantially during the past 30 years, with a 5-year relative survival rate estimated to be approximately 5 %. It is the fourth leading cause of cancer death in the USA, and it accounted for approximately 35,000 deaths in 2009. Similarly, the number of newly diagnosed cases in 2009 has been estimated to be close to 42,500 [3]. From an anatomical point of view, the trend in frequency of new pancreatic head cancer cases has remained stable, whereas the incidence of pancreatic body/tail cancers is rising [4]. Pancreatic cancer is associated with only a few known demographic and environmental risk factors. Multiple studies have established that advanced age, cigarette smoking and chronic pancreatitis are clear risk factors. Others factors, such as a diet high in meats and fat, low serum folate levels, diabetes mellitus and obesity also appear to confer increased risk. Increased risk has also been documented in relatives of pancreatic ductal adenocarcinoma (PDAC) patients. Individuals with a first-degree relative with pancreatic cancer have a 2.3-fold higher risk of developing the malignancy and specific germline mutations have been linked to familial PDAC [2]. Recently, the identification and isolation of pancreatic stem-like cells have been described [1]. These cells have high tumorigenic potential (they can recapitulate the cellular heterogeneity of the original tumor). Thus, a principal role for these cells in the origin of tumor formation has been hypothesized. Several studies have been performed to analyze the gene and protein expression patterns from pancreatic tumor samples; however, the heterogeneity of

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the cell types contained in pancreatic tumors has not been taken into account. Therefore, the development of new therapeutic agents against these cells, combined with standard therapies, would allow for substantial improvement in the pancreatic cancer patient's prognosis [5].

Pancreatic ductal adenocarcinoma is the most common pancreatic neoplasm and accounts for more than 85% of pancreatic tumor cases and more than 90% of malignant tumors of the exocrine pancreas [6,7]. Other types of pancreatic neoplasms, such as acinar-cell carcinomas, ampullary tumors, endocrine tumors, cystadenomas/cystadenocarcinomas and intraductal papillary mucinous tumors, are less common [8,9]. Array-comparative genomic hybridization, as well as other whole-genome analysis techniques, has revealed many amplifications and deletions in pancreatic tumors [10–12]. Many of these copy number alterations appear to be relevant to the pathogenesis of pancreatic cancer, as inferred from their highly recurrent nature and the presence of known cancer genes in some loci. Many of these loci have been identified, as well as their linked signaling pathways, and most of these DNA alterations could be associated with gene-expression changes. In this sense, works combining genomic with expression data are very informative [10,12]. Regarding pancreatic tumor formation and development, to date, three histologically different types of ductal premalignant lesions have been identified in the pancreas: pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasms (MCN) and intraductal papillary mucinous neoplasms (IPMN) [6,9]. Of these, the PanIN lesions are the best characterized in both genetic and pathologic terms.

Gemcitabine, a nucleoside analog, as a monotherapy is the standard treatment for advanced pancreatic cancer because of its clear clinical benefits. Erlotinib, a small molecule that reversibly inhibits EGFR, in combination with gemcitabine has been approved as first-line therapy in pancreatic cancer, in spite of its poor results at demonstrating a significant increase in survival time (median: 12 days). In addition, a multitude of novel therapeutic agents and strategies are currently under investigation [13], such as gemcitabine-based combinations, nongemcitabine cytotoxic-based chemotherapy, molecular targeted therapy, immunological strategies, genetic strategies and cell-mediated strategies.

Gene-expression techniques

Scientists from multiple fields have used many different approaches to discover differentially expressed genes. Studies have gone from a one-to-one gene approach to an overview of the entire transcriptome, with microarray technology. Transition has been very quick, but in this way different techniques, such as differential screening or subtractive libraries, were used as the only method to study hundreds of genes at a time; for example, Crnogorac *et al.* used macroarrays to study the differential expression between normal and tumorous pancreases [14]. The arrival of SAGE and, in particular, microarray technology has revolutionized the field of differential gene expression. Now, we can query the entire transcriptome looking for genes or alternatively spliced isoforms differentially expressed in an experimental condition or disease versus control/normal condition. One of the biggest promises of genomic technologies has been the application of this knowledge to diagnosis and prognosis of cancer. There are several examples of microarray-based gene-expression tests that have been applied to cancer. MammaPrint was the first US FDA-approved, gene-expression-based prognostic test that assesses patient risk for distant metastasis in women aged under 61 years with stage I–II lymph node negative breast cancer [101]. The Tissue of Origin test is a gene-expression-based diagnostic assay for determining the tissue of tumor origin for poorly differentiated or undifferentiated tumor specimens [102]. However, these are the only examples that have been applied to cancer. Often, results obtained with microarrays are limited for several reasons: different technologies were used, the microarray platforms contained different targets and the processes to generate the probes for microarrays were also different. These discrepancies were principally observed during the first decade that this technology was put into use. However, nowadays, as has been shown by the MicroArray Quality Control (MAQC) project, excellent correlation and reproducibility have been seen in experiments using the same platforms and between the most commonly used commercial methods [15]. Furthermore, most of the journals require authors to submit the raw data from microarray experiments to databases, making it necessary to include the Minimal Information About a Microarray Experiment (MIAME) [16]. All these collaborative initiatives have resulted in the ability to compare results from multiple microarray experiments performed in different laboratories all around the world. In this sense, it is important to mention the main gene-expression repositories, Gene Expression Omnibus (GEO) and ArrayExpress [17–19,103,104]. These public repositories archive and freely distribute microarray, next-generation sequencing and other forms of high-throughput functional genomic data submitted by the scientific community. In addition to data storage, a collection of web-based interfaces and applications are available to help users query and download the experiments and stored gene-expression patterns, as well as perform meta-analyses. In addition, specific repositories for cancer, called Oncomine [20,105], and even one specifically for pancreatic cancer, called the Pancreatic Expression Database [21,106], have been created in recent years and are of enormous utility for oncology researchers.

PDAC differential gene expression

Since the beginning of genomic technologies, many groups have tried to identify biomarkers of pancreatic cancer that would help classify progression of the illness, response to treatment, and of course, possible targets to develop new therapeutic drugs. Figure 1 shows how the number of Pubmed entries using the terms 'gene', 'expression', 'profiling', 'pancreatic' and 'cancer' has evolved in recent years. An exponential increase is observed from 2000 to 2007; however, after 2007, the number of entries has remained constant or even decreased. This could be interpreted that from 2000 to 2007 technology was improving and lead to an exponential growth in the field of gene expression in pancreatic cancer. Once microdissection and microarray methods were optimized, studies involving pancreatic cancer were less likely

to be innovative and therefore researchers utilized them to explore more precise objectives, such as obtaining biomarkers of specific situations.

One of the principal problems found in studying gene-expression profiles in pancreatic tissues has been obtaining high-quality RNA. In a recent review, Vilardell and Iacobuzio-Donahue described in detail a valid procedure to isolate RNA from the pancreas for gene-expression experiments [22]. In general, until 2005, works in gene-expression profiles for pancreatic cancers were carried out to identify genes deregulated in cancer versus nontumor samples, stroma versus epithelial tumor and so on. However, in the last 5 years, most notable studies in the field have used previous data to obtain a higher statistical probability of finding good biomarkers for diagnosis, prognosis or response to therapeutic agents.

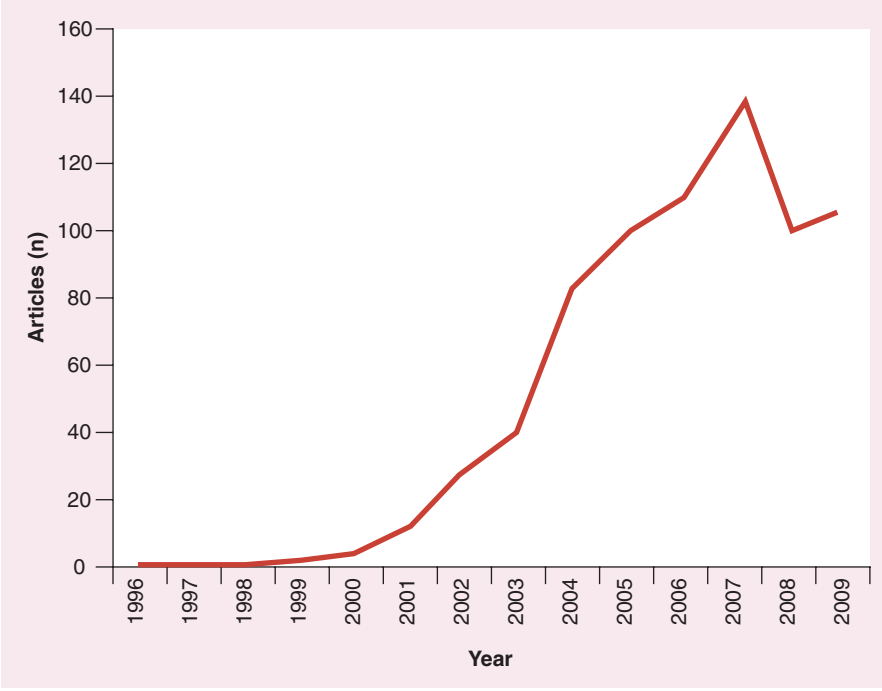

Figure 1. Number of published articles querying 'gene', 'expression', 'profiling', 'pancreatic' and 'cancer' in Pubmed [107] from 1996 to 2009.

Gene-expression profiles have been established using different techniques to look for genes differentially expressed in PDAC versus normal pancreatic tissue by using bulk tissues as well as microdissected ductal cells, pancreatic cancer cell lines or xenografts. One of the first studies published on gene-expression profiling in pancreatic cancer using bulk tissues and analyzing thousands of transcripts, was performed using SAGE [23]. The majority of published articles since then have used microarrays as the preferred technology. and pancreatic tumor cell lines have been used to identify regulated genes [24]. However, PDAC is difficult to study owing to the high proliferation of stromal cells that mask the neoplastic epithelial cells, which represent just a small fraction of the bulk tumor tissue [25]. To solve this problem, laser microdissection in combination with high-density microarrays has been performed achieving a more precise map of the gene-expression changes occurring in pancreatic cancer [26–29]. In 2002, characterization of gene-expression profiles from homogeneous populations of normal and neoplastic ductal cells by laser-capture microdissection was performed by cDNA [26] and oligonucleotide arrays [28]. Logsdon *et al.* identified genes differentially expressed between microdissected PDAC and chronic pancreatitis, and they showed that many deregulated genes were common in both situations compared with normal ductal cells, but with a higher fold change in PDAC samples [27]. Following initial characterization studies, projects involving comparisons of new and previous results have revealed new putative markers of pancreatic tumors. One of the first meta-analyses of gene-expression profiling data on pancreatic cancer was performed by Grutzmann *et al.* [30]. In this work, 568 genes were seen to be consistently and significantly deregulated in pancreatic cancer. The relevance of this study was that only 22% (127) were described in the published individual analysis. Badea *et al.* identified genes specifically overexpressed in tumor epithelia by microarrays [31], and combined their own data from whole tissue with those obtained from microdissected samples by others [28,32], such as epithelial and stromal cells from normal or PDAC samples, chronic pancreatitis stroma and PDAC cell lines. In that work, a list of differentially expressed genes is shown, which contains information about the frequency of identification of selected genes in 25 other previous publications on pancreatic cancer and microarrays, giving us an idea of the robustness of these genes as markers for PDAC. They obtained a clear TGF-β signature from the analyses of differentially expressed genes in tumor samples compared with normal samples. TGF-β signaling has been identified as one of 12 core signaling pathways and processes altered in pancreatic cancer, confirming the relevance of this pathway in the development of this type of cancer [12]. Jones *et al.* presented a very complete work integrating genomic data (sequencing, amplifications and deletions) with transcriptomic data (SAGE) in 24 PDAC patients [12]. The authors showed 12 core signaling pathways and processes altered in most pancreatic cancers, including apoptosis, DNA damage control, regulation of G_1/S phase transition, Hedgehog signaling, homophilic cell adhesion, integrin signaling, c-Jun N-terminal kinase signaling, KRAS signaling, regulation of invasion, small non-KRAS GTPase-dependent signaling, TGF-β signaling and Wnt/Notch signaling. This indicates that many genes can be affected in the case of pancreatic cancer. For this reason, the authors argue that we cannot talk of pancreatic cancer in general, but we have to talk of each individual patient's cancer. In this context, personalized medicine is being developed to treat a specific cancer in each individual patient, and it is only possible with a comprehensive point of view. However, to find common signatures for pancreatic cancer could be useful for diagnosis,

prognosis and treatment outcome, independently of the cancer origin. Thus, gene-expression profiles have helped, and will help, to further identify these biomarkers.

Use of microarray databases in pancreatic cancer

A web-based platform has been established to query pancreatic expression datasets [21,33,106]. This repository allows for the data mining of pancreatic cancer literature data. It contains 7636 gene-expression measurements from various pancreatic cancer types, precursor lesions and chronic pancreatitis, and includes proteomic information for urine and plasma [14,26–29,34–48]. It can be used for target discovery, identification and analysis of genes associated with the progression of cancer, cross-platform meta-analyses, single nucleotide polymorphism-association studies and cancer gene promoter analysis, as well as mining cancer ontology information. In the near future, this database will help to understand the pathobiology underlying pancreatic cancer initiation and progression, and therefore, help to develop diagnostic tools and new and more effective targeted drugs. Most of the molecules regulated in PDAC are also regulated in chronic pancreatitis, but to a different degree, probably because they share molecular and physiological pathways. We have used this database to obtain a list of differentially expressed genes in PDAC versus normal pancreas (microdissected and bulk tissue), and PDAC versus chronic pancreatitis tissues with the aim of summarizing the genes that can be putative biomarkers of pancreatic cancer. A total of 132 genes were retrieved from datasets included in the Pancreatic Expression Database. From those, 33 were revealed as differentially expressed in five or more studies (Table 1).

A second list was obtained using a meta-analysis by the Oncomine database (Figure 2). The 20 highest scored genes for over- and under-expression in PDAC versus normal pancreas using a set of six works containing 134 samples are shown [27–29,48–50]. We can observe the different output files obtained using these two databases when querying for pancreatic cancer-regulated genes. Comparison of gene lists obtained by the Pancreatic Expression Database (34 genes) and Oncomine (40 genes) reflects that seven genes were present in both lists: *S100P*, *FN1*, *THBS2*, *PNLIPRP1*, *CLPS*, *CPA2* and *AMY1A*. This strategy could help to identify good biomarkers for pancreatic cancer. Furthermore, S100P has recently been suggested as a useful biomarker for detecting all types of IPMN, a precursor of PDAC [51].

The clinical data submitted to public repositories are sometimes incomplete, which makes any future usage/reproducibility difficult. The combined use of specialized DNA microarray repositories and relevant publications has allowed for the identification of an almost complete list of molecules over- and under-expressed in PDAC [52]. As sources for this approach, Harsha *et al.* used databases such as GEO [103], ArrayExpress (EBI) [104], Oncomine [105] and PubMed [107] by looking for articles describing differential mRNA and/or protein profiling of neoplastic versus non-neoplastic pancreatic tissues and/or cell lines. After filtering the data, they selected 441 genes to be reported as overexpressed at both the mRNA and protein levels. More than 200 genes were reported to be overexpressed in pancreatic cancers in four or more studies, making them extraordinary candidates for biomarker validation. This compendium can be considered an excellent summary of gene-expression profiling for pancreatic cancer. A complete list with the results of this compendium is accessible for free [108].

Diagnostic biomarkers

Early detection of cancer is extremely important because an early diagnosis increases the probability of a healthy prognosis. Most patients diagnosed with pancreatic cancer die within 12 months and a 5-year survival rate is seen in less than 5% of cases [3,6,53]. Three PDAC precursor lesions have been histologically distinguished: PanIN (from grades 1–3), MCN and IPNM [54,55]. These precursor lesions provide an excellent opportunity to look for early detection biomarkers for PDAC. In 2002, a study on IPNM samples using microarrays identified four differentially expressed genes (*CAV1*, *GPC1*, *GAS6* and *CYR61*) as putative markers of tumor progression [46]. An aberrant expression of retinoic acid signaling components was identified in pancreatic cancer, including HOXB2, which was expressed in 15% of PanIN lesions [48]. Buchholz *et al.* used microarrays to compare the expression profiles of microdissected cells from normal pancreatic ducts, with PanINs of different grades and PDACs [29]. They found that most gene-expression changes were observed from stage 1b to 2, suggesting that this degree of tumor development is the first preneoplastic stage in PDAC. A search for upregulated genes in PanIN-2 lesions compared with normal ductal cells with a high expression maintained throughout the progression to PDAC resulted in the identification of 30 genes. In order to check the efficacy of the Pancreatic Expression Database at identifying biomarkers for early diagnosis of PDAC, we searched the database for genes differentially expressed in PanIN-2, PanIN-3 and microdissected PDAC, and compared the results obtained with the Buchholz list. A total of 80 genes were identified using these criteria, 15 of which were upregulated in the tumor stage. Comparison between both lists of upregulated genes showed four common genes: *IL-13RA1*, *IFI27*, *RABAC1* and *S100P*. S100P calcium-binding protein has been useful as a biomarker for the diagnosis of pancreatic tumors [56]. IFI27 has been suggested as a marker of epithelial proliferation and cancer [57]. Predictive studies using this gene hold a great deal of potential in the future given that pancreas ductal cells are a type of epithelial cells. Harsha *et al.* have also obtained a list of 1100 molecules overexpressed in PanIN and IPNMs, with most also showing elevated expression in PDAC [52]. All these data have revealed a lot of genes as potential biomarkers for the early diagnosis of PDAC. The step that remains is to check the validity of these genes to identify whether the tumor is in the early stages, and thus help reduce the mortality rate of these patients.

Although it is infrequent, clinicians sometimes have difficulties in distinguishing between PDAC and chronic pancreatitis. There are no good molecular biomarkers to distinguish between these situations, principally because most genes regulated in PDAC have also been shown to be regulated in chronic pancreatitis. Nevertheless, ANXA2 and IGFBP-2 have been seen to be more highly expressed in PDAC compared with chronic pancreatitis [58].

Table 1. Gene list obtained by querying the Pancreatic Expression Database.

Gene symbol	Gene name	Studies (n)	Ref./platform
S100P	Protein S100-P (S100 calcium-binding protein P)	11	[29]/13; [38]/1; [40]/9; [28]/5; [27]/1; [34]/3; [46]/8; [48]/5; [43]/2; [47]/5; [37]/14
FN1	Fibronectin precursor	10	[29]/13; [38]/1; [34]/3; [46]/8; [45]/4; [43]/2; [35]/3; [47]/5; [42]/5; [37]/14
AMY2A	Amylase, α2A	7	[38]/1; [28]/5; [34]/3; [41]/7; [39]/7; [44]/11; [47]/5
FSCN1	Fascin (singed-like protein)	7	[38]/1; [40]/9; [27]/1; [41]/7; [43]/2; [47]/5; [36]/10
PRSS1	Protease, serine, 2 (trypsin 2)	7	[28]/5; [34]/3; [41]/7; [39]/7; [35]/3; [44]/11; [47]/5
THBS2	Thrombospondin-2 precursor	7	[38]/1; [40]/9; [34]/3; [46]/8; [43]/2; [47]/5; [36]/10
CEL	Bile salt-activated lipase precursor	6	[29]/13; [38]/1; [34]/3; [41]/7; [39]/7; [47]/5
CLPS	Colipase precursor	6	[38]/1; [28]/5; [34]/3; [43]/2; [44]/11; [47]/5
COL3A1	Collagen α-1(III) chain precursor	6	[29]/13; [38]/1; [34]/3; [43]/2; [47]/5; [26]/6
CPA1	Carboxypeptidase A1 precursor	6	[38]/1; [41]/7; [39]/7; [43]/2; [44]/11; [47]/5
GATM	Glycine amidinotransferase, mitochondrial precursor	6	[38]/1; [34]/3; [41]/7; [39]/7; [45]/4; [43]/2
KRT7	Keratin, type II cytoskeletal 7	6	[40]/9; [27]/1; [39]/7; [41]/7; [47]/5; [26]/6
PNLIPRP1	Pancreatic lipase-related protein 1 precursor	6	[38]/1; [28]/5; [41]/7; [39]/7; [44]/11; [47]/5
SPARC	SPARC precursor	6	[38]/1; [45]/4; [43]/2; [35]/3; [47]/5; [42]/5
TFF3	Trefoil factor 3 precursor	6	[38]/1; [34]/3; [46]/8; [43]/2; [42]/5; [37]/14
VCAN	Versican core protein precursor	6	[38]/1; [34]/3; [45]/4; [43]/2; [47]/5; [26]/6
ACTB	Actin, cytoplasmic 1	5	[34]/3; [46]/8; [43]/2; [44]/11; [14]/12
AMY1A	Amylase, α1A	5	[38]/1; [28]/5; [34]/3; [39]/7; [47]/5
AMY1B	α-amylase 1 precursor	5	[38]/1; [28]/5; [34]/3; [39]/7; [47]/5
AMY1C	α-amylase 1 precursor	5	[38]/1; [28]/5; [34]/3; [39]/7; [47]/5
AMY2B	Amylase, α2B	5	[38]/1; [28]/5; [41]/7; [39]/7; [47]/5
CALD1	Caldesmon	5	[38]/1; [41]/7; [39]/7; [43]/2; [36]/10
COL1A1	Collagen α-1(I) chain precursor	5	[38]/1; [34]/3; [46]/8; [43]/2; [47]/5
COL1A2	Collagen α-2(I) chain precursor	5	[38]/1; [45]/4; [43]/2; [47]/5; [14]/12
CPA2	Carboxypeptidase A2 precursor	5	[38]/1; [28]/5; [39]/7; [43]/2; [44]/11
CPB1	Carboxypeptidase B precursor	5	[38]/1; [28]/5; [39]/7; [41]/7; [47]/5
CTRB2	Chymotrypsinogen B precursor	5	[38]/1; [28]/5; [41]/7; [39]/7; [47]/5
CYB5A	Cytochrome b5	5	[29]/13; [38]/1; [41]/7; [39]/7; [43]/2
LCP1	Plastin-2	5	[38]/1; [34]/3; [41]/7; [39]/7; [43]/2
PNLIP	Pancreatic triacylglycerol lipase precursor	5	[38]/1; [28]/5; [41]/7; [39]/7; [43]/2
RBP1	Retinol-binding protein 1	5	[38]/1; [41]/7; [39]/7; [48]/5; [43]/2
TRIM29	Tripartite motif-containing protein 29	5	[29]/13; [27]/1; [43]/2; [47]/5; [37]/14
TTR	Transthyretin precursor	5	[34]/3; [39]/7; [43]/2; [35]/3; [44]/11

The following filter criteria for genes differentially expressed were used: PDAC versus normal pancreas (bulk tissue), PDAC versus normal pancreas (microdissected normal ductal cells), and PDAC versus chronic pancreatitis (bulk tissue).
Note: 33 out of 132 genes identified in five or more studies were included. Platforms used were: 1: Commercial Array Platform:GeneChip HuGeneFL Affymetrix (7000 sequences representing 5600 full-length human genes); 2: cDNA Array (in house, Human Genome Centre Tokyo, Japan); 3: Mass spectrometry; 4: cDNA Array, United Gene technique, Ltd; 5: Commercial Array Platform: U133A Affymetrix GeneChip; 6: cDNA Array, Commercial Array Platform: Clontech Atlas Cancer Array, SAGE; 7: 2D-PAGE, MALDI; 8: cDNA Array, 5K1 slides used contained 5184 elements, Sanger UK; 9: Oligo Array, Commercial Array Platform: Affymetrix HG U95 A, B, C, D and E; 10: Commercial western blotting: BD PowerBlot; 11: 2D-PAGE, mass spectrometry, 12: cDNA Array, Commercial Array Platform: Clontech Atlas Cancer Array; 13: Oligo Array, Commercial Array Platform: The Human Genome Oligo-Set-Version 2.0 (Operon, Germany); 14: cDNA Array, version 1.2.1 human 7 K arrays contain 9932 elements, Sanger UK.
PDAC: Pancreatic ductal adenocarcinoma.

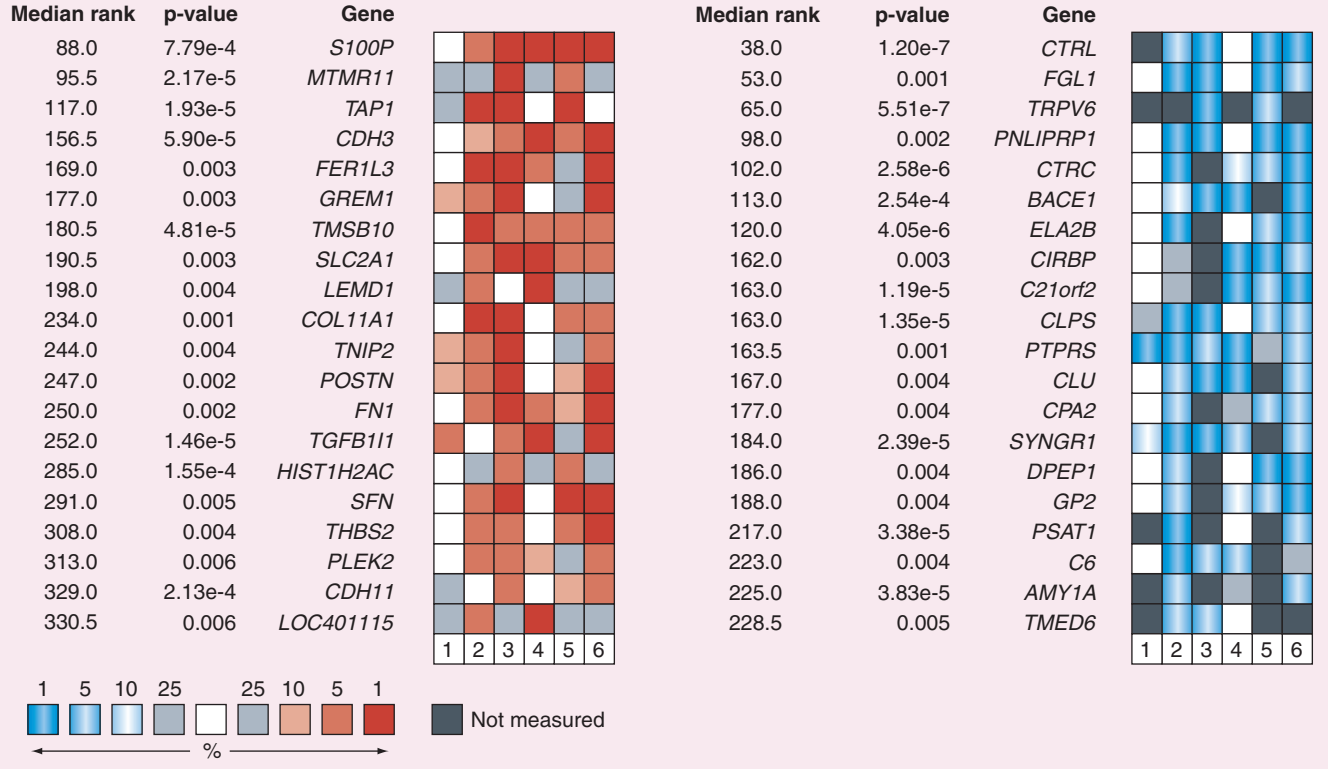

Figure 2. Up- and down-regulated genes obtained using the Oncomine database with the following filters: differential analysis, cancer versus normal analysis, and cancer type (pancreatic cancer). The rank for a gene is the median rank for that gene across each of the analyses. The p-value for a gene is its p-value for the median-ranked analysis. Data for comparison were as shown in the legend.
Correspondence of columns with studies: 1 [29], 2 [28], 3 [49], 4 [50], 5 [27] and 6 [48].

In terms of molecular diagnosis, protein arrays have also been used to identify signatures that discriminate between pancreatic cancer patients and healthy subjects [59,60]. The use of antibody microarrays distinguished cancer samples from healthy control samples with a 90–93% sensitivity and 90–94% specificity in duplicate experiment sets [59]. In addition, using human recombinant scFv antibody microarrays against 60 proteins, as few as 19 serum proteins were sufficient to identify pancreatic patients [60]. These are very promising results, which also possess the advantage of being a noninvasive method for diagnosis.

Biomarkers for progression & survival in pancreatic cancer

Another interesting goal of gene-expression profiling has been the search for gene predictors of a better prognosis. Pancreatic cancers with metastases have been seen to present extremely poor prognoses. Research was carried out to identify genes whose expression was different in an established pancreatic cancer cell line, SW1990, combined with a clone with a high potential for liver metastasis, SW1990HM [61]. A total of 40 genes were identified to be related to liver metastasis in pancreatic cancer. However, this is a very preliminary study that needs to be extended and validated with tumor samples.

Similarly, Campagna *et al.* compared the gene-expression profiles of primary carcinomas from five patients one-by-one versus their own matched metastases by microarrays [62]. No gene was regulated in all of the samples, but *PKC1* and *SFRP2* were selected in four out of five, and *COL10A1*, *FBX032*, *MFAP5* and *PDGFD* were selected in three out of five. They also analyzed the expression profiles associated with advanced tumor stages, finding 173 genes deregulated in the pathological stage pT4 compared with stage pT2/T3 carcinomas. Four of these genes were first validated by qRT-PCR with the same samples employed in microarrays and then tested with a new set of tumors. Only *MXI1* was confirmed to be overexpressed in pT4 compared with pT1–T3 tumors.

Using human recombinant scFv antibody microarrays, 21 proteins were sufficient to identify cancer patients with a life-expectancy of less than 12 months [60]. This is probably the most complete proteomic approach to define a signature capable of predicting the survival of cancer patients.

Gene-expression patterns & response to treatment

Gemcitabine is the standard chemotherapeutic agent for pancreatic cancer. Resistance to chemotherapy is a major problem in the treatment of pancreatic and other cancer types. Gene-expression profiling has tried to approach this problem using established cell

lines [63,64] or primary cultures [65]. Comparison by microarray was performed with MiaPaCa2, a pancreatic tumoral gemcitabine-sensitive cell line, and MiaPaCa2-RG, a gemcitabine-resistant variant [63]. From among 43 deregulated genes, the *RRM1* subunit was silenced by siRNA in MiaPaCa2-RG cells reducing its gemcitabine-resistance to the level of MiaPaCa2 cells. In a recent study, *ISG15* was identified as being related to intrinsic gemcitabine sensitivity in pancreatic cancer cell lines and is highly expressed in pancreatic cancer [64]. Bai *et al.* compared the gene-expression profiles of gemcitabine-resistant versus gemcitabine-sensitive pancreatic primary tumoral cells [65]. *GSTT1*, *TOP2A*, *CASP3* and *ABCC2* were identified as differentially expressed in gemcitabine-sensitive cancers using a microarray containing 1081 genes.

Src is a tyrosine kinase overexpressed in pancreatic cancer, which can be inhibited by bosutinib, a Src/Abl inhibitor. Analysis of gene arrays using a panel of human pancreatic xenografts containing three drug-sensitive and 12 drug-resistant samples, revealed a six-gene classifier that predicts resistance versus sensitivity [66]. This gene signature included *GRP116*, *CEACAM7*, *ALPK3*, *LQK1*, *ERLIN2* and *GUF1*. This result was validated using six new samples. The same authors also analyzed the predictive value for sensitivity/resistance of *CAV1* expression, a gene that was reported previously to negatively regulate the downstream activation of Src family tyrosine kinases [67]. They found that *CAV1* expression was higher in bosutinib-sensitive cases, but this difference was not statistically significant. Evidently, the number of samples used for validation was insufficient and the gene needs to be reanalyzed with a higher population. Nevertheless, these results are very promising.

Obviously, it is too early to have a biomarker that is suitably proven for clinical applications, but it is clear that in the coming years more pharmacogenomic biomarkers could be identified that would allow treatment response of PDAC patients to be accurately predicted.

MicroRNAs & pancreatic cancer

MicroRNAs (miRs) were first discovered in 1993 as negative regulators of gene-expression in *Caenorhabditis elegans* [68]. miRs are a class of small non-coding RNAs 19–24 nucleotides in length, with important regulatory functions in diverse biological processes. miRs bind complementary sequences in mRNAs, targeting them for degradation and/or inhibiting translation. Until now, 721 miRs have been identified [109], and differential expression of many of them has been related to cancer [69]. In particular, works have been published establishing the relationship between miR expression profiles and prostate cancer [70], breast cancer [71], colorectal cancer [72] and many other tumor types.

Not many works have been published specifically dealing with pancreatic cancer and miR profiling. The principal goal of such studies is to identify gene signatures for early prognosis, progression or for disease outcome. Moreover, array platforms of miRs are often different, making comparisons difficult. Bloomston *et al.* used a custom miR microarray from Ohio State University, USA, which contained 326 human miRs [73]. They identified 11 differentially expressed miRs differentiating pancreatic cancer from either normal pancreas or chronic pancreatitis. One of them, miR-21, has been suggested as a biomarker for disease outcome because it is overexpressed in pancreatic cancer, predicting limited survival time in patients with node-negative disease [74]. The expression of a subgroup of six miRs was also predictive of long-term (>24 months) survival [73]. Recently, results from a published study showed that tumors from patients demonstrating elevated expression levels of four miRs (miR-155, miR-203, miR-210 and miR-222) possessed a 6.2-fold increased risk of tumor-related death compared with patients whose tumors showed a lower expression of these miRs [75]. Good results have also been obtained using low-density miR microarrays based on quantitative PCR. A gene signature was established by real-time profiling of over 200 miR precursors (practically all known miRs at that time) in samples of human pancreatic adenocarcinoma, adjacent benign tissues, normal pancreas, chronic pancreatitis and cancer cell lines [76]. Most of the groups were clearly differentiated by an unsupervised hierarchical clustering using 112 miRs whose expression levels were altered. However, some adjacent benign tissue samples were classified in the PDAC group. A more recent and directed assay was performed analyzing the differential expression of 95 miRs chosen based on their potential functions related to cancer biology, cell development, and apoptosis [77]. Eight miRs were significantly upregulated in most pancreatic cancer tissues and cell lines. They were miR-196a, miR-190, miR-186, miR-221, miR-222, miR-200b, miR-15b and miR-95.

Nevertheless, the clinical application of these findings needs further supporting research before becoming a reality. Venny software [110] was used to compare four miR lists from different publications [73,76–78] in order to find common miRs from all four studies. Only miR-221 was found to be overexpressed in PDAC in all of them, while miR-222 was overexpressed in three out of four. This is not very informative because the number of tested miRs was extremely different between studies, but it gives us an idea that at least the deregulation of miR-221 and miR-222 are highly represented in PDAC. In addition to these data, miR-221 and miR-222 have also been related to many others types of cancer and in skeletal muscle differentiation [79].

Little is known about miRs as molecular biomarkers of treatment efficacy in pancreatic cancer. Li *et al.* compared the expression of miRs between gemcitabine-sensitive and gemcitabine-resistant pancreatic cancer cells [80]. They found that the expression levels of miR-200b, miR-200c, let-7b, let-7c, let-7d and let-7e were significantly downregulated in gemcitabine-resistant cells and that 3,3′-diindolylmethane and isoflavone could function as miR regulators.

Results obtained in miR expression profiles have potential therapeutic applications. For example, preliminary studies of the inhibition of miR-21 and miR-221 by antisense oligonucleotides reduced proliferation of pancreatic cancer cell lines and increased apoptosis [81].

In terms of molecular diagnostic applications of miR for pancreatic cancer, Wang *et al.* developed a noninvasive method for early detection of pancreatic cancer by isolating miRs from plasma [82]. In this work, expression of four miRs, miR-21, miR-210, miR-155 and miR-196a, were profiled in plasma differentiating pancreatic adenocarcinoma patients from healthy controls, with a sensitivity

of 64% and a specificity of 89%. Obviously, this diagnostic tool is not ready to be translated to the clinic, but at least it is a starting point that can be improved upon.

Endocrine pancreatic cancer

Little is known concerning gene-expression profiles of pancreatic endocrine tumors. The WHO classifies three categories of pancreatic endocrine tumors: well-differentiated endocrine tumors, well-differentiated endocrine carcinomas and poorly differentiated endocrine carcinomas, each with a different prognosis. Gene-expression profiling studies have identified several potential biomarkers for pancreatic endocrine tumors [42,83,84]. The most complete gene-expression profiling on this subject was performed using 72 pancreatic endocrine tumor patients [84]. The results suggested an important role for the PI3K–Akt–mTOR pathway in tumorigenesis and progression of pancreatic endocrine tumors. Namely, two genes in this pathway, *TSC2* and *PTEN*, were downregulated in most primary tumors, and their low expression was significantly associated with a shorter disease-free period and a decreased overall survival time. Genomic alterations in this pathway have also been found to be important in PDAC, suggesting overlapping mechanisms for endocrine and nonendocrine pancreatic tumors [12]. Moreover, *FGF13* was identified as a marker of progression in pancreatic endocrine tumors and its expression correlated with tumor aggressiveness and proliferation; again, this is evidence of another initial biomarker that must be validated before being applied in the clinic.

Expert commentary

Gene-expression techniques developed in the 1990s to analyze thousands of genes along with the sequencing of the human genome, promised definitive advances in multiple diseases. However, until now the utility of gene-expression tests is lower than expected in most fields in general and in pancreatic cancer in particular. For instance, MammaPrint and Tissue of Origin tests are two examples of only a few successful microarray-based gene-expression tests that have been applied to cancer. We are still a long way from having a similar test applied to pancreatic cancer. One reason that could explain this fact is that pancreatic cancer is a multifactor disease, which could be more complex and could affect more pathways than breast cancer. Thus, oncologists are starting to talk of each patient's pancreatic cancer instead of pancreatic cancer in general. All the assays to find a unique and universal biomarker have failed or at least have not given the expected results. However, multiple pathways have been identified to be altered in PDAC. Moreover, studies combining genomic with gene-expression data are very convenient and have shown potential biomarkers to be studied. Thanks to gene-expression profiles, hundreds of genes have been identified as regulated in pancreatic cancer. Many of these genes are potential biomarkers for diagnosis, prognosis, survival and response to treatment. The principal goal of these studies was to apply this knowledge to the clinic in order to provide a method for earlier diagnosis of the illness and to know the prognosis in order to apply the most adequate treatment, and to identify new targets. Unfortunately, all efforts made toward these objectives have not been completely successful. There have been promising but partial results, and the clinical application of these technologies for pancreatic cancer diagnosis and treatment is not a reality, yet. We know that through the exclusive use of gene-expression profiles, there are still many difficulties in dealing with pancreatic cancer. Furthermore, an individualized approach for each patient, defining the mutated, amplified or deleted genes, and those with regulated expression will be necessary. Only in this way will we be able to address a particular cancer and to provide the best treatment possible for each particular patient.

Five-year view

The accuracy of the data obtained in the last 5 years using integrative tools (genomic, transcriptomic and proteomic) and databases has helped to precisely define the putative genes that are upregulated or downregulated in pancreatic cancer and during different stages of the pancreatic tumor. The work published by Harsa *et al.* reported an extraordinary compendium of the acquired knowledge in molecular characterization of deregulated genes in pancreatic cancer [52]. It is an excellent base for further developing the validation of biomarkers for diagnosis, prognosis, pharmacogenetics and pharmacogenomics. However, an important effort must be made to submit the full clinical data of samples to the public repositories because it is essential to ensure that data generated across different organizations can be shared to maximize the impact of the research. Thus, in the next 5 years and once the regulated genes in pancreatic cancer have been described further, maximum efforts will be made to test and validate those that possess potential value for use in the clinic. This work has already been started with limited results. The miR research in pancreatic cancer will have special interest in the near future because of the new discoveries made recently. Results obtained in the last 5 years in this field are very promising and validation of putative biomarkers could be very successful. We need to assume that we will probably not have universal biomarkers and that gene signatures must include several components.

As far as diagnosis is concerned, we suppose that in a 5-year timeframe, noninvasive methods will probably be developed and advances will be achieved, reducing the time and improving the accuracy in the diagnosis of pancreatic cancer. Integrated approaches involving all the omics will be necessary to advance the knowledge and clinical applications for pancreatic cancer treatment. In the near future, pharmacogenomics, together with pharmacogenetics, in-depth sequencing of individual genomes and advanced cytogenetic analysis, will help in obtaining a deeper knowledge of the processes that lead to pancreatic cancer, thus improving diagnosis and prognosis, identifying predictive biomarkers, and developing new therapeutic strategies. We expect that in the next few years these improvements will help to reduce the enormous mortality rate from pancreatic cancer.

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Key issues

- Even though most molecular changes in pancreatic cancer have been elucidated using genomic technologies, concerning prognosis, the results are still very poor.
- The genomic characterization of pancreatic cancer precursor forms and gene signatures of different types of tumors in the pancreas is a challenge still faced.
- The use of results compiled during the last decade in gene-expression profiling in pancreatic cancer has provided a refined list of genes that can be tested as biomarkers for early diagnosis, prognosis and treatment response.
- Several molecules have been tested as diagnostic tools with promising results, but the number of patients in the studies has been too small in general, so the results must be confirmed with larger cohorts.
- MicroRNA profiling is a recently developed method of exploration that promises new findings in the field of pancreatic cancer research.
- There are no valid biomarkers or gene signatures for pancreatic cancer with clinical use in molecular diagnosis. However, this will probably change in the coming years, thanks to integrative approaches combining genomic, transcriptomic and proteomic data.

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