

Expression of Genes with Copy Number Alterations and Survival of Patients with Pancreatic Adenocarcinoma

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Abstract. *Background/Aim: Individual molecular information might improve management of pancreatic adenocarcinoma. To identify actionable genes, at the transcriptional level, we investigated candidate genes that we had previously identified using array-comparative genomic hybridization (aCGH). Materials and Methods: We collected 10 public gene-expression datasets, gathering a total of 524 pancreatic samples (105 normal and 419 malignant tissues). Based on our previous aCGH analysis, we searched for genes differentially expressed between normal and malignant samples and genes associated with survival. Results: Among genes amplified/gained by aCGH, 48% were overexpressed in malignant tissues. The majority of these genes were related to apoptosis, cell-cycle regulation and differentiation. Among genes located in areas of loss, 41% were underexpressed in malignant tissues; most of them were involved in ion transport, homeostasis maintenance and fatty acid metabolism. Survival analysis identified genes significantly related to shorter (n=17) or longer (n=29) survival. Conclusion: Some of these genes can be further investigated as potential prognostic markers.*

Pancreatic adenocarcinoma (PDAC) has the highest mortality rate of all human cancers. Early radical surgical resection of

the tumor is the only potentially curative treatment. However, fewer than 20% of patients are eligible for surgery. The median survival of patients with inoperable non-metastatic or metastatic pancreatic cancer is close to 6 months and 5-year survival is nil. Inoperability and poor prognosis are due to late diagnosis, rapid tumor progression (>80% of patients display metastases at diagnosis) (1), early recurrence after resection, and poor response to available therapies (1, 2). This poor clinical outcome and the heterogeneity of pancreatic cancer may be explained, at least in part, by molecular alterations present in the tumor at the DNA and RNA levels. Innovative approaches and a comprehensive characterization of these molecular alterations should help develop tools for early detection, in identifying new prognostic markers and in designing novel targeted therapies (3).

Progress has been made in the understanding of pancreatic cancer. Molecular analyses of cell lines and clinical tumor samples have identified frequent gains on chromosome arms 1q, 3q, 5p, 7p, 8q, 11q, 12p, 17q, 19q and 20q, and losses on chromosome arms 3p, 6, 8p, 9p, 10q, 13q, 15q, 17p and 18q (4-9). Inactivating mutations or deletions of tumor suppressor genes (TSGs), activating mutations of oncogenes, and epigenetic modifications have been identified (10-12). The most frequently affected genes are Kirsten rat sarcoma (*KRAS*), SMAD family member 4 (*SMAD4*), tumor protein p53 (*TP53*) and cyclin-dependent kinase inhibitor 2A (*CDKN2A/B*), but many genes such as AT-rich interaction domain 1A (*ARID1A*, *ARID2*), ATM serine/threonine kinase (*ATM*), mitogen-activated protein kinase kinase 4 (*MAP2K4*), splicing factor 3b, subunit 1 (*SF3B1*) and transforming growth factor beta receptor II (*TGFBR2*), and have been recently identified as being affected by next-generation sequencing (13, 14). A large number of gene expression studies have been published on PDAC (15-20). However, most of these analyses included limited numbers of samples. Furthermore, the current

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clinical applications are very limited, and considering the complexity of the genome, it is likely that most of molecular changes causing pancreatic cancer still need to be elucidated.

By using whole-genome high-resolution array-comparative genomic hybridization (aCGH), we previously studied the genomic alterations of 39 fine-needle aspirations from patients with PDAC. Recurrent losses were observed on chromosome arms 1p, 3p, 4p, 6, 8p, 9, 10, 11q, 15q, 17, 18, 19p, 20p, 21 and 22, and included several known or suspected TSGs. Frequent genetic gains or amplifications were found on 1q, 3q, 5p, 6p, 7q, 8q, 12q, 15q, 18q, 19q and 20q. We also identified for the first time mutations in *ARID1A* (3, 12). In the present study we focused on those genes that we found lost or gained using aCGH, and looked at their expression levels in a large pooled public dataset of malignant *versus* normal pancreatic tissues. In addition, we determined whether some of these genes exhibited de-regulated expression associated with survival.

Materials and Methods

Gene-expression datasets. We collected 10 retrospective public gene-expression datasets including clinical pancreatic cancer samples profiled using oligonucleotide-based microarrays (Table S1: http://disc.marseille.inserm.fr/PancADC_CGP2015/Table_S1.xlsx). Gene-expression and clinicopathological data were retrieved from Genbank GEO (<http://www.ncbi.nlm.nih.gov/gds/>) and Array Express databases (<https://www.ebi.ac.uk/arrayexpress/>). The 10 datasets comprised a total of 524 clinical samples, including 419 adenocarcinomas and 105 normal tissue samples. The main clinical features of the patients are listed in Table I.

Pre-analytic gene expression data processing. Before analysis, gene expression data were processed. Firstly, each dataset was normalized separately as follows: quantile normalization for the available processed data from Agilent-based and Illumina sets, and Robust Multichip Average (21) with the non-parametric quantile algorithm as normalization parameter for the raw Affymetrix data. Normalization was carried out in R using Bioconductor and associated packages. Secondly, we mapped hybridization probes across the technological microarray platforms represented in these datasets. We used NetAffx Annotation files (www.affymetrix.com; release from 01/12/2008) to update the Affymetrix gene chips annotations, and both SOURCE (<http://smd.stanford.edu/cgi-bin/source/sourceSearch>) and EntrezGene (Homo sapiens gene information db, release from 09/12/2008, <ftp://ftp.ncbi.nlm.nih.gov/gene/>) to retrieve and update the non-Affymetrix gene chips annotations. All probes were then mapped based on their EntrezGeneID. In the case of multiple probes mapped to the same EntrezGeneID, the one with the highest variance in a particular dataset was selected to represent the EntrezGeneID. Next, we corrected the 10 studies for batch effects using a z-score normalization. Briefly, for each gene-expression value in each study separately, all values were transformed by subtracting the mean of the gene in that dataset divided by its standard deviation, mean and standard deviation being measured on primary cancer samples only. The global dataset was obtained by concatenation of the 10 normalized sets by matching their EntrezGeneID. The final merged set included 19,824 genes in log₂-transformed data. The accuracy of normalization was verified with hierarchical clustering(22), which

Table I. *Clinicopathological characteristics of patients and tumors.*

Characteristic	N (%)
Median (range) age, years	62 (17-79)
Gender (M/F)	56/48
T in cTNM classification	
T1	3 (2)
T2	24 (19)
T3	98 (78)
T4	1 (1)
N in cTNM classification	
N0	41 (32)
N1	88 (68)
Median overall survival (range), months	15 (1-156)
2-Year overall survival (n=186) (95% CI)	37% (31-46%)
Median follow-up (range), months	21 (1-59)

CI: Confidence interval.

showed no correlation between the two main sample clusters and the dataset origin on primary cancer (Figure S1: http://disc.marseille.inserm.fr/PancADC_CGP2015/Figure_S1.ppt).

Gene expression analysis and statistical analysis. Analysis of gene expression data was then limited to the 692 genes that we had previously found as altered by aCGH¹³, including 352 amplified/gained (potential oncogenes), and 345 lost (potential TSGs). Supervised analysis searched for genes differentially expressed between the 419 cancer samples and the 105 normal samples (*i.e.* the gene-expression signature), using Student's *t*-test, unpaired for the whole series of 524 samples and paired in the subset of 81 matched cancer-normal tissue pairs. Genes with a *p*-value of 0.05 or less were considered as significant. To help in the interpretation, the lists of discriminator genes were interrogated using Ingenuity Pathway Analysis software (version 5.5.1-1002; Ingenuity Systems, Redwood City, CA, USA).

We also searched for correlation between gene-expression levels and survival. Clinicopathological data were available for 186 patients only, including 135 who died after a median of 13 (range=1-156) months. The median overall survival was 15 (range=1-156) months, with a 2-year survival rate of 37% (95% confidence interval=31-46%). The median follow-up was 21 (range=1-59) months. Univariate analysis was carried out using Cox regression analysis (Wald test) with survival analysis as continuous variable. For significant genes, the Cox model was used to define "high-risk" and "low-risk" groups based on gene expression level. Kaplan-Meier survival curves were then drawn and comparison between curves was performed using the log-rank test. All statistical tests were two-sided at the 5% level of significance. Analyses were carried out using the survival package (version 2.30), in the R software (version 2.15.2). Our analysis adhered to the REporting recommendations for tumor MARKer prognostic studies (REMARK) (23).

Results

Identification of potential oncogenes overexpressed in cancer samples. We surmised that whereas most genes previously identified by aCGH might correspond to passenger

Table II. List of top 20 overexpressed genes in malignant pancreatic samples.

Symbol	Entrez gene name	Fold Change	<i>p</i> -Value	Location	Type	Biomarker application	Drug
<i>FBXO32</i>	F-Box protein 32	13.49	6.24E-28	Cytoplasm	Enzyme		
<i>LY6E</i>	Lymphocyte antigen 6 complex, locus E	13.06	6.38E-27	Plasma Membrane	Other		
<i>SMURF1</i>	SMAD specific E3 ubiquitin protein ligase 1	11.46	9.96E-22	Cytoplasm	Enzyme		
<i>SQLE</i>	Squalene epoxidase	11.43	1.31E-21	Cytoplasm	Enzyme		Terbinafine, butenafine, naftifine
<i>TNFRSF11B</i>	Tumor necrosis factor receptor superfamily, member 11b	10.53	1.66E-20	Plasma Membrane	Transmembrane receptor	Diagnosis, efficacy	
<i>WISP1</i>	WNT1 inducible signaling pathway protein 1	10.83	5.06E-20	Extracellular Space	Other		
<i>LRRC6</i>	Leucine rich repeat containing 6	9.71	1.64E-19	Cytoplasm	Other		
<i>SOX4</i>	SRY (sex determining region Y-box 4	10.35	7.87E-19	Nucleus	Transcription regulator		
<i>SPAG1</i>	Sperm associated antigen 1	10.08	8.74E-19	Cytoplasm	Other		
<i>SAMD9</i>	Sterile alpha motif domain containing 9	10.25	5.45E-18	Cytoplasm	Other		
<i>ATAD2</i>	ATPase family, AAA domain containing 2	9.78	5.76E-18	Nucleus	Enzyme		
<i>ARPC1B</i>	Actin related protein 2/3 complex, subunit 1B, 41kDa	10.03	1.78E-17	Cytoplasm	Other		
<i>COL1A2</i>	Collagen, type I, alpha 2	9.81	1.25E-16	Extracellular Space	Other		Collagenase Clostridium histolyticum
<i>PSCA</i>	Prostate stem cell antigen	8.98	1.37E-16	Plasma Membrane	Other	Unspecified application	AGS-1C4D4
<i>CTHRC1</i>	Collagen triple helix repeat containing 1	9.72	1.88E-16	Extracellular Space	Other		
<i>CLDN12</i>	Claudin 12	9.29	2.02E-16	Plasma Membrane	Other		
<i>STK3</i>	Serine/threonine kinase 3	9.46	2.23E-16	Cytoplasm	Kinase		
<i>KIAA0196</i>	KIAA0196	9.2	2.42E-16	Cytoplasm	Other		
<i>YWHAZ</i>	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta	9.47	2.56E-16	Cytoplasm	Enzyme	Diagnosis	
<i>CDK6</i>	Cyclin-dependent kinase 6	9.02	1.86E-15	Nucleus	Kinase		Palbociclib, LEE011, alvocidib

alterations, driver genes or pathways should be affected at the transcriptional level and could be identified more easily when the analyzed dataset is large. Thus, among the genes found altered by aCGH, we looked at genes whose expression differed between normal (n=105) and malignant pancreatic tissues (n=419) in the 10 pooled public datasets. Of note, we did the same analysis with the expression data of the 81 matched pairs, that for each patient included the expression data of both malignant and normal pancreatic tissues, and found an extremely good correlation between the two analyses ($R^2=0.967$), which strengthened our results obtained by the entire cohort.

Among the 352 genes found amplified/gained by aCGH, 170 (48%) were overexpressed at the transcriptional level in malignant tissues compared to normal pancreatic tissues (Table S2: http://disc.marseille.inserm.fr/PancADC_CGP2015/Table_S2.xlsx). Among the 20 most highly overexpressed genes (Table II), we noted the majority of genes to be involved in apoptosis regulation [cyclin-dependent kinase 6 (*CDK6*), F-

box protein 32 (*FBXO32*), *KIAA0196*, prostate stem cell antigen (*PSCA*), sterile alpha motif domain containing 9 (*SAMD9*), tumor necrosis factor receptor superfamily member 11b (*TNFRSF11B*), serine/threonine kinase 3 (*STK3*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (*YWHAZ*)], with specific regulators of the TP53 pathway [ATPase family, AAA domain containing 2 (*ATAD2*), actin-related protein 2/3 complex subunit 1B (*ARPC1B*), SRY-box 4 (*SOX4*), and WNT1-inducible signaling pathway protein 1 (*WISP1*)], but there was also a significant proportion of genes involved in cell-cycle regulation [*ATAD2*, *CDK6*, leucine-rich repeat containing 6 (*LRRC6*), lymphocyte antigen 6 complex, locus E (*LY6E*), and *SOX4*], and the transforming growth factor beta 1 (TGF β) pathway [collagen, type I, alpha 1 (*COL1A1*), F-box protein 32 (*FBXO32*), SMAD specific E3 ubiquitin protein ligase 1 (*SMURF1*), and *SOX4*]. The top canonical Ingenuity pathways associated with these genes were involved in cell-cycle control of chromosomal replication and homologous

Table III. List of top 20 underexpressed genes in malignant pancreatic samples.

Symbol	Entrez gene name	Fold Change	p-Value	Location	Type	Biomarker application	Drug
<i>CYB5A</i>	Cytochrome b5 type A (microsomal)	-10.407	9.67E-19	Cytoplasm	Enzyme		
<i>P2RX1</i>	Purinergic receptor P2X, ligand-gated ion channel, 1	-9.457	8.83E-17	Plasma Membrane	Ion channel		
<i>ATP1B2</i>	ATPase, Na ⁺ /K ⁺ transporting, beta 2 polypeptide	-9.489	1.00E-16	Plasma Membrane	Transporter		
<i>SEC11C</i>	SEC11 homolog C (<i>S. cerevisiae</i>)	-9.412	1.57E-16	Cytoplasm	Peptidase		
<i>SAT2</i>	Spermidine/spermine N1-acetyltransferase family member 2	-8.617	1.00E-14	Plasma Membrane	Enzyme		
<i>ATP2A3</i>	ATPase, Ca ⁺⁺ transporting, ubiquitous	-8.211	8.06E-14	Cytoplasm	Transporter		
<i>RPH3AL</i>	Rabphilin 3A-like (without C2 domains)	-8.182	2.90E-13	Plasma Membrane	Other	Diagnosis, Prognosis	
<i>GGT6</i>	Gamma-glutamyltransferase 6	-8.117	3.18E-13	Plasma Membrane	Peptidase		
<i>CAMKK1</i>	Calcium/calmodulin-dependent protein kinase kinase 1, alpha	-8.047	3.83E-13	Cytoplasm	Kinase		
<i>CYB5D1</i>	Cytochrome b5 domain containing 1	-7.413	6.18E-12	Other	Other		
<i>RAX</i>	Retina and anterior neural fold homeobox	-7.4	1.52E-11	Nucleus	Transcription regulator		
<i>FHIT</i>	Fragile histidine triad	-7.24	2.28E-11	Cytoplasm	Enzyme	Efficacy	
<i>DNAH9</i>	Dynein, axonemal, heavy chain 9	-7.339	2.34E-11	Cytoplasm	Other		
<i>PHLPP1</i>	PH domain and leucine rich repeat protein phosphatase 1	-6.92	2.19E-10	Cytoplasm	Enzyme		
<i>SHPK</i>	Sedoheptulokinase	-6.831	2.39E-10	Cytoplasm	Kinase		
<i>GCGR</i>	Glucagon receptor	-6.785	4.52E-10	Plasma Membrane	G-protein coupled receptor		Glucagon
<i>RNMTL1</i>	RNA methyltransferase like 1	-6.607	9.39E-10	Cytoplasm	Enzyme		
<i>KCNAB3</i>	Potassium voltage-gated channel, shaker-related subfamily, beta member 3	-6.549	1.22E-09	Plasma Membrane	Ion channel		
<i>KCNNG2</i>	Potassium voltage-gated channel, subfamily G, member 2	-6.456	1.95E-09	Plasma Membrane	Ion channel		Nicorandil, Amiodarone
<i>FBXO15</i>	F-box protein 15	-6.375	2.47E-09	Other	Transcription regulator		

recombination ($p=8.87 \times 10^{-5}$), cell-cycle G₁/S checkpoint regulation ($p=2.38 \times 10^{-4}$), estrogen-mediated S-phase entry ($p=1.86 \times 10^{-3}$), and cell-cycle regulation by B-cell translocation genes (BTG) family proteins ($p=3.62 \times 10^{-3}$). These pathways were coherent with the following top molecular and cellular functions: DNA replication, recombination and repair ($p=4.62 \times 10^{-4}$), cell cycle ($p=6.56 \times 10^{-4}$), cell development ($p=7.66 \times 10^{-4}$), cellular function and maintenance ($p=7.66 \times 10^{-4}$), and cellular assembly and organization ($p=8.06 \times 10^{-4}$). Taking into account these functions, the most pertinent upstream modulators regarding PDAC were *TP53* ($p=2.81 \times 10^{-4}$), *mir26a-5p* ($p=2.98 \times 10^{-4}$), and histone deacetylase 2 (*HDAC2*) ($p=5.41 \times 10^{-4}$), corresponding to master regulators of apoptosis, microRNAome and epigenetic modulation, the last two often being related.

Identification of potential TSGs underexpressed in cancer samples. Among the 345 genes located in regions of losses identified by aCGH, 141 (41%) were underexpressed in

malignant tissues compared to normal tissues (Table S3: http://disc.marseille.inserm.fr/PancADC_CGP2015/Table_S3.xlsx). The top 20 underexpressed genes (Table III) were involved in ion transport (ATPase, Na⁺/K⁺ transporting, beta 2 polypeptide (*ATP1B2*), potassium channel, voltage gated subfamily A regulatory beta subunit 3 (*KCNAB3*), potassium channel, voltage gated modifier subfamily G, member 2 (*KCNNG2*)), notably calcium function regulation (ATPase, Ca⁺⁺ transporting, ubiquitous (*ATP2A3*), calcium/calmodulin-dependent protein kinase kinase 1, alpha (*CAMKK1*), purinergic receptor P2X, ligand gated ion channel, 1 (*P2RX1*), rabphilin 3A-like (*RPH3AL*)), in homeostasis maintenance (cytochrome b5 type A (*CYB5A*), glucagon receptor (*GCGR*), gamma-glutamyltransferase 6 (*GGT6*), retina and anterior neural fold homeobox (*RAX*), SEC11 homolog C, signal peptidase complex subunit (*SEC11C*), sedoheptulokinase (*SHPK*)), fatty acid metabolism (spermidine/spermine N1-acetyltransferase family member 2 (*SAT2*), *GGT6*) and pro-apoptotic genes (*CAMKK1*, *CYB5A*, fragile histidine triad (*FHIT*), PH

domain and leucine-rich repeat protein phosphatase 1 (*PHLPP1*), *SHPK*). The corresponding canonical Ingenuity pathways were involved in calcium signaling ($p=3.75\times 10^{-5}$), fatty acid oxidation ($p=5.3\times 10^{-3}$) and eicosanoid signaling ($p=1.03\times 10^{-3}$). These pathways were coherent with the following top molecular and cellular functions: lipid metabolism ($p=1.61\times 10^{-4}$), small-molecule biochemistry ($p=1.61\times 10^{-4}$), carbohydrate metabolism ($p=4.06\times 10^{-4}$), amino acid metabolism ($p=7.36\times 10^{-4}$) and cell-cycle regulation ($p=7.36\times 10^{-4}$). Taking into account these functions, the main upstream modulators were tissue differentiation-inducing non-protein coding RNA (*TINCR*) ($p=1.29\times 10^{-3}$), staufen double-stranded RNA binding protein 1 (*STAU1*) ($p=1.29\times 10^{-3}$), and OSI461 ($p=6.9\times 10^{-3}$), *i.e.* regulators of metabolism and cellular transport.

Candidate genes associated with survival. We next searched for correlations between survival and expression of genes located in gained ($n=352$) or lost ($n=345$) regions. Using survival information as continuous data, 46 genes were associated with prognosis (6.6% of all genes located in genomic alterations identified by aCGH). Among them, 17 (2.4%) were associated with “shorter survival” (high-risk) and 29 (4.2%) with “longer survival” (low-risk) (Table S4: http://disc.marseille.inserm.fr/PancADC_CGP2015/Table_S4.xlsx). Regarding the genes whose overexpression was found associated with shorter survival, half already had a well-established association with cancer [namely B-cell CLL/lymphoma 6, member B (*BCL6B*), family with sequence similarity 83 member A (*FAM83A*), methyl-CpG binding domain protein 1 (*MBD1*), MDM2 proto-oncogene, E3 ubiquitin protein ligase (*MDM2*), nucleoredoxin (*NXN*), runt-related transcription factor 1 (*RUNX1*), *RUNX2* and XIAP associated factor 1 (*XAF1*)], whereas the involvement of the other genes in oncogenesis remains unexplored [*C17orf85*, coiled-coil domain containing 102B (*CCDC102B*), ephrin-B3 (*EFNB3*), kelch-like family member 38 (*KLHL38*), neuropilin and tolloid-like 1 (*NETO1*), neuroligin 2 (*NLGN2*), otoconin 90 (*OC90*), phorbol-12-myristate-13-acetate-induced protein 1 (*PMAIP1*), and zinc finger protein 532 (*ZNF532*)] (Table S4: http://disc.marseille.inserm.fr/PancADC_CGP2015/Table_S4.xlsx). Figure 1A shows Kaplan–Meier curves for high-risk and low-risk groups based on the expression level of three genes: *PMAIP1*, *RUNX1* and *XAF1*.

Among the 29 genes whose overexpression was associated with longer survival in PDAC (Table S4: http://disc.marseille.inserm.fr/PancADC_CGP2015/Table_S4.xlsx), some were involved in the regulation of tumor-suppressive functions. Examples include ankyrin repeat domain 46 (*ANKRD46*), MAF1 homolog, negative regulator of RNA polymerase III (*MAF1*), retinoblastoma (RB1), ring finger protein 167 (*RNF167*), transmembrane protein 74

(*TMEM74*), WW domain containing E3 ubiquitin protein ligase (*WWP1*) and zinc finger protein 394 (*ZNF394*). Among the other genes associated with survival, some have an ambiguous or unknown involvement in oncogenesis depending on cancer type, such as C8orf37, cyclic nucleotide binding domain containing 1 (*CNBD1*), carnitine O-octanoyltransferase (*CROT*), eukaryotic translation initiation factor 3 subunit H (*EIF3H*), enhancer of yellow 2 homolog (*ENY2*), 3-ketodihydrosphingosine reductase (*KDSR*), matrilin 2 (*MATN2*), major facilitator superfamily domain containing 3 (*MFSD3*), platelet-activating factor acetylhydrolase 1b regulatory subunit 1 (*PAFAH1B1*), phosphatidylinositol transfer protein alpha (*PITPNA*), Rab-interacting lysosomal protein (*RILP*), serpin peptidase inhibitor, clade B (ovalbumin), member 12 (*SERPIN12*), solute carrier family 43 (amino acid system L transporter), member 2 (*SLC43A2*), syntabulin (syntaxin-interacting) (SYBU), transmembrane protein 130 (*TMEM130*), tissue-specific transplantation antigen P35B (*TSTA3*), thioredoxin-like 4A (*TXNLA4A*), vacuolar protein sorting 4 homolog B (*S. cerevisiae*) (*VPS4B*) and zinc finger protein 250 (*ZNF250*). Three genes with a known involvement in colorectal, gastrointestinal and prostate cancer, namely cadherin 17, LI cadherin (liver-intestine) (*CDH17*), copine III (*CPNE3*) and glucagon-like peptide 2 receptor (GLP2R), were surprisingly associated with a better prognosis in our study. Figure 1B shows Kaplan–Meier curves for high-risk and low-risk groups based on the expression level of three genes: *MFSD3*, *MAF1* and *TMEM130*.

Discussion

We previously performed aCGH of PDAC samples and identified potential driver genes involved in PDAC oncogenesis. Here, we identified which of those genes have consequences at the transcriptional level. We collected 10 retrospective public datasets of clinical PDAC samples profiled using oligonucleotide microarrays, representing a total of 419 cases associated with 105 normal tissue samples. To our knowledge, this is the largest series of PDAC analyzed using genomics tools. Analysis was performed in two directions: identification of genes differentially expressed in cancer *versus* normal tissues, and identification of genes associated with survival.

A total of 697 genes were found altered at the genomic level in our previous aCGH analysis. Among the 352 genes amplified/gained, half were overexpressed at the transcriptional level in malignant tissues compared to normal pancreatic tissues. Our results suggest that some of the genes gained in aCGH contribute to driving PDAC oncogenesis through the activation of three major pathways, namely cell-cycle regulation (probably related to CDKN2A, a major actor in PDAC) (24), regulation of/escape from apoptosis (notably

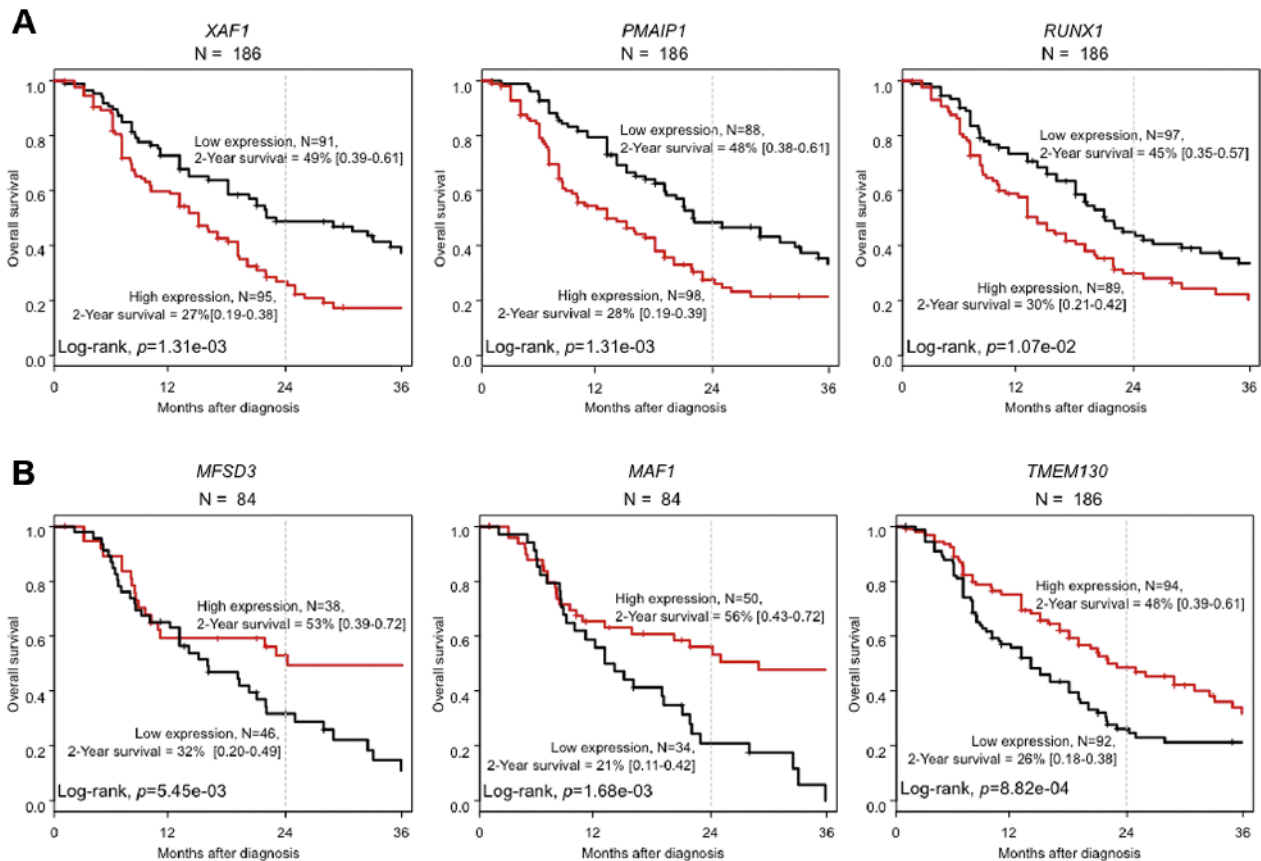


Figure 1. Survival of patients with pancreatic cancer according to gene expression. Kaplan–Meier curves of patient groups according to mRNA expression of genes whose overexpression is associated with shorter survival (A) and of genes whose overexpression is associated with longer survival (B). XAF1: XIAP associated factor 1; PMAIP1: phorbol-12-myristate-13-acetate-induced protein 1; RUNX1: runt-related transcription factor 1; MFSD3: major facilitator superfamily domain containing 3; MAF1: MAF1 homolog, negative regulator of RNA polymerase III; TMEM130: transmembrane protein 130.

through the TP53 pathway, induced in response to DNA damage and genomic instability), and tumor dedifferentiation (most certainly involving TGF β). Among the candidate genes involved, we identified known major actors of PDAC oncogenesis, such as *SMURF1*, encoding an ubiquitin-ligase specific for receptor-regulated SMAD proteins in the TGF β signaling pathway (25, 26). TGF β is known to promote pro-invasive and pro-metastatic microenvironment playing a central role in stroma production, angiogenesis, and tumor-induced immunosuppression (27). The TGF β signaling pathway is a highly interesting pathway to target in PDCA. In this regard, the antisense oligodeoxynucleotide trabedersen AP 12009 monotherapy, which specifically inhibits TGF β 2 expression, has led to markedly enhanced survival in PDCA and other cancer types (28). We also identified several other novel candidates, closely related to this pathway, namely *SOX4*, *SPAG1* and *COL1A*, that could

serve as complementary targets, notably *SOX4* (29). Among newly identified candidates, another interesting up-regulated gene was *WISP1*; this gene encodes a member of the WNT1 inducible signaling pathway protein subfamily, which belongs to the connective tissue growth factor family. It also attenuates TP53-mediated apoptosis in response to DNA damage through activation of v-Akt murine thymoma viral oncogene kinase. Targeting *WISP1* would impact several major pathways involved in PDAC oncogenesis (30).

Among the 345 genes located in regions of losses, around 40% were underexpressed in malignant tissues compared to normal tissues. Altogether, the pathways involved by these genes suggested a deregulated metabolic activity, sustained in normal pancreatic cells and lost in the less-differentiated malignant cells. These pathways are related to ion transport, notably calcium function regulation, homeostasis maintenance, pro-apoptotic genes and fatty acid metabolism.

Most disturbances of these pathways are probably related to changes in cellular composition of the pancreas (malignant and stromal cells) and differentiation (notably with enhanced triggering of the TGF β pathway). Nevertheless, several teams are now focusing their attention on fatty acid metabolism in order to understand its role in PDAC oncogenesis and as potential pharmacological targets. Indeed, defects in this pathway exert growth-inhibitory effects in human PDAC as compared to healthy pancreas (31). With our study, we identified two candidates involved in the regulation of this pathway (*SAT2* and *GGT6*) that deserve further investigations. Another affected pathway that related to ion channels and transporter expression is a recognized hallmark of cancer because of its known involvement in the development of therapeutic resistance. However, most ion channels have not been investigated yet as drug resistance-associated channels, but their expression has closely been linked to pathogenesis, invasion, metastasis, and prognosis of other cancer types (32). Some of the molecules identified in our study might thus represent interesting novel therapeutic targets for future studies of PDAC.

We also identified genes whose expression was associated with shorter or longer survival. Refinement of pathological assessment has recently provided prognostic tools following pancreaticoduodenectomy, such as the lymph node ratio (33), number of positive lymph nodes (34), and site of margin involvement (35). However, even in patients with similar clinicopathological factors, the clinical outcome is heterogeneous in terms of survival (36). A better molecular characterization might help explain such differences (20, 37). Some studies have already linked gene-expression profiles with lymph node status or advanced PDAC stage, but the results remain inconsistent (38, 39). Preoperative identification of patients with a poor prognosis is still necessary to aid appropriate clinical decision-making and estimate the potential of existing and novel therapies. We identified certain genes related to shorter survival. Some have well-established association with cancer (*BCL6B*, *FAM83A*, *MBD1*, *MDM2*, *NXN*, *RUNX1*, *RUNX2* and *XAF1*), including pancreatic cancer. Among these, some have already a well-established association with cancer (including pancreatic, but mostly gastrointestinal, hepatic, breast and lung cancer) (Table S4). Notably high *MBD1* expression has been correlated with lymph node metastasis and poor survival in pancreatic cancer (40). This gene is thus a major candidate involved in pancreatic progression, a result that we confirmed here on a larger dataset ($n=186$ tumors, $p=6.1\times 10^{-3}$). Two genes associated with survival are involved in the remodeling of the central nervous system (*EFNB3*, $p=0.02$; *NLGN2*, $p=0.004$). These genes might be interesting targets, or at least indicators of 'worst' prognosis because of their ectopic expression. A recent study showed that genomes in PDAC have alterations in axon guidance

genes (41). In addition to these known candidates, we identified three novel markers related to survival (*XAF1*, *PMAIP1* and *RUNX1*). *XAF1* and *PMAIP1* encode proteins that can exert pro-apoptotic function in specific diseases (42). Their respective overexpression correlating with shorter survival is thus counterintuitive, and their role in PDAC oncogenesis remains unclear. However, in the case of *XAF1*, its up-regulation may confer sensitivity of cancer cells to cisplatin-mediated apoptosis, which may offer an interesting therapeutic perspective for this group of patients (43). Regarding *RUNX1*, a major oncogene involved in hematopoiesis, recent studies highlighted its importance in solid tumors both as a tumor promoter and a suppressor (44). Given its central role in cancer development, *RUNX1* is an excellent candidate for targeted therapy (45). In our population of PDAC, *RUNX1* overexpression was associated with shorter survival, suggesting that its involvement as a tumor promoter exceeds its suppressive role.

Among the 29 genes associated with longer survival in our series, some are involved in the regulation of tumor-suppressive functions, notably *RBI*, a major negative regulator of the cell cycle. The other genes include *MAF1*; *RNF167* and *WWP1*, two E3 ubiquitin ligases (46, 47); *ZNF394*, which codes for a zinc finger protein regulating jun proto-oncogene (*JUN*) transcriptional activity (48); *TMEM74*, which codes for a protein promoting functional autophagy during stress conditions (49); and *ANKRD46*, known to inhibit *in vivo* growth of breast cancer cells (50). *MAF1* is particularly interesting because of its known repressor activity of RNA polymerase III transcription (51, 52). This function is coherent with the fact that *MAF1* was overexpressed in patients with long survival. The E3 ubiquitin ligase *WWP1* gene is located in 8q21, a region frequently amplified in human cancer, including prostate and breast cancer. Recent studies have shown that *WWP1* negatively regulates the TGF β tumor-suppressor pathway by inactivating its molecular components, including *SMAD2*, *SMAD4* and *TGFBR1*; its underexpression was associated with reduced cancer cell migration and bone marrow metastasis (47, 53). These findings suggest an oncogenic role of *WWP1* in carcinogenesis. Many genes associated with longer survival were related to various biological functions, notably hepatocyte function and differentiation or reticulum activity: *SLC43A2*, *MFSD3*, *TSTA3*, *MATN2*, *SYBU*, *ENY2*, *CNBD1*, *TMEM130*, *CROT*, *TXNL4A*, *KDSR*, *PITPNA* and *RILP*. Three genes with a known involvement in colorectal, gastrointestinal and prostate cancer, namely *CDH17*, *GLP2R* and *CPNE3*, were surprisingly associated with a better prognosis in our study. Finally, other genes (*SERPINB12*, *ZNF250* and *C8orf37*) were associated with survival, but their involvement in oncogenesis remains unexplored. Among the other genes associated with better survival, some have a different role in oncogenesis depending on the cancer type. For example,

EIF3H is an indicator of poor prognosis in prostate cancer, whereas its overexpression improves the response and survival of patients with non-small cell lung cancer treated with gefitinib (54); *PAFAH1B1* is down-regulated in hepatocellular carcinoma, whereas its overexpression is indicative of poor prognosis in lung carcinoma (55); *VPS4B* promotes cell division in non-small cell lung cancer, whereas high-grade or relapsed breast carcinomas exhibit lower *VPS4B* expression than do low-grade (56, 57).

In conclusion, by integrating transcriptional data on a large series of PDAC, we highlighted certain genes that were previously identified among many other genes by aCGH. Our approach is validated by the identification of already known players of PDAC oncogenesis (*SMURF1*, *ARPC1A*, *HIC1*, *MBD1*, *NBN* and *ZIP4*). Importantly, we reveal novel interesting targets, with prognostic or therapeutic potential (such as *MTDH*, *LAPTM4B*, *BOP1*, *EIF3H*, *ATAD2*, *DPH1* and *MAFI*). Further clinical and functional validations are now warranted, notably at the protein level.

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