The role of the wider peritumoral tissue environment of pancreatic cancer in tumour survival

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ABSTRACT

Transcriptional profiling was performed on 452 pancreatic tissues, with a focus on peritumoral samples. Pancreatic ductal adenocarcinomas (PDAC) and cystic tumours were most different in these non-tumorous, adjacent tissues, whereas the actual tumours exhibited more similar patterns. The environment of cystic tumours was transcriptionally nearly identical to normal tissue. In contrast, the tissue surrounding PDAC behaved a lot like the tumour, indicating some kind of field defect, and showed far less molecular resemblance to both chronic pancreatitis and healthy tissue. This suggests that the pathogenic difference between cystic and ductal tumours may also be influenced by their different cellular environment rather than variation between the tumours themselves. Little correlation between DNA methylation and transcript levels makes it unlikely that the field defect in PDAC peritumoral tissues is solely controlled by such epigenetic regulation. Functionally, a strikingly large number of autophagy genes was uniquely changed in both PDAC and its peritumoral tissue. A transcription signature of 15 autophagy genes permits a survival prognosis with high accuracy, highlighting the important role of autophagy in tumour biology.

Keywords:

Disease prognosis / pancreatic cancer / peritumoral tissue / survival / transcript variations.

INTRODUCTION

Pancreatic cancer is one of the most aggressive tumours at all. Despite an incidence that represents only 3% of all cancer cases recorded in industrialised countries, it is the fourth most common cause of tumour-related deaths in the Western world (Ferlay et al., 2015; Siegel et al., 2016). Among the different forms of pancreatic cancers, pancreatic ductal adenocarcinoma (PDAC) is accounting for more than 90% of all cases and has the worst prognosis; the other types are less lethal. Most PDAC patients die within a year of diagnosis; the overall five-year survival rate is about 5%. PDAC patients often and rapidly develop resistance to chemotherapy; the reasons for this are still largely unclear (Neoptolemos et al., 2012; Werner et al., 2013; Habermehl et al., 2013). There is currently no efficient treatment available except surgery, which can only be applied to 10 to 20% of cases, however (Gurusami et al., 2014). For an improvement of the clinical situation, prognostic markers are required that allow predicting clinical progression more accurately (Costello et al., 2012). With the objective of understanding the molecular basis of pancreatic tumours, several studies of the RNA expression variation in pancreatic cancer have been performed (e.g., http://www.pancreasexpression.org/cgi-bin/pancexp/DataSets.pl). Substantial variations have been recorded between different studies, documenting technical variance as well as tumour heterogeneity (for meta-analyses see: Brandt et al., 2004; Bhasin et al., 2015). Also, several of these studies dealt with relatively few RNA samples and material from one hospital source only. In addition, only relatively few data sets are available on pancreatic tumour types other than PDAC. The same is true for non-tumorous pancreatic tissues and especially so for peritumoral samples from cancer patients. An accurate molecular differentiation of these tissue entities and a detailed understanding of their relation to the actual tumours are missing.

In order to provide a more reliable source of information at several molecular levels on a large set of tumour and control samples – in particular including tissue samples that were close to tumour but not part of it – we performed extensive analyses on originally more

than 1000 pancreatic tissue samples collected by surgery at three major European pancreas clinics. At the DNA level, results on the mutational status of the *KRAS* and *CDKN2A* genes and their prognostic significance have been reported (Rachakonda et al., 2013). Also, the effect of several common polymorphisms on pancreatic cancer susceptibility and their possible impact on patient survival was published (Rizatto et al., 2011). Furthermore, suitability of microRNA variations and DNA methylation for diagnosis has been studied (Bauer et al., 2012; Keller et al., 2014, Moskalev et al., 2015) and was followed up by detailed investigations of their functional contributions to the disease (e.g., Botla et al., 2016).

Here, we report about an analysis of transcriptional variations at the mRNA level performed on the basis of this large dataset. We identified significant variations between the various tumour forms, but also detected unexpected similarities between the mRNA expression patterns. Particularly striking was a substantial degree of similarity in transcriptional regulation between PDAC and the surrounding peritumoral cellular environment, indicating some kind of field defect. Interestingly, the transcriptional variation did not much coincide with changes in the DNA-methylation levels, which have been implicated in field defects (e.g., Wolff et al., 2010). Comparing cystic tumours and PDAC, most transcriptional differences took actually place in this peritumoral environment, while the transcriptional patterns in the tumour tissues were rather similar, suggesting a possible involvement of the wider cellular environment of a tumour in its pathology. Looking at the data from a functional angle, we inferred relevant pathways and possible functional consequences. Our findings highlighted the importance of autophagy-related transcript expression in the peritumoral environment of pancreatic tumours and the potential role of autophagy genes for prognosis and as legitimate targets for therapeutic intervention schemes.

RESULTS

Comparison of transcriptional variations in different tissue types

The study was performed on pancreatic tissue samples collected at three clinics in Heidelberg (Germany), Liverpool (UK) and Verona (Italy) by resection from cancer patients or donors, who had pancreatic tissue removed for reasons other than cancer. Sample analysis was performed in one central laboratory, following the same protocols throughout. From each sample, three tissue slices were evaluated by pathologists prior to further analysis, estimating the percentages of normal, tumour and stromal cells as well as the degree of inflammatory infiltration. In total, 452 RNA preparations met our quality standards and were included in the transcriptional profiling analysis. We studied a variety of tissue types. In particular, we looked at samples of non-tumorous tissue that had been located next to the actual tumour. These peritumoral tissues had been in a distance of up to 10 mm from the tumour and did not exhibit any tumour cell content in the histochemical analyses. They consisted of normal parenchyma and stroma and are referred to as “macro-environment” below. The 452 high-quality samples represented 195 cases of PDAC, 30 cases of PDAC macro-environment (N.PDAC), 24 cystic tumours (TC), 22 macro-environmental tissues from next to cystic tumours (N.TC), 59 samples of chronic pancreatitis (CP), 15 tissues from the macro-environment of CP (N.CP), and 41 healthy pancreatic tissues from non-cancer patients (N). Other neoplasms, for which RNA was isolated but ignored in the analysis reported below, were 18 endocrine tumours, 2 macro-environmental samples of endocrine tumours, 31 other pancreatic tumours as well as 15 related macro-environment tissues. Information about clinical patient parameters is given in **Tab. 1**.

Many genes exhibited changes at the transcript level in the various tissue types compared to samples from healthy donors (**Fig. 1**) (see also **Tab. EV1**). Also, a large number of changes were found that were common between different tissues (**Fig. 2**). It is noteworthy that this means a variation in the same direction (up or down, respectively) as compared to the normal tissue and not an increase in one and a decrease in the other tissue. As a matter

of fact, an analysis revealed that all genes, which showed a significant variation in two tissues, were similarly regulated either up or down in both when comparing N.PDAC, PDAC and cystic tumours (**Fig. 2E**). This complete lack of inverse regulation suggests that functionally similar cellular effects are triggered by these common transcriptional changes.

Particularly interesting results were obtained from the macro-environments of PDAC and cystic tumours, which histologically showed a similar cell composition. Compared to the transcript profiles obtained from normal tissue, a large number of genes were found differentially transcribed in the PDAC macro-environment, indicating that the phenotypically non-tumorous appearance does not represent the actual molecular status (**Fig. 2A**). In addition, there was a substantial overlap of 2,641 genes with the results obtained from PDAC, of which 1,997 were also changed in CP. At a transcriptional level, the PDAC macro-environment and tissue of patients with CP were similarly different to normal tissue. However, PDAC macro-environment exhibited significantly more molecular resemblance with tumour tissue than with CP. For cystic tumours, there was a rather different picture. The overlap with the transcriptional pattern of CP was identical in number to the overlap of PDAC and CP (**Fig. 2B**). However, in contrast to the PDAC result, the macro-environment of cystic tumours behaved very similar to normal pancreatic tissue with only 343 differentially transcribed genes as opposed to 2909 genes in the PDAC macro-environment. In a comparison of the macro-environments of PDAC and cystic tumours (**Fig. 2C**), only 45 differentially expressed transcripts were found to be specific for the macro-environment of cystic tumours.

Variations that are specific to PDAC and its macro-environment

Since PDAC represents the vast majority of clinical pancreatic cancer cases and has the worst prognosis, we focussed our analysis on this tumour type. More than half of the 5,196 genes that exhibited significant variations in PDAC compared to healthy tissue were also differentially expressed in PDAC macro-environment or CP tissues **(Fig. 2A)**. The 1,997 regulated genes shared between the three tissue types mostly represent changes that are

associated with inflammation. The most overrepresented canonical pathways defined by these genes are relevant for immunological and inflammatory response **(Fig. EV1).** Next to the shared transcript variations, there were (marker) genes that exhibited expression changes that were unique to each tissue type. The six most significant biological functions associated with the 2,373 unique PDAC expression markers are: *cellular growth and proliferation*; *cellular movement*; *cell death and survival*; *cancer*; *cell cycle;* as well as *organismal injury and abnormalities* **(Fig. 3)**. There were no over-represented functions that are associated with inflammation. In contrast to this, the 127 and 162 markers that were unique to the PDAC macro-environment or CP, respectively, are genes over-representing functions associated with both inflammation and cancer.

Of particular interest are the 644 regulated transcripts shared between PDAC and its macro-environment, but not regulated in CP (**Fig. 2A; Tab. EV2)**. The fact that the non-tumorous macro-environment tissue exhibited a large number of variations, which are common with PDAC only, suggests the possibility of a cancer field defect at the transcript level. A cancer field defect is defined as a biological cancerisation process in which tissue in relatively large areas beyond the actual tumour is exhibiting epigenetic changes similar to the ones of the actual tumour (Shen et al., 2005; Yan et al., 2006; Wolff et al., 2010; Botla et al., 2012). Methylation changes are expected to result in variations at the transcript level, too. To reveal the degree by which promoter methylation may be responsible for the observed variations at the RNA level, we analysed the genomic DNA of 24 randomly selected samples, 12 each from healthy donors and PDAC patients, from which we had also obtained mRNA profiles. In the set of 644 transcripts shared by PDAC and its macro-environment, 154 genes exhibited an inverse correlation of promoter methylation and mRNA expression: 115 hyper-methylated promoters could be linked to down-regulation of the mRNA level, 39 hypo-methylated promoters coincided with transcripts that were present in higher abundance. However, for the majority of genes, 490, there was no such correlation of promoter DNA methylation and gene expression. For 76 genes, there was even a concurrent increase or decrease, respectively, of both methylation and mRNA level.

Pathways and gene networks affected in the macro-environment of PDAC

Transcripts showing significant expression differences in the macro-environment of PDAC as compared to healthy control samples were submitted to a functional bioinformatics examination using Ingenuity pathways analysis. In this analysis, one particular network of genes was identified**.** It consists of 73 directly linked genes **(Fig. EV2; Tab. EV3).** Many genes show a gradual change of expression from healthy via CP to PDAC macro-environment and finally PDAC. Two typical examples are the chemokine receptor *CXCR4* (chemokine (C-X-C motif) receptor 4) and *NUPR1* (nuclear protein transcriptional regulator 1). *CXCR4* is a prognostic marker in various types of cancer (Furusato et al., 2010) and a biomarker of migrating pancreatic cancer-initiating cells in mice (Wang et al., 2013). It was up-regulated compared to normal tissue by a factor of 3.51 in CP, 4.38 in the macro-environment and 6.68 in PDAC. *NUPR1* interacts with numerous partners to regulate cell cycle, apoptosis, autophagy, chromatin accessibility, and transcription (Cano et al., 2011) and reduced expression promotes pancreatic cancer development (Hamidi et al., 2012). It was down-regulated by -1.80 (CP), -2.10 (macro-environment) and -2.41 (PDAC). Numerous genes within the network are essential in Pancreatic Adenocarcinoma Signalling, as well as the Integrin-Linked Kinase (ILK) and mTOR Signalling pathways. ILK is known to transmit mechanical stimuli to the mammalian target of the mTOR Signalling pathway (Dwayne et al., 2002). mTOR is an evolutionarily conserved serine/threonine protein kinase that integrates both intracellular and extracellular signals and serves as a central regulator of cell metabolism, growth, proliferation, survival, and autophagy (Neufeld, 2009). The Akt/mTOR pathway mediates oncogenesis and controls tumour cell growth (Shaw and Cantley, 2006).

Based on these findings, we took a closer look at these pathways (**Fig. EV3**). Interestingly, in the Pancreatic Adenocarcinoma Signalling pathway, 35 of the 106 genes (33%) are annotated as relevant to autophagy. For ILK/mTOR Signalling, the absolute number of autophagy-related genes was even higher with 54 molecules out of 186, although the percentage was slightly lower with 29%. The observed considerable overrepresentation

of autophagy-associated genes within the pathways suggested a strong link between autophagy and pancreatic cancer. This was corroborated by the overall number of autophagy genes found to be regulated in PDAC. Of the genes that were assayed in the transcriptional profiling experiments, 512 are annotated to be associated with autophagy processes according to public autophagy databases (Homma et al., 2010; Moussay et al., 2011). Of these, 88 genes were regulated in CP compared to normal tissue. In the macro-environment, this number increased to 108 genes, while a total of 208 genes were regulated significantly in PDAC **(Tab. EV4).** Given the obvious importance of autophagy for pancreatic cancer and the fact that about 40% of all autophagy genes were significantly regulated in PDAC or in the PDAC macro-environment, we focussed further analyses on those genes.

Autophagy genes in PDAC, cystic tumours and macro-environment

In our data set, there were several autophagy genes that are known markers for pancreatic cancer. Examples are *ATG3, ATG4A, ATG4B, ATG4C and ATG5* (Devenish et al., 2012); they showed down-regulation in PDAC and its macro-environment. Also the ductal transcription factor HNF6 (*ONECUT1*) was found to be less expressed. Such a reduction in HNF6 expression correlates with human pancreatic cancer progression (Pekala et al., 2014). HNF6 has also been described as biomarker for acinar-to-ductal metaplasia (Prevot et al., 2012) suggesting that a phenotypic switch converting pancreatic acinar cells to duct-like cells could lead to pancreatic intraepithelial neoplasia and eventually to invasive PDAC. Furthermore, HNF6 is a transcription regulator of the UDP glucuronosyltransferase (UGT) family members *UGT2B11* and *UGT2B15*, which were also less expressed in PDAC and its macro-environment. Interestingly, variations in the UGT genotype are associated with an altered risk to pancreatic cancer (Ockenga et al., 2003). None of the above genes was regulated in the macro-environment of cystic tumours. As mentioned before, this is actually true for the majority of regulated mRNAs. Many were significantly differentially transcribed in CP, PDAC macro-environment, PDAC and cystic tumours but not in the macro-environment of cystic tumours (**Fig. 2C**). As cystic tumours are known to have a much better prognosis in

comparison to PDAC (Basturk et al., 2009), factors that are regulated in the macro-environment of PDAC but not in the macro-environment of cystic tumours might be relevant for the much more aggressive nature of PDAC.

Autophagy-related prognostic markers

Given the apparent importance of autophagy genes as specific markers of the tumour and the macro-environment of PDAC, we wondered whether they may act as a clinically relevant surrogate for disease and might have prognostic significance. In order to investigate this, the relationship was explored between patient survival and several explanatory variables. First, we performed a multivariable analysis to learn about the influence of the factors age, gender, tumour grading, treatment, smoking, and alcohol consumption. Their risk coefficients were insignificant, except for tumour-stage with a p-value of 0.0018. Then, the effect of gene expression was investigated for each gene individually. In total, 35 autophagy-related transcripts were differentially transcribed in PDAC and statistically linked to survival time **(Tab. EV5**). Eighteen genes were regulated in both PDAC and its macro-environment (**Tab. 2).** The top-candidates were *PRAF2, PLK4*, *ACTB* and *PKM2*. The transcript levels were confirmed by qRT-PCR, fitting to the microarray data in all cases **(Fig. EV4)**. Interestingly, neither *PRAF2* nor *PLK4* had been connected to PDAC so far. With respect to prognosis, the expression of the two genes corresponded well with survival time and matched the performance of hypoxia-inducible factor 1 alpha (*HIF1A*) **(Fig. 4)**, whose change in expression has been shown to have a strong impact on the prognosis of patients with PDAC (Sun et al., 2007; Hoffmann et al., 2008).

Based on the analysis of the prognostic value of the expression of individual genes and by cross-referencing this with the list of autophagy-related genes that are differentially expressed in PDAC and its macro-environment, we identified the most-informative signature, which consists of 15 genes: *ACTB, ANTXR1, CAMK1G, DLG4, DNAJB9, EIF2AK3, ITPR1, MPDZ, MYO5C, NLE1, P4HB, PKM2, PLK4, PRAF2* and *WDFY2* (**Fig. EV5**). In combination, the selected genes showed a strong statistical linkage to survival in a Cox regression model

(p-value = $8x10^{-15}$). Next, we classified patients by a support vector machine as described in the Methods section: our classifier discriminated “good” and “poor” prognosis patients. As a basis, they were divided into two groups based on their survival in relation to the median survival time. The classification was performed 1000 times yielding an average accuracy of 82.6±0.1%. The discriminating power of the signature is shown in a Kaplan-Meier plot (**Fig. 5)**, indicating the substantial difference in survival between the patients with a good prognosis and the group with bad prognosis.

DISCUSSION

We performed transcriptional profiling on various types of pancreatic tissue samples, focussing particularly on changes in tissues that were located next to actual tumours. Comparison of the expression data documented that PDAC and cystic tumours are not that different at the transcriptional level, even though 892 and 516 genes, respectively, had expression patterns that were unique to either tumour type. However, variation in 4179 genes was in common. In addition, both tumour types shared a rather similar set of transcriptional variations with CP tissues, indicating the substantial contribution of inflammatory aspects that are relevant for tumour pathology. The most significant difference between PDAC and cystic tumours was actually found in their surrounding, non-tumorous tissues. Histologically, they exhibited a similar cell composition. However, while the PDAC macro-environment behaved in part like the actual tumour, the macro-environment of cystic tumours was transcriptionally nearly identical to normal pancreas tissue. This suggests that major differences between cystic tumours and PDAC may not be solely intrinsic to the actual tumours, but be triggered indirectly by the way tumours influence or are influenced by their wider cellular environment.

Assuming that a field defect could be responsible for the large number of genes, which were identically regulated in PDAC and its macro-environment, and that it may be

resulting from DNA methylation processes, which have been implicated in field defects (Shen et al., 2005; Yan et al., 2006; Wolff et al., 2010; Botla et al., 2012), we studied how the degree of promoter methylation correlated with the transcriptional variations. Surprisingly, the degree of inverse correlation – hyper-methylation and low expression, hypo-methylation and high expression – was rather low overall. One possible explanation for this observation could be the presence of processes other than DNA methylation, which regulate transcription. Any such mechanism must be rather efficient in transporting the information, since the effect could be detected over distances of several millimetres. MicroRNA transport in cellular vesicles, such as exosomes, and absorption by recipient cells has been shown to have such effects (e.g., Costa-Silva et al., 2015). In the intracellular space, one would expect a gradient in the concentration of exosomes radiated by tumours and a related gradient in the degree of cellular transcriptional variations. The actual distance of the macro-environmental samples analysed in this study was inadequately annotated so as to identify such an effect.

Alternatively, it could be that only very few genes and their methylation may be initiating the field defect. Gene *NR5A2* (encoding the nuclear receptor subfamily 5, group A, member 2) was found to be one of the genes, which was both hyper-methylated and down-regulated in PDAC and PDAC macro-environment. *NR5A2* had not been described as a methylation marker of PDAC before. However, single nucleotide polymorphisms in the vicinity of *NR5A2* have been linked through genome-wide association studies to the risk of developing PDAC, suggesting a broader role of this gene in pancreatic homeostasis and disease (Petersen et al., 2010). It was shown that *NR5A2* heterozygosity correlates with pancreatic damage in the progression of mutant *KRAS*-driven preneoplastic lesions, suggesting that *NR5A2* could contribute to PDAC through its role in the recovery from pancreatitis-induced damage (Flandez et al., 2015). The gene might be an interesting candidate for exploring a potential cancer field defect in pancreatic cancer. With respect to clinical utility, a field defect in the non-tumorous tissue and the identification and validation of relevant molecular variations at the RNA level or the degree of DNA methylation could allow

the establishment of effective disease biopsy markers, since they would also be present in a wider distance to the actual tumour and therefore be easier to collect.

One cannot rule out that other mechanisms than the ones above might be responsible for the field defect. For example, surgeons and pathologists observe that peritumoral tissues in patients with PDAC are apparently altered phenotypically, while tissues adjacent to cystic or neuroendocrine tumours often look quite normal. The mere degree of expansion of PDAC and the resulting obstruction of the macro-environment could be triggering transcriptional variations that also occur in PDAC tissue itself but not in or around the slowly growing cystic tumours thus providing a simple mechanical explanation for the differences. Further studies are required to define the relevant molecular processes in more detail.

Our findings particularly indicate the importance of autophagy transcripts in the macro-environment of pancreatic tumours and their potential role as prognostic markers. Autophagy is an evolutionarily conserved catabolic process, by which a cell digests its own cytoplasmic content. Autophagy is activated in reaction to multiple stress factors during cancer progression, such as hypoxia and poor nutrient supply (Fortunato et al., 2009) and has an important role in tumour development (Kondo et al., 2005; Newman et al., 2007). Overall, the dynamic role of autophagy in cancer appears to be complex and context-dependent. On the one hand, it could function as a tumour suppressor, whose inactivation promotes tumorigenesis (Liang et al., 1999; Mathew et al., 2009), on the other hand, it may act as a pro-survival pathway that helps tumour cells to handle metabolic stress and to resist chemotherapeutic agents (Kimmelman, 2011). In pancreatic cancer, autophagy is required for tumour growth (Yang et al., 2011; 2014) and mediates survival of pancreatic-tumour-initiating cells in a hypoxic microenvironment (Rausch et al., 2012). Inhibition of autophagy reduced pancreatic cancer growth independent of the *p53* status (Yang at al., 2014). Furthermore, autophagy was shown to be essential for oncogenic *KRAS*-induced malignant cell transformation (Kim et al., 2011). There are several early-phase clinical trials in progress targeting the autophagic machinery, among them a study of MEK1/2 and AKT inhibitors in

patients with *KRAS*-driven pancreatic tumours (Tolcher et al., 2015). Other studies have suggested that autophagy is involved in a broad crosstalk to multiple pathways that determine cell fate, including apoptosis (Marino et al., 2014).

The increased recognition of the active role of autophagy in tumorigenesis has led to the identification of novel autophagy markers for prognosis prediction (Lazofa et al., 2012). Prognostic significance of autophagy-related protein expression in resected pancreatic ductal adenocarcinoma has been described (Ko et al., 2013). In our study, the regulation of several autophagy-associated genes correlated with survival-time. Of special interest are the prognostic factors that were particularly regulated in pancreatic cancer and its macro-environment, such as *PRAF2* and *PLK4*, as these transcripts might be useful in distinguishing inflamed versus oncogenically transformed regions of the pancreas. PRAF2 (Prenylated Rab acceptor 1 domain family, member 2) is a small transmembrane protein with a putative role in transport from the endoplasmic reticulum to the Golgi apparatus (Ruggiero et al., 2008). It induces apoptotic cell death upon expression and is counteracted by Bcl-xL (Vento et al., 2010). It stimulates cell proliferation and migration and predicts poor prognosis in neuroblastoma and glioma (Borsics et al., 2010; Yco et al., 2013). PLK4 (Polo-like kinase 4) is a conserved upstream regulator of centriole duplication (Habedanck et al., 2005). It is aberrantly expressed in different tumour types (Chng et al., 2008; Salvatore et al., 2007), causing a loss of centrosome numeral integrity, thereby promoting genomic instability (Holland et al., 2010). Recently, a drug discovery programme identified a potent and selective small molecule inhibitor of PLK4 (Sampson et al., 2015), which may have therapeutic implications for PDAC as well.

Already some individual differentially expressed autophagy transcripts allowed a disease prognosis that is equivalent to markers reported before. By combining 15 autophagy genes, we obtained a signature, which allowed an even better classification of patients into groups with good or bad prognosis. Even though some of these transcripts exhibited an only modestly differential expression in PDAC, they documented a significant prognostic power. An analysis of these transcripts may offer a means for a better prognosis after tumour

resection, although limitations apply for utilising actuarial probabilities for a prediction of the actual survival of an individual patient (Grunkemeier et al., 2007). However, the analysis highlights the importance of autophagy for tumour pathology and indicates that this process is likely be highly relevant for future treatment strategies and monitoring of its success.

MATERIALS AND METHODS

Tissue samples and histopathology

Human pancreatic tissue samples were collected during surgery. In all cases, written informed consent was obtained from the patients. The study was approved by the local ethics committees at the universities of Heidelberg, Verona and Liverpool. The samples were snap-frozen in liquid nitrogen directly after resection and subsequently stored at -80°C until being used in the analysis. All samples were analysed at DKFZ following identical procedures. The frozen tissue was cut into slices of 15 µm thicknesses with a Leica CM 1850 UV cryotome at -34°C; three slices were picked from the top, middle and bottom third of a tumour and immediately used for histopathology. All remaining slices were mixed to assure equal representation of the entire tissue sample and split into three aliquots, which were used for separate preparations of DNA, RNA and protein.

For a histopathological assessment of each sample's cellular composition, the three tissue sections were stained with hematoxylin and eosin (H&E staining). They were scanned with a ScanScope GL system (Aperio Technologies, Vista, USA) and visualised using the accompanying ImageScope software. For each tissue sample, pathologists evaluated independently the histology and estimated the percentages of normal, tumour and stroma cells.

DNA methylation profiling

DNA was isolated with the AllPrep Isolation kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. For DNA methylation analysis, we performed on 12 normal and 12 PDAC samples the Infinium Human Methylation 450 BeadChip assay of Illumina (San Diego, USA), which interrogates 485,000 methylation sites across the human genome, using 1 µg of DNA per sample. The procedure followed the manufacturer's standard workflow, starting with the bisulfite conversion of the DNA samples using the EpiTect Bisulfite kit (Qiagen). Bisulfite-converted DNA acted as template for whole-genome amplification, enzymatic digestion, followed by a DNA clean-up process and hybridisation to the BeadChip. The samples were washed and scanned with the BeadArray Reader (Illumina). From the signal intensities, the DNA methylation was analysed using the Illumina Bead studio software.

Transcriptional profiling

For RNA isolation, the aliquot of frozen tissue slices allocated for RNA preparation was submerged in liquid nitrogen and gently ground by three turns with a polypropylene micropestle (Eppendorf, Hamburg, Germany) in a 2 ml Eppendorf tube. Total RNA was isolated with the AllPrep Isolation kit (Qiagen), following the manufacturer's protocol. RNA integrity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). Only samples with an RNA integrity number of at least seven were used for further analyses.

The total RNA prepared from individual samples was analysed on Sentrix Human-6v3 Whole Genome Expression BeadChips (Sentrix Human WG-6; Illumina). To synthesize first and second strand cDNA and for amplifying biotinylated cRNA, the Illumina Totalprep RNA Amplification kit was used. Hybridisation to the BeadChip was performed according to the manufacturer's instructions. In short, a maximum of 10 µl cRNA was mixed with 20 µl GEX-HYB hybridisation solution. The 30 µl sample was preheated, dispensed into the large sample-port of each array and incubated on the array at 58°C for 18 h. Subsequently, the

arrays were washed and scanned with a BeadArray Reader (Illumina). An application of low quality samples yielded higher background combined with weaker signal intensities. Such results were significantly less accurate and reproducible and therefore ignored in the discovery phase.

Data analysis

Preprocessing and quality control. Raw data were exported from the Illumina Beadstudio software and processed by R/Bioconductor scripts (Ritchie et al., 2011). The data was quantile normalised and $\log_2$ transformed. Distribution and quality of the expression data was performed using principle component analysis (PCA) and hierarchical clustering. The raw and normalised data are accessible at the public database ArrayExpress (DNA methylation profiling ID: Reviewer E-MTAB-3855; password "pyzqdbii"; transcriptional profiling ID: Reviewer_E-MTAB-1791; password: "rpqqrysi").

Differential expression analysis. Significant differentially expressed transcript features were detected using the *LIMMA* package of R/Bioconductor (Smyth et al., 2004) by pairwise comparisons of the groups (e.g., PDAC vs. normal, chronic pancreatitis vs. normal, etc.). The resulting p-values were adjusted for multiple testing using Benjamini-Hochberg's false discovery rate (FDR) method (Benjamini and Hochberg, 1995); features with a FDR<0.01 and an absolute $\log_2$-fold change $|\log_2 FC|>0.5$ were considered significant. Transcript features were annotated by gene symbols; we only report results for distinctly annotated genes, in order to avoid bias caused by genes, which are represented on the array with multiple probes. For a functional enrichment analysis, the Ingenuity PA software tool (Ingenuity Systems, http://www.ingenuity.com) was applied.

Survival analysis. Cox's proportional hazards models were used for exploring the relationship between the survival of a patient and several explanatory variables, including phenotypic parameters and gene expression. Analysis was performed on PDAC patients utilising the *survival* package of R/Bioconductor (Terry et al., 2000). This analysis returns statistical significance of prognostic variables included into the model and allowed us to

estimate the risk of death for individuals. A positive regression coefficient for an explanatory variable at high significance level means that the hazard is higher, and thus the prognosis is worse. Conversely, a negative regression coefficient implies a better prognosis for patients. We started with checking the effect of phenotypical parameters: age, gender, cancer stage, smoking and alcohol intake. Then the effect of gene expression was investigated for each gene independently and FDR-adjusted p-values of a Wald test were assigned to them.

Signature selection amongst autophagy genes. We used the intersection among significant genes in order to identify autophagy genes, which can be used as prognostic signature for PDAC. Before analysis, we summarized microarray features to the gene level in order to merge data from features targeting the same gene. Genes were selected that were differentially expressed, linked to survival by Cox regression (FDR<0.01) and annotated as autophagy-related genes in relevant literature and databases (Homma et al., 2010; Moussay et al., 2011). In order to transform expression intensity values of genes to a single value, we used an approach presented before (Collura et al., 2013). Gene expression values were median-centred, and expression values of genes with a negative Cox's coefficient were inverted by multiplication by minus one in order to account for a survival effect. Then, gene expression of each patient was summed up forming a single score. Predictive power of the signature was characterised using support vector machine (SVM) as a classifier. Similar to Lu and colleagues (Lu et al., 2006), cancer patients were divided into two groups with "good" and "poor" prognosis based on their survival in relation to median survival time of the patients. Gene expression was used as input to a support vector machine classification; output was considered as a binary signal for "good" or "poor" survival. During performance testing, 80% of the patients were randomly selected and divided into two groups, keeping a correct proportion of good/poor prognosis patients. This operation was repeated 1000 times in order to characterise confidence intervals for accuracy (ratio between numbers of correctly classified patients to total number).

Pathway analysis of differentially expressed genes

Differentially expressed transcripts were mapped onto a molecular network developed from information contained in the Ingenuity knowledge base (Ingenuity Systems, http://www.ingenuity.com). Networks of these genes were generated based on their interconnectivity. Ingenuity Pathway Analysis ranks the resulting networks by calculating a significance score corresponding to the negative log of p-value. Furthermore, pathway core analysis identified the pre-specified canonical pathways that were most over-represented in the data set. Fisher's exact test was used to calculate a p-value for the association between the genes in the data set and the canonical pathway or network.

RT-PCR confirmation

For RT-PCR, 1 µg total RNA of 20 samples per group (normal pancreas, PDAC and its macro-environment) was reverse-transcribed using the ProtoScript M-MuLV First Strand cDNA Kit (New England Biolabs, Frankfurt, Germany). Quantitative RT-PCR was performed in triplicate on a LightCycler 480 (Roche Diagnostics, Mannheim, Germany) with a pre-amplification incubation of 95°C for 10 min, followed by 45 cycles of 95°C for 10 sec and 55°C for 30 sec. The following molecules of the QuantiTect Primer Assays (Qiagen) were used as primers: Hs_ACTB_1_SG, Hs_PKM_1_SG, Hs_PLK4_1_SG, Hs_PRAF2_1_SG and Hs_BUD13_1_SG. *BUD13* was used as reference gene as it showed constant mRNA expression in all 452 analysed pancreatic tissue samples and an adequate expression level. Data were analyzed using the LightCycler software (Roche) and relative fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method (Bookout et al, 2006).

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AUTHOR CONTRIBUTIONS

ASB, AS, MWB, JPN and JDH conceived the study; ASB, NG, SB, AH and MB performed experiments; ASB, PVN, NG, WG, EC, AM, OS, TH, LV, AS and SK analysed and interpreted data; NG, OS, TH, AS, MWB and JPN provided vital reagents; ASB, PVN, NG and JDH wrote the manuscript; all authors contributed to manuscript writing and editing.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

THE PAPER EXPLAINED

Problem

Pancreatic ductal adenocarcinomas (PDAC) and cystic tumours of the pancreas differ widely in their mortality. In most cases, the former kills patients within few months after diagnosis, while patients with the latter tumour have a much better prognosis. Also, non-tumorous samples from areas adjacent to the tumour are considered to differ from the tumour as much as healthy tissues do, but for the personal genetic background. Looking at the transcriptional variations in both tumour types and by comparing the transcriptional signatures of peritumoral tissues, we aimed at identifying molecular differences and similarities between the different tissue types and thus studying the influence of the peritumoral tissue on tumour development and prognosis.

Results

The peritumoral environment of cystic tumours was transcriptionally nearly identical to normal tissue. In contrast, the tissue around PDAC behaved much like the tumour, indicating some kind of field defect. At the same time, it was as different to chronic pancreatitis as to

healthy tissue. Very little correlation between the degree of methylation of promoter regions and the regulation of the respective genes was observed, suggesting a mechanism for the field defect other than methylation. Functionally, a strikingly large number of autophagy genes was uniquely changed in both PDAC and its peritumoral tissue. The relevance of autophagy for tumour pathology was demonstrated by the ability to predict patient survival with good accuracy on the basis of the expression of 15 autophagy genes.

Impact

The results suggest that the pathogenic difference between cystic and ductal tumours could – at least in part – be due to their cellular environment rather than variation between the tumours. Also, the importance of autophagy for PDAC pathology was highlighted.

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FIGURES LEGENDS

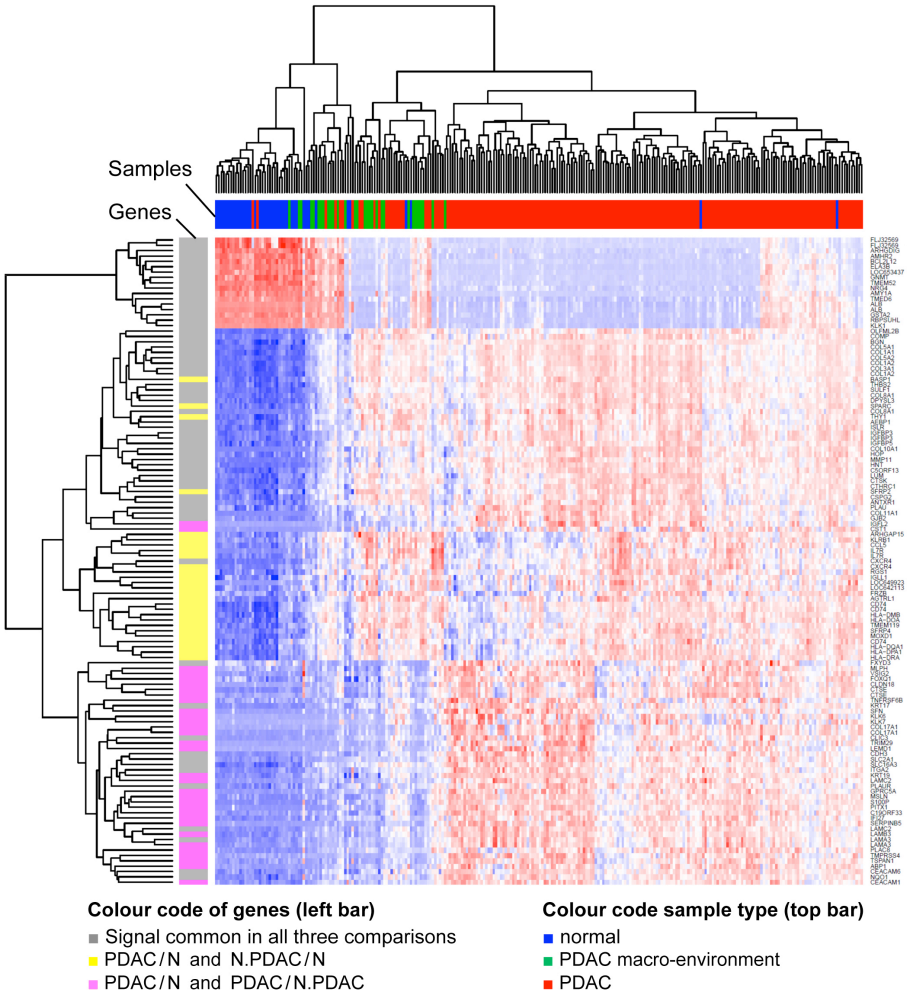

Figure 1. Heatmap representing the relative signal intensities of the most differentially expressed genes in all analysed samples. The complete dataset is provided in Tab. EV1; here, the top 50 genes of each comparison are shown: PDAC/N; PDAC/N.PDAC; N.PDAC/N. Columns represent individual samples; the bar at the top indicates their clinical annotation. In the rows, the genes are shown. If a gene name is listed more than once, it was represented on the microarrays by different features. The median of all normalised signal intensities was determined and is represented by white cross-sections. The intensity of the red and blue colour at cross-sections indicates the degree by which gene expression in this sample was higher or lower (at a $\log_2$ scale), respectively, than the median value.

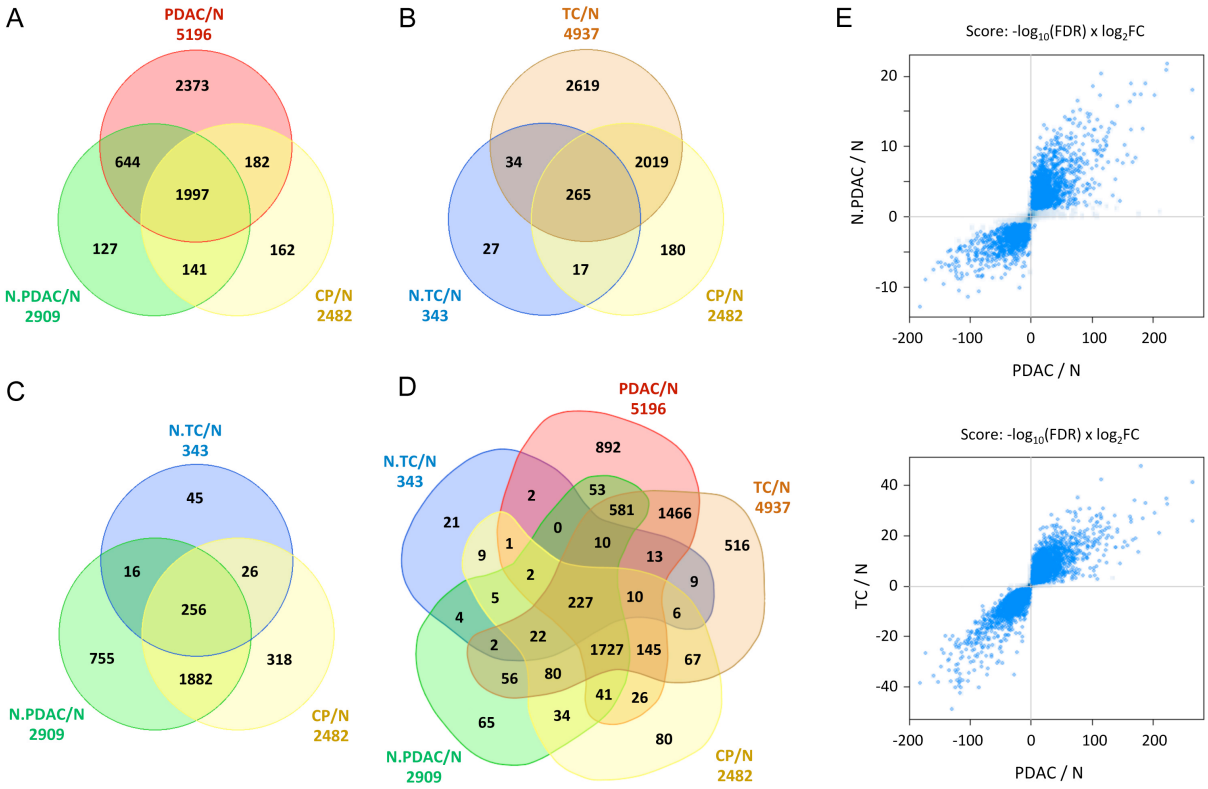

Figure 2. Tissue specificity of mRNA level variations. For each tissue type, the number of mRNAs is shown that were significantly differentially expressed in comparison to normal pancreas tissue (N). The numbers in overlap regions stand for genes, regulated similarly in the relevant tissues. (**A**) Results are presented for PDAC, the related macro-environment (N.PDAC) and chronic pancreatitis (CP), marked in red, green and yellow, respectively. (**B**) The panel presents the same for cystic tumours (TC; brown), the related macro-environment (N.TC; blue) and again chronic pancreatitis (CP; yellow). (**C**) The macro-environment of cystic tumours (N.TC) exhibited relatively few variations at the mRNA level that were specific. (**D**) Presentation of the result of a comparison of all five data sets. (**E**) Correlation in the direction of variation observed for N.PDAC/N (top panel) or TC/N (bottom panel), respectively, in comparison to PDAC/N. Both axes represent the score shown above the panels, thus focussing on the most significant variations (shown in blue). Grey dots, mostly close to the centroid, represent insignificant changes.

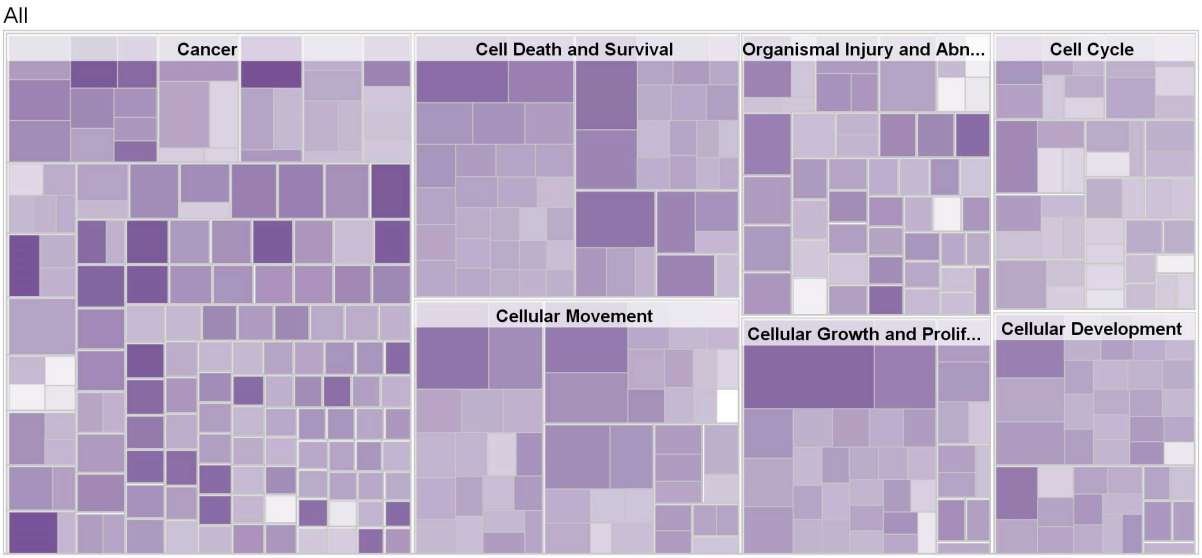

Figure 3. Most overrepresented biological functions associated with the 2,373 unique marker genes of PDAC. The intensity of the purple colour of the squares is proportional to the number of genes that are associated with each function. The size of the squares reflects the associated negative $\log_{10}$ of the assigned p-value. Larger squares indicate a more significant overlap between the genes perturbed in the dataset and the respective function.

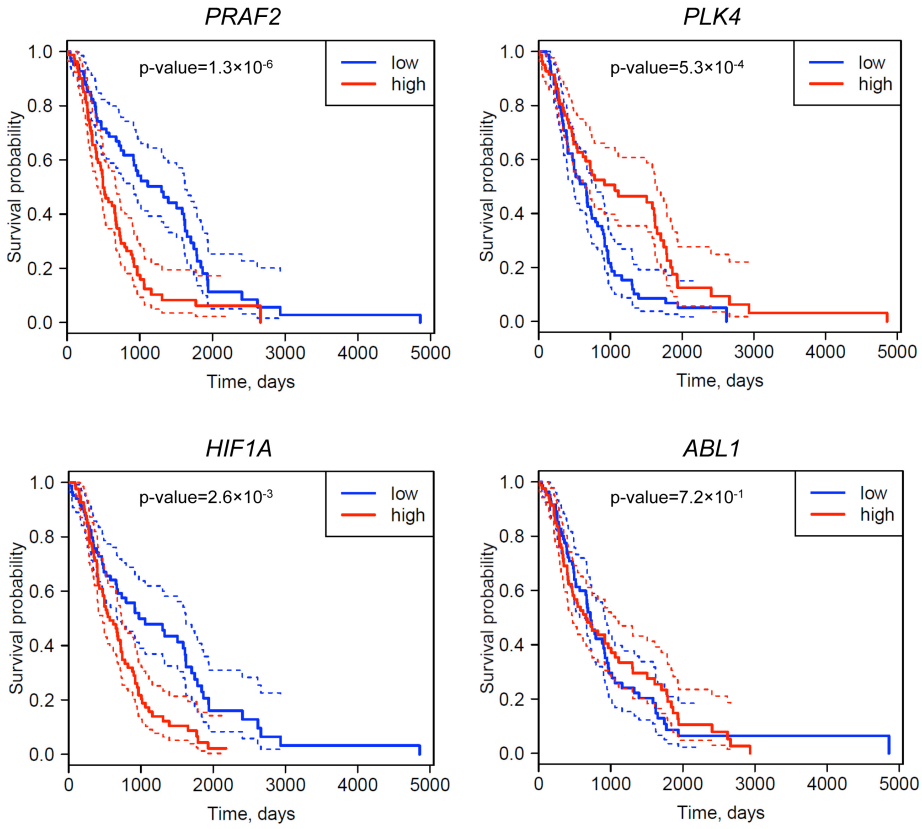

Figure 4. Linkage of gene expression levels in PDAC and patient survival time. Four typical Kaplan-Meyer curves with 95% confidence (dotted lines) are shown. An increase in expression of *PRAF2* is linked to poor survival, whereas stronger *PLK4* expression predicts better survival. In the third panel, the result is shown for the established prognostic marker *HIF1A*, which is linked to poor survival. The expression of *ABL1* is not correlated to survival time at all and shown as a reference. The p-values shown are based on a Cox regression of continuous $\log_2$ gene expression.

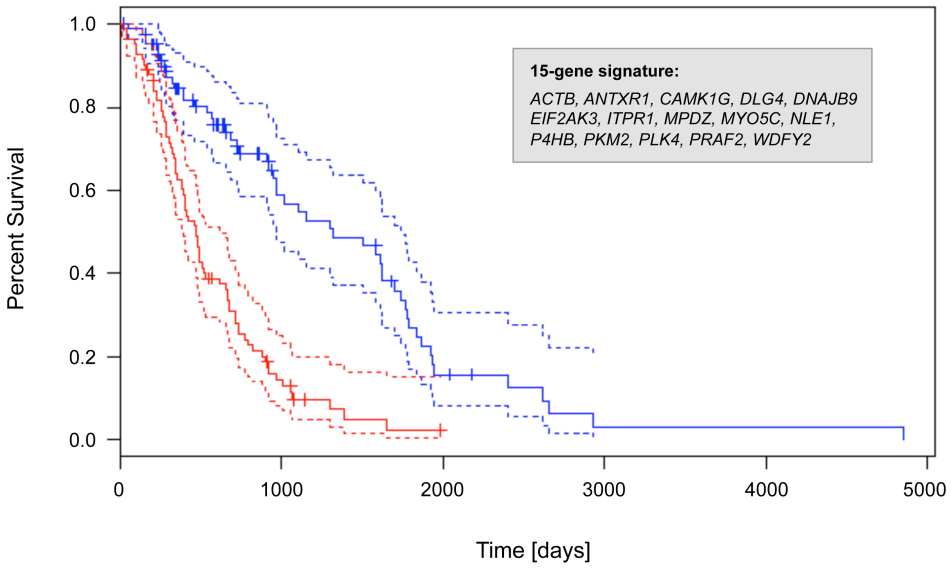

Figure 5. Prognosis of patient survival. Kaplan-Meier curves were calculated based on an expression signature in PDAC of the 15 genes named in the figure (logRank p-value of Cox model = 8.44×10^{-15}). In blue, the survival of the patients with good prognosis is shown; the red line represents the result of the patients with poor prognosis. The dotted lines correspond to 95% confidence intervals.

TABLES

Table 1. Clinical characteristics of patient cohort. Details are listed of the clinical parameters of the patients from whom the 452 RNA-preparations were isolated and subsequently analysed. N = healthy tissue; CP = chronic pancreatitis; PDAC = pancreatic ductal adenocarcinoma; N.PDAC = macro-environment of PDAC; TC = cystic tumour; N.TC = macro-environment of cystic tumour; n/a = not applicable.

	N	CP	PDAC	N.PDAC	TC	N.TC	Others
No. of patients	41	58	195	30	24	22	82
Gender (male / female)	n/a	48 / 10	109 / 86	21 / 9	7 / 16	4 / 18	50 / 32
Age at surgery, median (range)	n/a	47.1 (13-73)	63.4 (40-85)	60.8 (34-84)	62.0 (23-75)	57.1 (38-75)	55.7 (13-86)
Stage							
0	n/a	n/a	-	-	1	-	-
IA	n/a	n/a	-	-	1	-	1
IB	n/a	n/a	1	-	1	-	1
IIA	n/a	n/a	21	5	-	3	5
IIB	n/a	n/a	123	18	4	1	24
III	n/a	n/a	7	-	-	-	2
IV	n/a	n/a	17	2	2	-	4
Median survival-time in months, (range)	n/a	n/a	24.7 (1-159)	19.7 (1-65)	14.7 (3-36)	22.2 (4-53)	18.22 (1-54)

Table 2. Result of a Cox regression of 18 autophagy transcripts statistically linked to survival time. A positive coefficient indicates a worse prognosis, a negative coefficient a protective effect. In addition, the regulation in PDAC and PDAC macro-environment (N.PDAC) as compared to healthy tissue is shown. FRD: false discovery rate; log_2FC: logarithm of fold change.

Gene	Cox Coeff.	Cox FDR	PDAC log_2FC	PDAC FDR	N.PDAC log_2FC	N.PDAC FDR
PKM2	0.617	0.0004	1.475	0.000	0.702	0.000
ANTXR1	0.601	0.0088	1.373	0.000	0.843	0.000
ACTB	1.087	0.0023	1.298	0.000	0.768	0.000
CAMK1G	-0.410	0.0008	1.140	0.000	1.015	0.001
PLK4	-0.633	0.0034	0.991	0.000	0.529	0.000
VIM	0.421	0.0166	0.846	0.000	0.865	0.000
DLG4	0.570	0.0001	0.761	0.000	0.902	0.000
ITPR1	-0.704	0.0017	0.741	0.000	0.816	0.000
MPDZ	-0.685	0.0038	0.613	0.000	0.729	0.000
PRAF2	1.068	0.0000	0.588	0.000	0.595	0.000
EIF2AK3	0.846	0.0014	-0.715	0.000	-0.501	0.001
SH3GLB2	-0.550	0.0198	-0.726	0.000	-0.713	0.000
NLE1	1.153	0.0050	-0.728	0.000	-0.532	0.000
MYO5C	-0.407	0.0036	-0.818	0.000	-0.780	0.001
MAP2K7	0.697	0.0179	-0.901	0.000	-0.608	0.000
P4HB	0.550	0.0057	-1.079	0.000	-0.592	0.003
WDFY2	-0.822	0.0052	-1.192	0.000	-0.915	0.000
DNAJB9	-0.598	0.0069	-1.300	0.000	-0.904	0.000

EXPANDED VIEW FIGURES

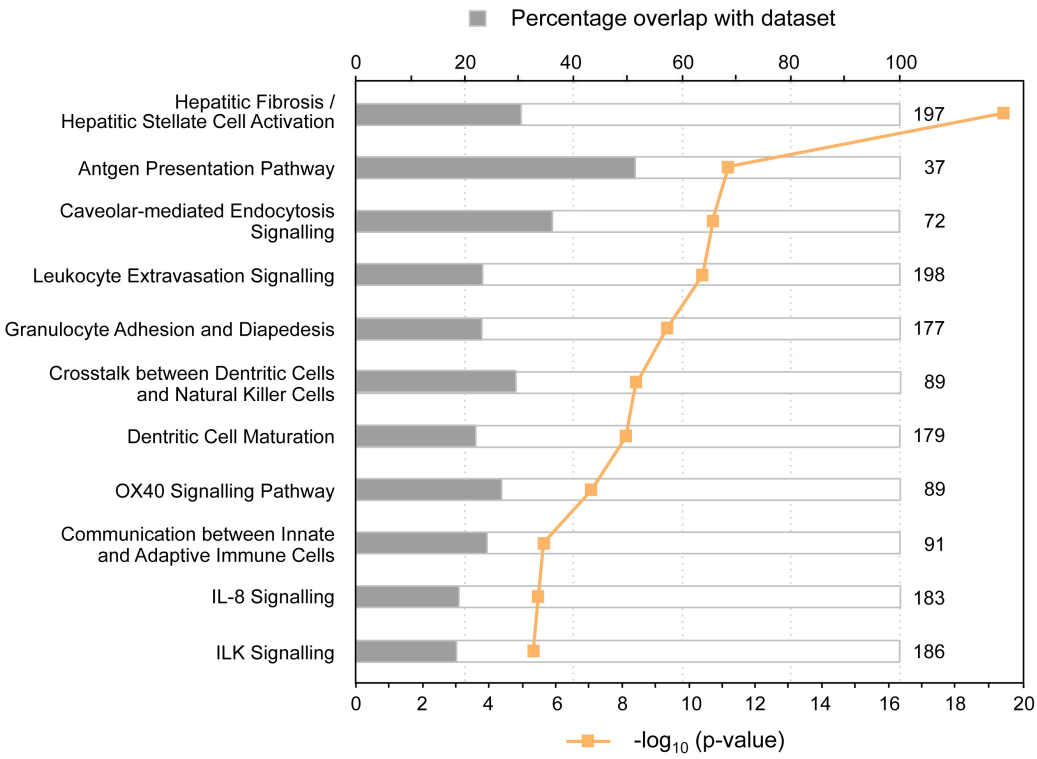

Figure EV1. List of the most overrepresented canonical pathways shared between PDAC, chronic pancreatitis and PDAC macro-environment. The bar chart displays for each canonical pathway the percentage of genes in the pathway that were found overlapping with the dataset of 1,997 regulated genes shared between PDAC, PDAC macro-environment and chronic pancreatitis. The numerical value to the right of each bar represents the total number of genes in the canonical pathway. The Benjamini-Hochberg method was used to adjust the right-tailed Fisher's Exact t-test p-value, displayed as negative $\log_{10}$ value by the orange squares.

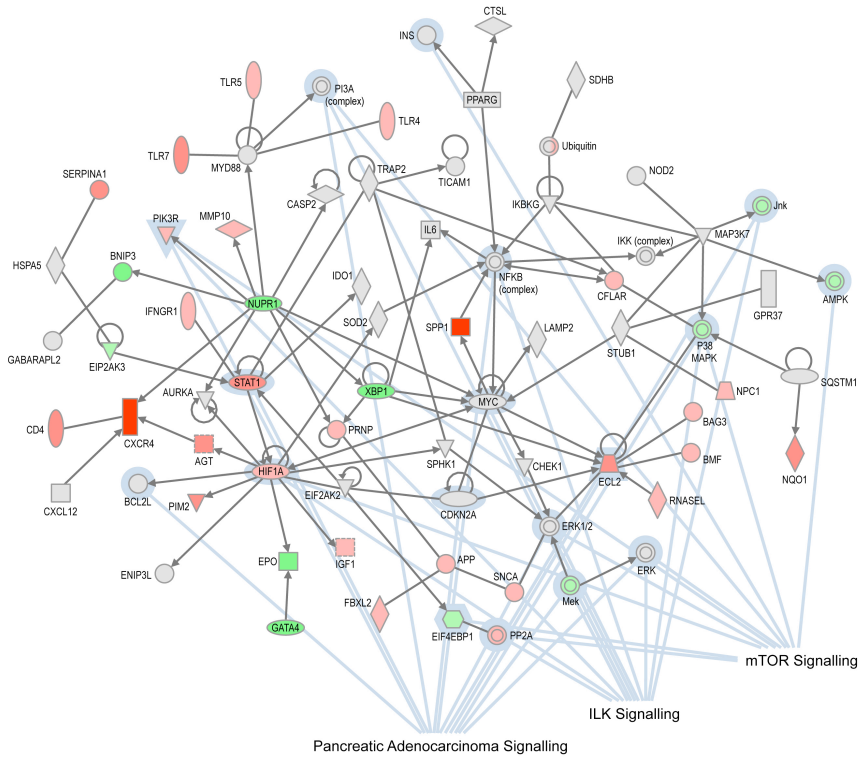

Figure EV2. Gene network strongly associated with transcriptional variations in the PDAC macro-environment. The network was generated by Ingenuity pathway analysis of the genes regulated in the PDAC macro-environment. A red node indicates that a gene is up-regulated compared to normal pancreas, while green represents down-regulation. Darker colours indicate stronger regulation. A grey colour stands for genes that did not show any apparent variation of their transcript levels. Proteins that are associated with the pathways of Pancreatic Adenocarcinoma Signalling, ILK Signalling and mTOR Signalling are highlighted in light blue.

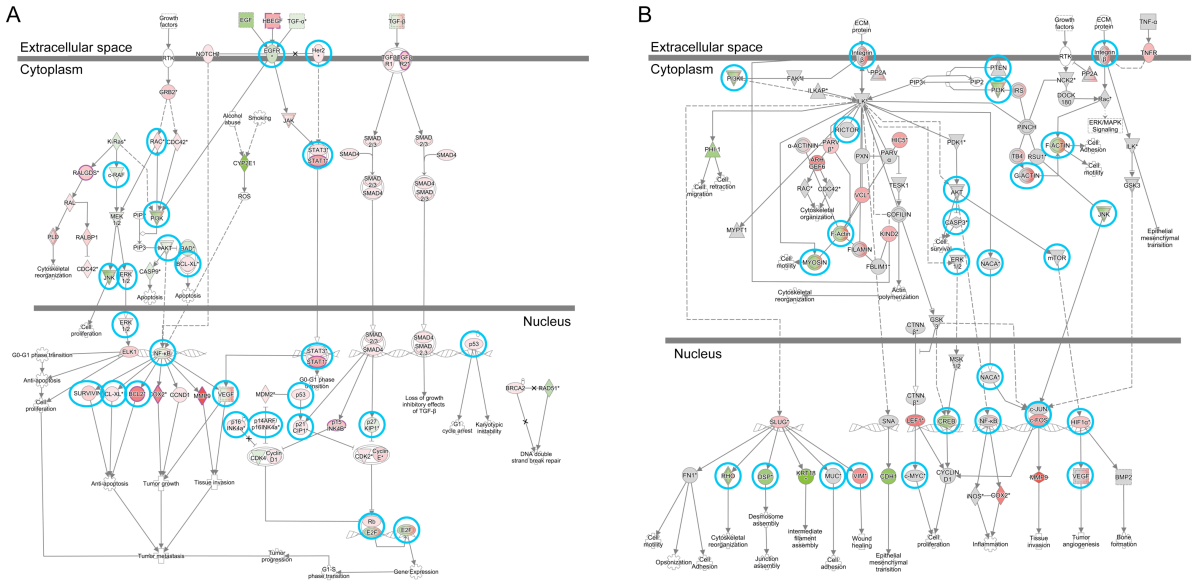

Figure EV3. The frequency of autophagy genes in pathways regulated in the macro-environment of PDAC. Using the Ingenuity pathway finder software, genes are shown that contribute to the Pancreatic Adenocarcinoma Signalling (**A**) and the ILK (**B**) pathways. Genes that are functionally associated with autophagy are marked by blue circles. Genes labelled in green (down-regulation) or red (up-regulation) exhibited significant changes in their expression level in the macro-environment of PDAC compared to healthy tissue (**Tab. EV4**). Solid lines indicate direct interactions between proteins, dotted lines stand for indirect interactions.

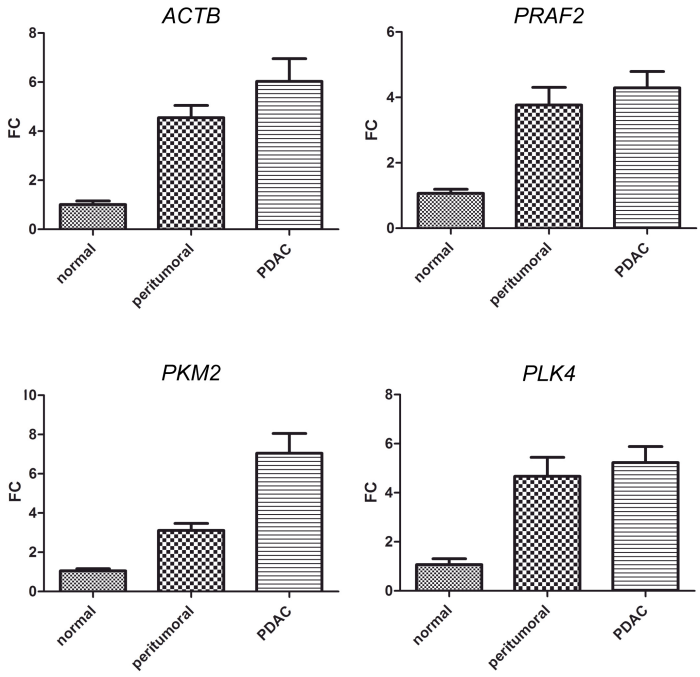

Figure EV4. *ACTB*, *PKM2*, *PRAF2* and *PLK4* mRNA expression in normal, peritumoral and PDAC tissue obtained by qRT-PCR. Each bar represents the results from 60 measurements: three replicate analyses each performed on 20 randomly selected samples of the respective tissue type. The values were normalised by reference gene *BUD13* and relative fold-changes (FC) were calculated in comparison to the mean of all normal tissues.

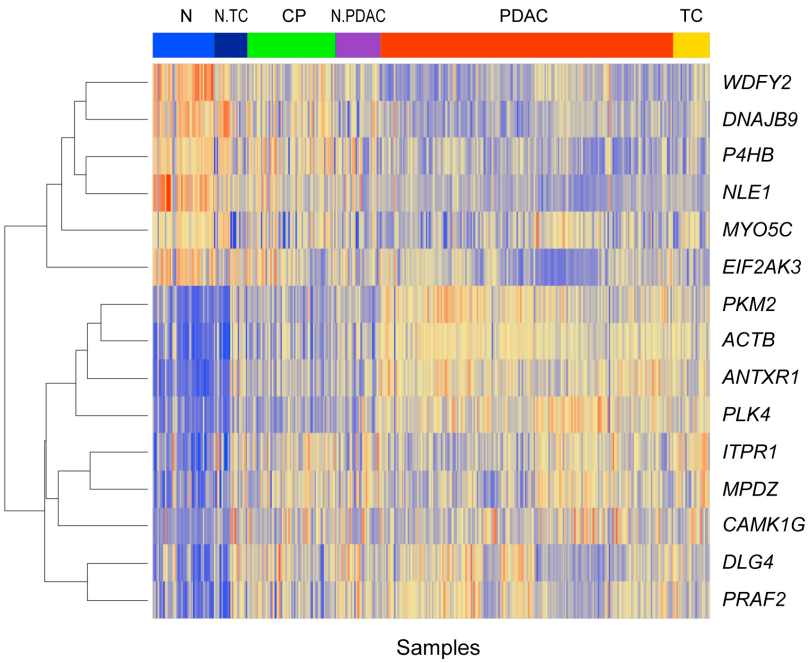

Figure EV5. Heatmap representing the colour-coded expression levels of the 15 genes of the prognostic gene signature. Columns represent individual samples; each row stands for a gene. Blue and red indicates up- and down-regulation, respectively. The sample types are indicated at the top: normal pancreas (N; blue), macro-environment of cystic tumours (N.TC; dark-blue), chronic pancreatitis (CP; green), macro-environment of PDAC (N.PDAC; purple), PDAC (red) and cystic tumour (TC; yellow).

EXPANDED VIEW TABLES

Table EV1. Genes that exhibited significant changes at the transcript level in the various kinds of tissue compared to samples from healthy donors.

Table EV2. List of transcripts that were regulated in PDAC and its macro-environment but not in chronic pancreatitis.

Table EV3. Transcript variation of the 73 genes associated with the gene network of the **PDAC macro-environment (Fig. EV2)**. Their expression variations are shown in PDAC, its macro-environment and in CP, respectively, in comparison to healthy tissue**.**

Table EV4. Transcriptional variations of autophagy-related genes in PDAC, cystic tumours, their respective cellular macro-environments and chronic pancreatitis.

Table EV5. List of all differentially expressed autophagy transcripts that were statistically linked to survival time.