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Gene expression profiling of morphological subtypes of pancreatic ductal adenocarcinoma using surgical and EUS-FNB specimens

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Abstract

Background/Objectives: Various classifications of pancreatic ductal adenocarcinoma (PDAC) based on RNA-profiling resulted in two main subtypes. Kalimuthu and coworkers proposed a morphology-based classification that concurred with these subtypes. Immune therapy approaches were so far disappointing in PDAC. Morphological PDAC subtypes may differ regarding key immune-oncology pathways. We aimed to examine the reproducibility and prognostic value of Kalimuthu's subtypes, and to evaluate differences regarding gene expression related to tumor biology and immune-oncology.

Methods: 108 consecutive chemotherapy-naïve surgical specimens and 88 endoscopic ultrasound-guided fine needle biopsies (EUS-FNBs) from 196 PDAC patients were evaluated by two pancreatic pathologists as per Kalimuthu, resulting in Group A and Group B tumors. Digital mRNA expression profiling was performed, on the surgical cohort using the NanoString IO360 panel of 770 key tumor biology related and 30 custom-genes, and on the EUS-FNB cohort using a targeted panel of 123 genes.

Results: Morphological subtyping reached substantial agreement. In the surgical and EUS-FNB cohorts, 44.4% and 38.6% were Group A, which was associated with improved survival. Hierarchical clustering based on genes significantly different expressed in Group A and Group B revealed clusters with prognostic value. One of these showed accumulation of Group A tumors and upregulation of genes related to immune system and cytokine/chemokine/interleukin signaling.

Conclusions: Morphological subtyping according to Kalimuthu is reproducible and holds prognostic value, in surgical as well as EUS-FNB specimens. As upregulation of immune regulatory genes was found in Group A, future studies should evaluate the potential of immune therapy in this particular subtype of PDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal cancer with an overall 5-year survival rate of 5% and median survival of 5.9 months [1]. PDAC has been estimated to be the third- and second-leading cause of cancer-related death in the European Union and US by 2025 and 2030 [1]. In the last decade, subtyping of PDAC has been investigated in various studies, aiming to stratify patients regarding treatment and outcome. One important obstacle to the management of PDAC is the lack of predictive biomarkers for effective personalized treatment, including immunotherapy.

So far, classification of PDAC has been mainly based on transcriptional profiling, resulting in several prognostically relevant classification systems [2-5]. Most studies have used RNA sequencing (RNAseq) and RNA array techniques, which are time consuming and relatively difficult to implement in clinical practice. Recently, Kalimuthu et al. proposed a histomorphology-based classification of PDAC, the two subtypes of which showed considerable overlap with the two main molecular subtypes as per Bailey, Collison, and Moffitt [6]. However, our knowledge regarding the potential therapeutic implications of this morphological classification is very limited.

In contrast to other types of cancer, immunotherapeutic strategies for the treatment of PDAC have so far not been successful [7-9]. It is tempting to speculate whether the morphological subtyping of PDAC as per Kalimuthu may be able to help identify subsets of patients where targeted treatment, such as immunotherapy, may be useful, but this has so far not been elucidated. A hurdle for the implementation of a transcription-based classification in clinical practice is the fact that RNA sequencing requires access to frozen tissue samples, which are more difficult to obtain than formalin-fixed paraffin embedded (FFPE) tissues. Digital mRNA gene expression profiling may overcome this hurdle, as it is specifically designed to detect RNA molecules in FFPE tissue samples. Compared to RNA sequencing, it also has the advantage of measuring directly without the need for prior amplification or cloning, thereby avoiding introduction of no gene-specific or 3′ biases. In addition, the reaction is driven to completion, allowing for a higher level of sensitivity than in micro-arrays across many target genes with lower amounts of starting material. Moreover, the system provides a digital readout of the amount of transcript in a sample. Finally, the time, effort, and sample requirements of dedicated digital mRNA gene expression are more scalable than real-time PCR or microarrays [10-12].

The aims of this study were to examine the reproducibility and prognostic value of the morphological subtyping of PDAC as per Kalimuthu, using surgical specimens as well as histological endoscopic ultrasound-guided fine-needle biopsies (EUS-FNBs). We further aimed to

evaluate whether the morphological subtypes differ regarding canonical pathways related to tumor biology and key immune-oncology pathways.

Materials and methods

Study cohort

A series of 108 consecutive patients with chemotherapy-naïve PDAC who underwent surgical resection at Odense University Hospital (OUH), Odense, Denmark, was identified by searching the Danish Pathology Registry for the period 01.01.2015-30.04.2018. We also included a cohort af 88 patients who had been diagnosed with PDAC using EUS-FNB in the period from 01.01.2018–31.12.2019. The inclusion criteria were a tissue area ≥8 mm^2 and a tumor/normal tissue ratio ≥55 %.

This study was approved by the Ethics Committee of the Region of Southern Denmark (project-ID: S-20190175) and the Danish Data Protection Agency (project-ID 19/45478). Patients had not advocated against the use of their tissue in the Danish registry for the use of tissue in research ('Vævsanvendelsesregisteret').

Digitalization of H&E-stained slides

H&E-stained slides were scanned using a 20x objective on a NanoZoomer 2.0HT whole-slide scanner (Hamamatsu Photonics, Hamamatsu, Japan). One of the pathologists (SDE) measured the tumor-bearing areas on each slide, by marking them using the NDP.view 2.7.25 software (Hamamatsu Photonics). The digitalized slides were uploaded to a pseudonymized web library, with personalized access.

Morphological subtyping according to Kalimuthu et al.

All H&E-stained slides with tumor tissue from the 108 surgical PDAC and 88 EUS-FNB specimens were re-evaluated by two pancreatic pathologists, referred to observer A and observer B, and subtyped as described by Kalimuthu et al. [6]. This classification recognizes four different morphological patterns (conventional, tubulopapillary, composite, and squamous), giving rise to two different morphological subtypes (Group A and Group B).

The conventional morphological pattern shows well-differentiated glands with a tubular, stellate configuration. The tubulopapillary morphological pattern shows rounded and dilated glands that are generally larger and often lined by a combination of foveolar-gastric type and pancreaticobiliary-type epithelium. The composite morphological pattern shows a range of morphological features traditionally associated with poor differentiation, such as angulated glands, cribriform complexes,

sheets, nests/islands, ribbons, cords, angulated glands, or scattered buds and single cells. The squamous morphological pattern consists of well-to-poorly differentiated squamous structures. The conventional and tubulopapillary patterns are merged into Group A and, correspondingly, given the lack of well-formed glands, the composite and squamous patterns are merged to represent Group B [6].

Each resection specimen with tumor was semi-quantitatively assessed for the presence of the four patterns as follows: The area of tumor-containing tissue on each slide was divided into four quadrants. For each quadrant, the dominant pattern was identified. Subsequently, the percentage of quadrants dominated by each of the four morphological patterns was calculated [6]. Group A is defined as dominance of conventional and/or tubulopapillary pattern in at least 60% of the quadrants. Group B is defined as dominance of the composite and/or squamous pattern in at least 40% of the quadrants. With other words, tumors showing a dominant conventional/tubulopapillary pattern in 0% to 59% or 60% to 100% of the quadrants were categorized as Group A or Group B [6].

Tumor differentiation grading

The grade of histological differentiation was assessed for all resection specimens by both pathologists based on the grading system recommended by the WHO [13, 14]. The tumor grade was determined by the lowest grade. For the purpose of this study, it was decided that this lowest grade had be present in at least 20% of a given tumor.

RNA extraction

For the surgical cohort, the FFPE block most representative of the morphological subtype was selected for RNA isolation and macrodissected, for removal of non-tumor tissue. Sections of 10 µm thickness were cut, their number depending on the size of the tumor area on the slide. At least 150 mm^2 total tumor area was included from all tumors/EUS-FNBs. The sections were prepared and processed according to the Prosigna® Breast Cancer Prognostic Gene Signature Assay package insert (2016-09 LBL-C0223-06). Total RNA was extracted using High Pure FFPE RNA Isolation kit (Roche Diagnostics GmbH, Mannheim, Germany, 06650775001), according to the Prosigna® protocol. RNA concentration was measured by Thermo Scientific™ NanoDrop™ One^C Spectrophotometer (Thermo Scientific) to meet the quality control of RNA concentration (≥ 12.5 ng/µL) and purity (A260/A280 ratio between 1.7 and 2.3). If the RNA concentration exceeded 200

ng/µL, they were diluted with molecular grade RNase- and DNase-free water (Sigma-Aldrich, 7732-18-5) prior to downstream hybridization assay according to NanoString protocol. The RNA samples were stored at -80°C.

Digital mRNA expression profiling and data processing

For the surgical cohort, mRNA gene expression levels were assessed using 100 ng RNA and the PanCancer immune-profiling panel, Immuno-Oncology 360 (IO-360, NanoString Technologies, Seattle, WA), designed to give a unique 360 degree view of gene expression in the tumors. The panel consists of 770 genes (hereof, 20 housekeeping genes), falling into eight functional categories: tumor immunogenicity, tumor sensitivity to immune attack, inhibitory immune mechanisms, stromal factors, inhibitory metabolism, anti-tumor immune activity, inhibitory immune signaling, and immune cell population abundance. Thirty custom genes were added, related to fibroblasts, endothelial cells, and extracellular matrix (ECM), resulting in a total of 800 genes: *ACTA2*, *ANO1*, *CALD1*, *CD34*, *CEACAM5*, *COL3A1*, *COL4A1*, *CYGB*, *FN1*, *GPC1*, *HAS2*, *INS*, *KCNH2*, *KRT7*, *KRT8*, *LGALS1*, *MME*, *MUC1*, *NES*, *PDPN*, *POSTN*, *PRSS1*, *S100A4*, *SLC16A3*, *SMAD4*, *SPARC*, *SYP*, *TNC*, *VCL* and *VIM* [15]. *CALD1, INS*, *KRT8*, *PRSS1*, and *SYP* were not included in the hierarchical clustering analysis.

Normalization of RNA was performed using the geometric mean of internal negative controls, positive controls, and 20 housekeeping genes. RNA samples were aligned with a synthetic panel standard and hybridized in a 12-strip tube well with gene-specific probes (reporter and capture) following the manufacturer´s protocol. Subsequently, the target-probe complexes were read, counted, and processed within the Counter Digital Analyzer (NanoString Technologies, Seattle, WA) [11]. The raw digital counts of expression were exported to the nSolver v4.0 software (NanoString) for downstream analysis following the manufacturer's protocol. The raw data from the NanoString nCouter Digital Analyser were imported to the nSolver software, where the data was investigated to check quality of reads, according to manufacturer´s instructions. The data was normalized and grouped according to the morphological subtype. Differential gene expression was analyzed, where the normalized data was used to calculate the false discovery rate (FDR) for each gene. The genes showing significantly different expression between Group A and Group B were used to create a heat map with hierarchical clustering data, and the expression of these genes and of Moffitt´s top 50 basal-like and classical genes was examined in the EUS-FNB cohort [4].

Pathway analysis

The significant differently expressed genes (FDR<0.05) identified in the surgical cohort were evaluated for over-representation of gene-sets or pathways in the Molecular Signatures Database (MSigDB). The over-representation analysis compares a reference set of genes to a test gene-set using the hypergeometric test. The probability of finding the number of significant genes belonging to a particular gene-set or pathway can be calculated using hypergeometric distribution. The analysis was performed using the analytical tool provided by Gene Set Enrichment Analysis (GSEA) (http://www.broadinstitute.org/gsea/index.jsp) [16, 17].

Tissue microarrays and immunohistochemistry (IHC)

Multi-punch tissue microarrays (TMA) were used to validate the level of four representative proteins encoded by differently expressed genes. The TMAs had been produced in a previous study, as described elsewhere [18]. Included in the IHC analysis were 64/108 randomly chosen PDACs (32 each of Group A and B). TMA sections were immunostained for CD5, CD10, FOXP3, and TNC using the BenchMark Ultra immunostainer (Ventana Medical Systems, Tucson, AZ). Antibody specifications, dilutions, incubation times, and epitope retrieval procedures are specified in Supplementary Table 1. Stained slides were scanned using a 40x objective on a Hamamatsu NanoZoomer 2.0–HT whole slide scanner (Hamamatsu Photonics, Hamamatsu, Japan). Automated digital image analysis was performed using the Visiopharm Image Analysis Software, version 2020.01 (Hoersholm, Denmark) as previously described [15].

Statistical analysis

The interobserver agreement of pattern assignment, subtype classification, and WHO grading was investigated by utilizing Krippendorrf's alpha, according to Landis J.R. and Koch G.G [19], which calculates coefficients 0 (or <0 in extreme cases) to 1. A coefficient 0 indicates no agreement, while coefficient 1 represents perfect agreement. The coefficient values are interpreted as follows: <0: poor/systematic disagreement, 0 - 0.2: slight agreement, 0.21 - 0.40: fair agreement, 0.41 - 0.60: moderate agreement, 0.61 - 0.80: substantial agreement, and 0.81 - 1.0: almost perfect agreement [19]. Survival was analyzed using Kaplan-Meier and Cox regression using gender, age, tumor stage, and area of tumor as covariates. Survival was calculated from date of surgery or date of diagnosis. Patients alive on February 1, 2020 (surgical cohort) or December 1, 2020 (EUS-FNB cohort) were

censored at this date. Median survival and categorical data are presented with 95% confidence intervals. A *P*-value < 0.05 was considered significant. Analysis was made using Stata v. 16, StataCorp, USA.

The normalized gene expression data were first transformed by taking the logarithm with base 2. The log transformation has the advantage of making the data approach a normal distribution. Association between gene expression and subtypes was investigated using the binomial logistic regression to the log odds of subtype as a function of gene expression. Significance of the gene expression was determined by the likelihood ratio test comparing the likelihood of the current model with that of the reduced model without gene expression variable, with the test statistic distributed as a chi-squared random variable with 1 degree of freedom. The logistic regression analysis was performed by the glm function in R (https://www.r-project.org) with a binomial link function. To adjust for the statistical significance due to multiple testing, a false discovery rate (FDR) was calculated for each gene tested using the Benjamini and Hochberg method [20]. Statistical significance was assigned to genes with FDR<0.05.

The two-tailed Mann-Whitney test was used to compare the immunohistochemical expression of the selected markers in the gland-forming vs. non-gland-forming subtypes, and scatter plots were generated in GraphPad Prism, version 5.01.

Results

Clinical data

Characteristics of the patients included in the surgical cohort of 108 PDACs and general pathological findings are presented in Table 1.

Morphological subtyping as per Kalimuthu et al.

In average, 7.50±3.40 (range 2-23) slides containing tumor tissue were included in the morphological scoring. The average tumor-containing area per slide was 94.26±87.88 mm^2 (range 0.24-744.3 mm^2). There was substantial agreement between both observers regarding morphological subtypes (Krippendorrf's alpha coefficient 0.68±0.07; 95%CI 0.54-0.82). For assignment of the dominant morphological patterns, the agreement was moderate (Krippendorrf's alpha coefficient 0.58±0.07; 95%CI 0.44-0.71). Considering that agreement was substantial for the

subtype classification, further analyses were conducted based on observer A's scoring, as decided during the design of this study.

Observer A classified 48/108 of the tumors as Group A and 60/108 as Group B. Obviation of slides that contained very small amounts of tumour (less than 20 mm^2 and 30 mm^2, respectively) resulted in minimal changes in the ratio between Group A and Group B tumors (Supplementary Table 2). A cut-off of 20 mm^2 and 30 mm^2 resulted in the change of 2 tumors from Group A to Group B and of 3 tumors from Group B to Group A and the Group A/Group B ratio was 49/59 for both cut-off values. The dominant morphological pattern was conventional in 46/108 (42.59%), tubulopapillary in 10/108 (9.26%), composite in 46/108 (42.59%), and squamous in 6/108 (5.56%). Morphological heterogeneity was observed, as two or more patterns were present in 95/108 (87.96%) (Fig. 1A). Thirteen of 108 tumors (12.04%) showed exclusively one morphological pattern. These patterns were conventional (n=2), tubulopapillary (n=1), and composite (n=10). In some instances, small cancer cell clusters or abortive glands were associated with larger glandular formations but were less conspicuous than the latter (Fig. 1B-E).

WHO grading

Regarding the grade of differentiation, interobserver agreement was substantial, with a Krippendorff's alpha coefficient of 0.78±0.05 (95% CI 0.68-0.89). Given this result, further (survival) analyses were based on observer A's grading, as decided during the design of this study. Twenty of 108 (18.52%) tumors were well-differentiated (G1), 38/108 (35.18%) moderately differentiated (G2), and 50/108 (46.30%) poorly differentiated (G3).

Survival analysis

The median survival for the entire surgical cohort was 18.83±1.56 months (95% CI 15.31-25.82) (Fig. 1F). At the time of survival analysis, 87/108 patients (80.56%) had died. Group A (n=48) and Group B (n=60) showed a median survival of 25.82±8.65 months (95% CI 15.34-38.28) and 17.15±2.84 months (95% CI 12.75-19.78). There was a significantly longer survival of patients in Group A compared to Group B (P = 0.026, Fig 1G). Median survival associated with the four morphological patterns is given in Figure 1H. The squamous pattern was associated with a significantly worse survival (P = 0.020), and there was a trend towards poorer outcome for the composite pattern (P = 0.052) (Fig. 1H). Median survival based on the WHO differentiation

grading is shown in Fig. 1I: Tumors showing G3 differentiation had a significantly shorter survival than G1 ($P = 0.044$), whereas G2 tumors did not differ significantly ($P = 0.71$) (Fig. 1I).

Gene expression profiling

In the surgical cohort, 53 genes were significantly different expressed in Group A versus Group B (FDR<0.05) (Supplementary Table 3). Thirty-nine genes were upregulated in Group A: *CCR2*, *CD2*, *CD27*, *CD40LG*, *CD5*, *CD6*, *CD74*, *CX3CR1*, *FOXP3*, *FUT4*, *GLI1*, *ICOSLG*, *IL10RA*, IL22RA1, *JAK2*, *KLRB1*, *LY9*, *MMP1*, *MMP7*, *NLRC5*, *PDZK1IP1*, *PIK3CG*, *PRKACB*, *PRLR*, *PSMB10*, *P2RY13*, *RASAL1*, *RIPK3*, *RORC, SHC2*, *SLAMF7*, *SYK*, *TICAM1*, *TLR3*, *TNFRSF11A*, *TNFRSF17*, *TRAF1*, *WNT2B*, and *ZAP70*. Fourteen genes were upregulated in Group B: *ANGPTL4*, *ANLN*, *BIRC5*, *CCNB1*, *CDK2*, *ENO1*, *FLNB*, *IL11*, *MME*, *NT5E*, *PLOD2*, *PTGS2*, *TNC*, and *UBE2C*.

Expression of these 53 differentially expressed genes served as the basis for hierarchical clustering, resulting in a heat map, where four clusters could be identified (Fig. 2A). Cluster 1 contained 64 cases, 43 (67.19%) of which were Group A. Cluster 2 contained 27 tumors, 22 (81.48%) of which were Group B. Clusters 3 and 4 contained 10 and 7 tumors, all of which (100%) were in Group B. Six tumors (case 6, 60, 64, 73, 93, and 107) were in Group B with a dominant squamous pattern, three of which were located in cluster 4 (42.86%, 3/7) and one case each in clusters 1 (1.56%), 2 (3.70%), and 3 (10.00%). Cluster 1 and 3 had the best survival, whereas cluster 2 and cluster 4 had significantly poorer survival, with hazard ratios of 2.3 and 12 (Fig. 2B-C).

GSEA analysis identified the top-20 canonical pathways for each morphological subtype (Table 2). Group A showed higher expression of immune-related genes and cytokine/chemokine/interleukin signaling, whereas Group B showed higher expression of genes related to cancer cell proliferation and cell cycle regulation.

Microsatellite deficiency

The tumors included in the present study were also evaluated in a previous study, revealing that one tumor was microsatellite instable (case 33) and another showed loss mismatch repair protein MSH6 (case 96) [18]. All other tumors were microsatellite stable. Case 33 was assigned to cluster 2 and case 96 to cluster 1.

Immunohistochemical protein expression

CD5 protein was expressed significantly higher in the Group A than Group B, in accordance with the corresponding gene expression levels (P = 0.037, Fig. 3A-B). There was a similar trend for FOXP3 (P = 0.12, Fig. 3C-D). Likewise, the protein expression of both CD10 (P = 0.042) (Fig. 3E-F) and TNC (P < 0.0001) (Fig. 3G-H) was significantly higher in the Group B than in Group A.

Morphological subtyping, survival analysis and gene expression profiling in 88 EUS-FNBs from patients with pancreatic ductal adenocarcinoma

Eighty-eight EUS-FNBs were included, where 46/88 (52.3%) were from males, mean age 73 years (range 53-88). There was substantial agreement between both observers regarding morphological subtypes (Krippendorrf's alpha coefficient 0.79±0.06; 95%CI 0.66-0.92). Observer A classified 34/88 (38.6%) and 54/88 (62.4%) of the tumors as Group A and B (Fig. 4A). The survival analysis showed an improved survival for Group A vs. Group B (Fig. 4B). Twenty of the top 53 genes identified in the surgical cohort were significantly different expressed in Group A vs. Group B. Hierarchical clustering of these genes identified gene clusters similar to those in the surgical cohort (Fig. 4C-D), albeit with different relative frequencies, probably due to the clinically very different populations studied. Figure 4E illustrates the survival analysis regarding the clusters in the EUS-FNB cohort.

When we examined Moffitt's top 50 genes for the classical and basal-like subtypes of PDAC, 26 of these genes were significantly different expressed between Group A and Group B. Hierarchical clustering showed that Group A tumors were more often classical (28/34, 82%) and that Group B tumors were more often basal-like (35/54, 65%) (Fig. 5A).Of the 41 basal-like tumors, 35 (85%) were Group B. Of the 47 classical tumors, 28 (60%) were Group A. A survival analysis showed a tendency towards poorer survival for the basal-like tumors (P=0.026) (Fig. 5B).

Discussion

In the present study, we were able to reproduce the morphological subtyping of PDAC, as per Kalimuthu and coworkers, with substantial agreement between two pancreatic pathologists, in surgical and EUS-FNB specimens. Our data confirm that the morphological subtyping holds prognostic value. Using digital RNA expression analysis, we found that tumors categorized in Group A had a higher expression of genes related to the immune system and cytokine/chemokine/interleukin signaling, whereas Group B had a higher expression of genes related to cancer cell proliferation and cell cycle regulation. Hence, it may be hypothesized that immune therapeutic approaches may be more promising in some patients with the Group A subtype of PDAC.

This appears to be the first study testing the reproducibility of the histology-based classification of PDAC as per Kalimuthu et al in surgical specimens, and the first study using this approach in EUS-FNBs. In the surgical cohort, we identified a slightly higher relative number of tumors located to Group B (n=60, 55.6%), compared to the original report (n=37, 43%) [6]. A possible explanation could be the selection of included tumors. IPMN-associated PDACs, for example, were excluded from the current study, in contrast to Kalimuthu et al [6]. There may also be differences between the populations from which the cases were selected. The interobserver agreement was substantial, indicating the robustness and applicability of the morphological criteria that define the patterns and subtypes. Regarding the grade of histological tumor differentiation, we found a higher rate of G3 tumors (46.3%), in contrast to Kalimuthu et al. (18.6%), but in agreement with other studies [6, 21, 22]. In our cohort, more than 15% were G1, in agreement with Kalimuthu et al. and Moffitt et al. [4, 6]. Given the histomorphological criteria that define both subtypes of the classification, low-grade PDACs are likely to be assigned to the gland-forming subtype, and high-grade PDACs to the non-gland-forming subtype. The co-variation between morphological and molecular subtypes, as revealed by the Kalimuthu study, seems therefore to bring us a step closer to the molecular basis for the morphological concept of grade of tumor differentiation, which hitherto has remained unexplored [6].

Group A showed a better median overall survival than Group B. The squamous morphological pattern conferred the worst prognosis (statistically significant) and was associated with a poorer overall survival compared to the conventional pattern, while there was a trend towards poorer prognosis for the composite pattern. Also Kalimuthu and coworkers reported a trend for difference

in outcome between both subtypes, a significant worse outcome for the composite pattern, and a trend towards worse outcome for the sqaumous pattern [6]. We found that poorly differentiated tumors (G3) were associated with a worse outcome. In the study conducted by Kalimuthu et al., the association of G3 with poor outcome did not reach full statistical significance[6].

We used the morphological subtyping as per Kalimuthu as framework for hierarchical clustering-based gene expression analysis. Fifty-three significantly different expressed genes were identified in the surgical cohort. Ten of these 53 genes were also included in several gene sets that have been reported in earlier studies, which have investigated RNA-based PDAC classification [3, 4, 6, 23, 24]: *ICOSLG*, *IL22RA1*, *MME*, *NT5E*, *PDZK1IP1*, *RASAL1*, *TNC*, *TNFRSF11A*, *ANGPTL4*, and *MMP7*. Thirty-nine genes were expressed at significantly higher levels in Group A, related to immune system and to cytokine/chemokine/interleukin signaling. Kalimuthu et al. demonstrated that Group A shared most commonalities with the classical subtype as described by Moffitt and Collison, respectively, and the pancreatic progenitor subtype proposed by Bailey et al. [3-5]. Our own data support this, as 82% of Group A tumors were classical. Interestingly, also Bailey et al. (immunogenic subtype) and Puleo et al. (immune classical subtype) identified a subgroup of PDAC that was characterized by high expression of genes related to immunosuppressive checkpoint pathways and gene signatures of multiple executors of immunity [2, 5]. Some these factors can potentially be targeted with immune modulators [2]. Despite the wide range of upregulated genes, only anti-PD-1 and anti-CTLA4 treatments have been tested in PDAC so far, with poor results [7-9]. In accord with this, we also found a trend for higher expression of *CTLA-4* in the group of gland-forming tumors. However, the relative proportion of the immunogenic and immune classical tumors in Bailey et al.'s and Puleo et al.'s studies were much lower than Group A in the present study [2, 5]. Le at al. observed that MSI PDAC was responsive to PD-1/PDL-1 antagonist treatment [7]. MSI has, however, a low frequency in PDAC with about 1-3%, which also was found in our cohort (2/108 (1.85%)) [18]. It may be hypothesized that immune therapeutic approaches may have potential in some patients, particularly in those with the Group A subtype. For example, we found that *FOXP3, ICOSLG*, *IL10RA*, *RORC, TICAM1,* and *TLR3* are expressed at higher levels in this subtype, and these genes were considered treatable targets [25-30]. *RORC* encodes the proteins RORγ and RORγt, hormone receptors that upon stimulation function as transcription factors. Low expression of RORγ has been associated with poorly differentiated tumors and negative outcome of aggressive basal-like breast cancer and bladder cancer, in agreement with our findings on PDAC [31-33]. Several studies indicate that targeting of RORγ and RORγt may be promising as a new

immunotherapeutic approach [25, 31-33]. Cancer cells are able to activate TLR3 and TICAM-1 to increase release of cytokines and chemokines that stimulate immune suppressive cells leading to tumor progression [29, 30, 34]. A phase 1 study investigated a TLR3 agonist and dendritic cell-based vaccine, and found an increased median survival of 2.8 months [35]. Future studies should evaluate whether the individual RNA expression profile is able to predict response to certain therapeutic approaches, for instance immune therapy. This could be based on digital mRNA oncogenic profiling, the approach used in the present study, including an EUS-FNB cohort in addition to the surgical cohort. Only one previous study used a similar approach, examining 13 EUS-FNBs with PDAC and identifying possible targets for combined immunomodulatory therapeutics [12].

Fourteen genes were expressed significantly higher in Group B, related to cancer cell proliferation and cell cycle regulation, in agreement with Kalimuthu et al., who reported that Group B was similar to the subtypes known as basal-like, squamous or QM-PDA [3-6]. Also the present study found that 85% of the basal-like tumors in the EUS-FNB cohort were Group B subtype. Moffitt et al. reported that basal-like tumors had faster growth rates than the classical subtype [4]. Collisson et al. reported a higher proliferation in QM-PDA tumors [3]. QM-PDA/squamous/basal-like PDACs had a worse outcome compared to classical/pancreatic progenitor, immunogenic, ADEX/classical subtypes [3-5]. Early results from the COMPASS trial suggest that fist-line chemotherapy is associated with significantly better outcome in the classical subtype, whereas basal-like tumors tend to be more resistant [24]. We found *ANGPTL4*, *ENO1*, *PLOD*, *PTGS2*, *TNC*, and *UBE2C* among the genes that were upregulated in Group B, some of which are under scrutiny for targeted therapy [36-41].

While the high degree of interobserver agreement testifies to the applicability of the morphological classification system proposed by Kalimuthu et al., it has several weaknesses. While the quadrant-based approach considerably facilitates scoring, the fact that scoring is done irrespective of the tumor area may be a limitation. The patterns in small tumor areas have a disproportionally strong impact on the overall scoring of the tumor. In addition, the “size” of a tumor area in a section may be entirely fortuitous, determined by the way the specimen slices were divided to fit into the tissue cassettes. A small rim of tumor along the edge of tissue block has as much impact on the overall score as a block consisting mainly of tumor. While identifying the dominant tumor pattern seems fairly straightforward, several practical issues make this at times challenging. First, it is not clearly defined whether “dominance” is determined by the area occupied by tumor of a certain pattern or

by the number of tumor cells. As cancer cell density often varies significantly from one spot to the next, it is difficult to compare the “burden” of tumor of various morphologies within a given quadrant. Furthermore, the presence of small cancer cell clusters or abortive glands less conspicuous than larger glandular formations are easily underestimated at low power. In order to identify all of them, one needs higher magnification, but then the quantification, *i.e.*, assessment of the proportion with respect to the remainder of the tumor, becomes difficult. It is tempting to speculate whether artificial intelligence could be used for this quantification in the future.

The Kalimuthu classification takes into account morphological heterogeneity by semiquantitative assessment of the different morphologies in a given tumor. By translating this into a binary system, the subtypes themselves do not reflect the wide morphological variety that exists in PDAC but serve as a framework that may ease the translation into clinical practice. The RNA-based classifications with discrete categories, including morphological subtyping, are probably also somewhat rigid, and one should probably rather think of a "molecular grading" system, with each PDAC having more or less of either subtypes (i.e. a sliding scale between the extremes of pure classical and pure basal-like) [42]. This approach is supported by Kalimuthu et al.’s and our own data, showing a high level of morphological heterogeneity within most of the PDACs. These findings are in line with studies based on single-cell sequencing, showing that basal-like and classical tumor cells frequently co-exist [23, 43].

Regarding the morphological subtyping as per Kalimuthu, it should be mentioned that the term “non-glandular” may appear a bit confusing, as tumors categorized as “composite” do often show lumina. A further challenge lies in the change in growth pattern that not uncommonly occurs when tumor cells invade particular microanatomic compartments. For example, a cancer of composite pattern in the bulk of the tumor mass, often assumes a conventional pattern when invading the duodenal muscle layer, a well-known phenomenon coined as "intestinal mimicry” [44]. Foci of so-called duct cancerization, that is, the growth of invasive PDAC along the wall of preexisting pancreatic ducts often present a cystic-papillary pattern, irrespective of the pattern in the remainder of the tumor mass. While these observations likely represent cancer “plasticity”, i.e. the reversible transition from one transcriptional subtype to the other, it is not clear whether this has the same oncological implications as the tumor phenotype outside these microcompartments.

In conclusion, our data indicate that the morphological classification as per Kalimuthu et al. is reproducible and holds prognostic value, in surgical as well as EUS-FNB specimens. Using digital mRNA expression profiling, we identified key canonical pathways that are differentially regulated

when comparing the two morphological subtypes of PDAC. These findings may have therapeutic implications, as immunotherapeutic strategies may be more promising in at least some patients with Group A subtype. Future studies should evaluate the potential of immune therapy with special emphasis on this subtype of PDAC, and these could also include the use of EUS-FNB specimens.

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Legends

Fig. 1. Morphological subtyping of 108 consecutive, chemotherapy-naïve, surgically resected pancreatic ductal adenocarcinomas (PDACs) as per Kalimuthu. **A**. Bar chart visualizing the morphological pattern composition of each of the 108 tumors (in % of all tumor areas of a given tumor). Each bar represents one tumor. **B–E**. Risk of underestimation of Group B. **B**. At low magnification, the presence of larger glandular tumor formations characteristic of the Group A subtype is conspicuous (H&E). **C**. Higher magnification is required to identify small tumor cell clusters with a solid or abortive glandular pattern consistent with Group B, and to distinguish them from disrupted remnants of non-neoplastic parenchyma. **D–E**. Immunohistochemical staining for broad-spectrum cytokeratin CKAE1/AE3 helps with identifying the less conspicuous non-gland-forming tumor component, but semiquantification, which requires an overview at low power, remains difficult. **F-I**. Survival data for the cohort of surgically resected PDACs, based on Kaplan-Meier and Cox regression analysis. **F**. Survival of the entire cohort. **G**. Survival related to morphological subtypes Group A vs. Group B. **H**. Survival related to the four morphological patterns as per Kalimuthu.**I**. Survival related to WHO differentiation grades.

Fig. 2. **A**. Hierarchical clustering based on gene expression analysis of 108 surgically resected pancreatic ductal adenocarcinomas (PDACs). The analysis is based on 53 genes, 39 and 14 of which were upregulated in Group A and Group B of PDAC. Bar A) indicates the four identified clusters: Cluster 1 (n=64), Cluster 2 (n=27), Cluster 3 (n=10) and Cluster 4 (n=7). Bar B) indicates the WHO differentiation grades: G1 (grey), G2 (green), and G3 (brown). Bar C) indicates the dominant morphological pattern: Conventional (orange), Tubulopapillary (green), Composite (blue), and Squamous (brown). Bar D) indicates the morphological subtype as per Kalimuthu: Group A (red) and Group B (blue). Case numbers are stated at the bottom of the heat map. **B-C**.

Survival data for the surgical PDAC cohort, based on Kaplan-Meier and Cox regression analysis. **B**. Survival related to the gene clusters 1-4. **C**. Survival related to the clusters 1+3 vs. 2+4.

Fig. 3. Immunohistochemical expression of four representative proteins encoded by genes differentially expressed when comparing Group A with Group B subtypes of PDAC. (A) CD5 is strongly expressed in immune cells and neoplastic glands in a case of the Group A subtype, but almost lacking in Group B (CD5 immunostaining). Scale bar = 250 μm. (B) CD5 levels are significantly increased in Group A compared to Group B. (C) Higher frequency of FOXP3-positive cells in a Group A case, compared to a Group B case (FOXP3 immunostaining). Scale bar = 250 μm. (D) FOXP3 tends to be higher expressed in Group A compared to Group B tumors. (E) Strong expression of CD10 in cancer-associated fibroblasts in a Group B, while a Group A cases are almost negative (CD10 immunostaining). Scale bar: 100 μm. (F) CD10 protein expression levels are significantly higher in Group B compared to Group A. (G) Strong expression of tenascin c (TNC) in cancer-associated fibroblasts and extracellular matrix in a Group B compared to a Group A tumor (TNC immunostaining). Scale bar: 250 μm (upper panel) and 100 μm (lower panel). (H) TNC protein expression levels are significantly higher in the Group B compared to the Group A. *Horizontal lines* indicate the median. * signifies $P < 0.05$.

Fig. 4. Morphological subtyping, survival analysis, and gene expression profiling of 88 patients who underwent endoscopic ultrasound-guided fine needle biopsy (EUS-FNB) with a diagnosis of pancreatic ductal adenocarcinoma (PDAC). **A**. EUS-FNBs showing Group A features (upper panel) and Group B features (lower panel) (H&E). **B**. Survival data for the EUS-FNB PDAC cohort, based on Kaplan-Meier and Cox regression analysis (Group A vs. Group B). **C**. Hierarchical clustering of genes in the EUS-FNB PDAC cohort, based on 21 genes, 15 and 6 of which are were upregulated in Group A and Group B subtypes of PDAC. **D**. Relative expression of the 21 genes, shown as mean values for each cluster, in the EUS-FNB cohort (n=88) and the surgical PDAC cohort (n=108). For each gene and each cohort, the cluster with the highest and lowest expression is highlighted in green and red. Clusters in-between are highlighted in white/light green/light red. **E**. Survival data for the EUS-FNB PDAC cohort (Cluster 1 vs. Cluster 2/4 vs. Cluster 3). Twelve patients who underwent surgery after pancreatic biopsy were excluded from the survival analyses.

Fig. 5. Expression profiling of genes characteristic of the basal-like and classical subtypes of

pancreatic ductal adenocarcinoma (PDAC) in 88 patients who underwent endoscopic ultrasound guided fine needle biopsy (EUS-FNB). **A**. Hierarchical clustering shows that most Group A tumors correspond to the classical subtype (28/34, 82%). Most Group B tumors, on the other hand, correspond to the basal-like subtype (35/54, 65%). **B**. Survival data for the EUS-FNB PDAC cohort, based on Kaplan-Meier and Cox regression analysis (basal-like vs. classical). Twelve patients who underwent surgery after pancreatic biopsy were excluded from the survival analyses.

Tables

Table 1 - Clinicopathological characteristics of the study cohort of 108 preoperative chemotherapy-naïve pancreatic ductal adenocarcinomas (PDACs), specified for the entire cohort and per subtype.

		Entire cohort		**Group A**		**Group B**	
		n	**%**	n	**%**	n	**%**
Subjects (n)		108	100.00%	48	44.44%	60	55.56%
Gender	Male	52	48.15%	22	45.83%	30	50.00%
	Female	56	51.85%	26	54.17%	30	50.00%
Age in years – average, (range):		68.8±8.7 (47-86)		69.75±8.62 (49-86)		67.05±8.47 (47-81)	
Tumor site	Pancreatic head	81	75.00%	37	77.08%	44	73.33%
	Pancreatic body	2	1.85%	1	2.08%	1	1.67%

	Pancreatic tail	25	23.15%	10	20.84%	15	25.00%
Resection type	Whipple	71	65.74%	32	66.67%	39	65.00%
	Distal pancreatectomy	27	25.00%	12	25.00%	15	25.00%
	Total pancreatectomy	10[a]	9.26%	4	8.33%	6	10.00%
Tumor stage [45]	T1	1	0.93%	1	2.08%	0	0.00%
	T2	3	2.78%	0	0.00%	3	5.00%
	T3	103	95.37%	46	95.84%	57	95.00%
	T4	1	0.93%	1	2.08%	0	0.00%
Nodal stage [45]	N0	26	24.07%	13	27.08%	13	21.67%
	N1	82	75.93%	35	72.92%	47	79.33%
Grade of differentiation [13, 14]	Well differentiated (G1)	20	18.52%	18	37.50%	2	3.33%
	Moderately differentiated (G2)	38	35.18%	24	50.00%	14	23.33%

	Poorly differentiated (G3)	50	46.30%	6	12.50%	44	73.33%
Perineural invasion	Yes	64	59.26%	27	56.25%	37	61.67%
	No	10	9.26%	5	10.42%	5	8.33%
	Not available	34	31.48%	16	33.33%	18	30.00%
Vascular invasion	Yes	59	54.63%	25	52.08%	34	56.67%
	No	19	17.59%	9	18.75%	10	16.67%
	Not available	30	27.78%	14	29.67%	16	26.67%
Pancreatic transection margin	Positive	2[b]	2.78%	1	2.08%	1[b]	1.67%
	Negative	106[c]	97.22%	47[c]	97.92%	59	78.33%
Shortest distance to one of the four resection margins[d]	0	36	33.33%	14	29.17%	22	36.67%
	0.5	20	18.52%	12	25.00%	8	13.33%
	1	24	22.22%	12	25.00%	12	20.00%

	1.5	3	2.78%	3	6.25%	0	0.00%
	2	12	11.11%	3	6.25%	9	15.00%
	2.5	1	0.93%	0	0.00%	1	1.67%
	3	11	10.19%	4	8.33%	7	11.67%
	Not available	1	0.93%	0	0.00%	1	1.67%
Mismatch repair status	Mismatch repair deficient	2	1.85%	0	0.00%	2	3.33%
	Mismatch repair stable	106	98.15%	48	100.00%	58	96.67%
Postoperative adjuvant chemotherapy	Gemcitabine[e,f]	50	46.30%	26	54.17%	24	40.00%
	Gemcitabine/S1[e]	18	16.67%	6	12.50%	12	20.00%
	Gemcitabine/capecitabine[g]	3	2.78%	1	2.08%	2	3.33%

	Gemcitabine <4 months[h,i]	11	10.18%	4	8.33%	7	11.67%
	None[j]	26	24.07%	11	22.92%	15	25.00%
Overall survival - median (months)		18.83±1.56 (95% CI; 15.31-25.82)		25.8+8.65 (95% CI; 15.34-38.28)		17.15±2.85 (95% CI; 12.75-19.78)	

a: Six total pancreatectomies were Whipple resections followed by left-sided resection, due to a positive lateral transection margin at frozen section. b: One patient had high grade dysplasia in the lateral transection margin, but not PDAC. c: One patient had a NET G1 at the lateral margin, but not PDAC. d: The resection margins are the anterior, posterior, superior mesenteric vein (SMV) and superior mesenteric artery (SMA) margins, according to Verbeke et al. [46]. e: Median duration was 6 months. f: One patient received gemcitabine plus nab-paclitaxel and one patient received 6 months of preoperative chemotherapy. g: Median duration was 4 months. h: Median duration was 2 months. i: Six patients received gemcitabine, three patients received gemcitabine/S1, and 2 patients received gemcitabine/capecitabine. j: Patients did not receive therapy because of poor performance or very old age. Two patients decided not to receive adjuvant therapy.

Table 2 - The top-20 canonical pathways identified in the Group A and Group B subtypes of PDAC using expression analysis of 800 genes.

Description	**Number of Genes in Overlap (k), gene name(s)**	**k/K**	**p-value**	**FDR q-value**
Group A subtype of PDAC				
Cytokine Signaling in Immune system	15 *CCR2*, *CD27*, *CD40LG*, *IL10RA*, *IL22RA1*, *JAK2*, *MMP1*, *PRLR*, *PSMB10*, *RASAL1*, *RORC*, *SHC2*, *SYK*, *TNFRSF11A*, *TNFSF17*	0.1215	2.33E-15	5.21E-12
Cytokine-cytokine receptor interaction	9 *CCR2*, *CD27*, *CD40LG*, *CX3CR1, IL10RA*, *IL22RA1, PRLR*, *TNFRSF11A*, *TNFSF17*	0.2361	5.47E-12	6.11E-9

Signaling by Interleukins	8 *CCR2*, *IL10RA*, *IL22RA1*, *JAK2*, *MMP1*, *PSMB10*, *RORC*, *SYK*	0.1201	1.83E-8	1.08E-5
TLR3-mediated TICAM1-dependent programmed cell death	3 *RIPK3*, *TICAM1*, *TLR3*	3.4722	1.93E-8	1.08E-5
Chemokine signaling pathway	6 *CCR2*, *CX3CR1*, *JAK2*, *PIK3CG*, *PRKACB*, *SHC2*	0.2201	3.74E-8	1.67E-5
TNFR2 non-canonical NF-kB pathway	5 *CD27*, *CD40LG*, *TNFRSF11A*, *TNFSF17*, *PSMB10*	0.3438	6.1E-8	2.27E-5
Adaptive Immune System	9 *CD40LG*, *CD74*, *ICOSLG*, *PRKACB*, *PSMB10*, *KLRB1*, *SLAMF7*, *SYK*, *ZAP70*	0.0764	1.03E-7	3.29E-5

Jak-STAT signaling pathway	5 *IL10RA*, *IL22RA1*, *JAK2*, *PIK3CG*, *PRLR*	0,2243	5.17E-7	1.44E-4
RIP-mediated NFkB activation via ZBP1	3 *RIPK3*, *TICAM1*, *TLR3*	1.2257	6.52E-7	1.62E-4
TICAM1, RIP1-mediated IKK complex recruitment	3 *RIPK3*, *TICAM1*, *TLR3*	1.0965	9.28E-7	2.07E-4
ZBP1(DAI) mediated induction of type I IFNs	3 *RIPK3*, *TICAM1*, *TLR3*	0.9924	1.27E-6	2.58E-4
Hemostasis	7 *CD2*, *CD74*, *JAK2*, *MMP1*, *PIK3CG*, *PRKACB*, *SYK*	0.0715	4.88E-6	9.07E-4
GMCSF-mediated signaling events	3 *JAK2*, *PRKACB*, *SYK*	0.5785	6.75E-6	1.16E-3
Cell adhesion molecules (CAMs)	4 *CD2*, *CD6*, *CD40LG*, *ICOSLG*	0.2090	1.03E-5	1.64E-3

Natural killer cell mediated cytotoxicity	4 *PIK3CG*, *SHC2*, *SYK*, *ZAP70*	0.2028	1.16E-5	1.7E-3
MAPK family signaling cascades	5 *JAK2*, *SHC2*, *PIK3CG*, *PSMB10*, *RASAL1*	0.1174	1.22E-5	1.7E-3
Innate Immune System	8 *CCR2*, *NLRC5*, *PRKACB*, *PSMB10*, *RIPK3*, *SYK*, *TICAM1*, *TLR3*,	0.0072	1.34E-5	1.76E-3
Intestinal immune network for IgA production	3 *CD40LG*, *ICOSLG*, *TNFSF17*	0.4340	1.62E-5	1.91E-3
Class I PI3K signaling events	3 *PIK3CG*, *SYK*, *ZAP70*	0.4340	1.62E-5	1.91E-3
Pathways in cancer	5 *GLI1*, *MMP1*, *PIK3CG*, *TRAF1*, *WNT2B*	0.1069	1.91E-5	2.14E-3

Genes not related to top 20 canonical pathways	7 *CD5*, *FOXP3*, *FUT4*, *LY9*, *MMP7*, *PDZK1IP1*, *P2RY13*	-	-	-

Description	**Number of Genes in Overlap (k), gene name**	**k/K**	**p-value**	**FDR q-value**
Group B subtype of PDAC				
FOXM1 transcription factor network	3 *BIRC5*, *CCNB1*, *CDK2*	0.5208	3.78E-7	8.44E-4
Validated targets of C-MYC transcriptional activation	3 *BIRC5*, *CCNB1*, *ENO1*	0.2639	3,00E-06	2.16E-3
Cell Cycle Checkpoints	4 *BIRC5*, *CCNB1*, *CDK2*, *UBE2C*	0.0951	3.04E-6	2.16E-3

APC/C-mediated degradation of cell cycle proteins	3 *CCNB1*, *CDK2*, *UBE2C*	0.2424	3.88E-6	2.16E-3
Estrogen-responsive protein Efp controls cell cycle and breast tumors growth	2 *CCNB1*, *CDK2*	0.8681	1.48E-5	6.59E-3
Phosphorylation of the APC/C	2 *CCNB1*, *UBE2C*	0.7313	2.1E-5	7.82E-3
Cyclins and Cell Cycle Regulation	2 *CCNB1*, *CDK2*	0.6042	3.11E-5	8.67E-3
APC/C:Cdc20 mediated degradation of Cyclin B	2 *CCNB1*, *UBE2C*	0.6042	3.11E-5	8.67E-3

Cyclin A/B1/B2 associated events during G2/M transition	2 *CCNB1*, *CDK2*	0.5556	3.68E-5	8.9E-3
Cell Cycle, Mitotic	4 *BIRC5*, *CCNB1*, *CDK2*, *UBE2C*	0.0071	3.99E-5	8.9E-3
Nicotinate metabolism	2 *NT5E*, *PTGS2*	0.4479	5.7E-5	1.16E-2
Mitotic Metaphase and Anaphase	3 *BIRC5*, *CCNB1*, *UBE2C*	0.0889	7.83E-5	1.4E-2
Cell Cycle	4 *BIRC5*, *CCNB1*, *CDK2*, *UBE2C*	0.0059	8.18E-5	1.4E-2
FoxO family signaling	2 *CCNB1*, *CDK2*	0.2833	1.44E-4	2.14E-2

TP53 Regulates Transcription of Cell Cycle Genes	2 *CCNB1*, *CDK2*	0.2833	1.44E-4	2.14E-2
Pathways in cancer	3 *BIRC5*, *CDK2*, *PTGS2*	0.0092	2.04E-4	2.72E-2
Cytokine Signaling in Immune system	4 *BIRC5*, *FLNB*, *IL11*, *PTGS2*	0.0047	2.07E-4	2.72E-2
HIF-1-alpha transcription factor network	2 *ENO1*, *NT5E*	0.2104	2.61E-4	3.2E-2
p53 signaling pathway	2 *CCNB1*, *ENO1*	0.2042	2.77E-4	3.2E-2
Transcriptional Regulation by TP53	3 *BIRC5*, *CCNB1*, *CDK2*	0.0082	2.87E-4	3.2E-2
Genes not related to top 20 canonical pathways	5 *ANGPTL4*, *ANLN*, *MME*, *TNC*, *PLOD2*	-	-	-