Transcriptional Profiling of Peripheral Blood Mononuclear Cells in Pancreatic Cancer Patients Identifies Novel Genes with Potential Diagnostic Utility

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Abstract

Background: It is well known that many malignancies, including pancreatic cancer (PC), possess the ability to evade the immune system by indirectly downregulating the mononuclear cell machinery necessary to launch an effective immune response. This knowledge, in conjunction with the fact that the transcriptome of peripheral blood mononuclear cells has been shown to be altered in the context of many diseases, including renal cell carcinoma, lead us to study if any such alteration in gene expression exists in PC as it may have diagnostic utility.

Methods and Findings: PBMC samples from 26 PC patients and 33 matched healthy controls were analyzed by whole genome cDNA microarray. Three hundred eighty-three genes were found to be significantly different between PC and healthy controls, with 65 having at least a 1.5 fold change in expression. Pathway analysis revealed that many of these genes fell into pathways responsible for hematopoietic differentiation, cytokine signaling, and natural killer (NK) cell and CD8+ T-cell cytotoxic response. Unsupervised hierarchical clustering analysis identified an eight-gene predictor set, consisting of SSBP2, Ube2b-rs1, CASB, FS, TBC1D8, ANXA3, ARG1, and ADAMTS20, that could distinguish PC patients from healthy controls with an accuracy of 79% in a blinded subset of samples from treatment naive patients, giving a sensitivity of 83% and a specificity of 75%.

Conclusions: In summary, we report the first in-depth comparison of global gene expression profiles of PBMCs between PC patients and healthy controls. We have also identified a gene predictor set that can potentially be developed further for use in diagnostic algorithms in PC. Future directions of this research should include analysis of PBMC expression profiles in patients with chronic pancreatitis as well as increasing the number of early-stage patients to assess the utility of PBMCs in the early diagnosis of PC.

Introduction

Pancreatic cancer (PC) remains a lethal malignancy with an overall five-year survival rate of only about 5% [1]. A significant contributor to the poor prognosis of PC patients is the failure to detect the tumor at an early and potentially resectable stage. It is estimated that only 8% of PC cases are diagnosed with tumors localized to the pancreas, while only 15–20% are considered resectable. Further, of those patients who have had their tumor resected, only 20% live more than 5 years post-diagnosis [2]. The most common cause of death post-resection is distant metastases; local recurrence is rare. Although studies showing prolonged survival in PC patients are rare, it is unquestionable that early detection and resection of PC, especially in a localized state, would likely yield a significant increase in survival.

Designing an early diagnostic test for PC however, presents a particular challenge owing to the relative rarity of the disease and the fact that the disease often remains asymptomatic until an advanced stage. Ideally, an early diagnostic test for PC would be minimally invasive, and relatively inexpensive, while being sufficiently sensitive to identify all or most cases of PC. When combined with a highly specific confirmatory test, it could potentially permit the early identification of patients with resectable disease.

CA19-9 is currently the only marker approved by the FDA for use in PC. However, while CA19-9 is useful as a marker of disease burden, it lacks both sensitivity and specificity (approximately 80% and 73% respectively) as a diagnostic marker [3–8]. Nonetheless, it remains the gold standard against which every potential biomarker
Study population

The study of blood-based biomarkers in PC was approved by the Institutional Review Board (IRB) at the University of Nebraska Medical Center (UNMC) (IRB number 209-00). Written informed consent was obtained from all patients and controls before enrollment into the study. For this study, 26 PC patients and 33 age, race, and gender matched healthy controls were recruited. A total of 35 samples were obtained from the PC and 33 from the healthy group. Baseline demographic information for both groups is detailed in Table 1.

The diagnosis of PC was based on a positive biopsy of a pancreatic mass or a metastatic lesion. The PC patients were further classified as localized (stage 1 and 2a) or non-localized (stage 2b and higher), pre-or post-surgery, and pre- or post-chemotherapy. A patient was classified as being post-surgery if they had undergone a pancreaticoduodenectomy before the sample was drawn. All other samples, including samples from patients who never had surgery during the course of their disease, were classified as pre-surgery. Any sample drawn before the patient had undergone any chemotherapy for PC was classified as pre-chemotherapy. If the patient had ever had chemotherapy for PC, regardless of whether or not that patient was undergoing chemotherapy at the time the sample was drawn, the sample was classified as being post-chemotherapy. For patients in whom multiple samples were drawn on different dates, all samples were used in the data analysis unless explicitly stated in the results section.

PC staging was based on one of four criteria: 1) pathological staging post-surgery, 2) MRI/CT/ultrasound staging, 3) endoscopic staging, or 4) biopsy of metastatic disease.

Isolation of total RNA from PBMCs

PBMCs were isolated from whole blood using the PharmLyse RBC lysis solution (BD, San Jose, CA) according to the manufacturer’s instructions. Total RNA was extracted using the Qiagen RNeasy RNA isolation kit (Qiagen, Valencia, CA, USA) and then converted to cDNA using the SuperScript II cDNA synthesis kit (Invitrogen, Carlsbad, CA) according to a previously published protocol [19].

cDNA microarray analysis of global gene expression profile of PBMCs

Microarray analysis was performed by the UNMC microarray core facility using established lab protocol on a Phalanx whole genome cDNA microarray containing 30,275 features probing for approximately 22,000 unique genes. A universal human reference (Stratagene, Cat: 740000, Cedar Creek, TX) was used as the reference against which all samples were normalized.

Statistical Analysis

Log2 transformation was applied to all ratios followed by normalization to “center” each array using Lowess smoother through BRB ArrayTools developed by Dr. Richard Simon and Amy Peng [20]. Any gene in which the percent of spots missing or filtered out exceeded 50% was excluded. Duplicate spots were not averaged but treated as separate genes for analysis. Mixed effects models were then used to determine which genes were significantly differentially expressed between the PC and the healthy control groups, allowing for a 10% false discovery rate. Diagnosis group (cancer vs. normal) was included in the model as a fixed effect and a random subject effect was also included to account for multiple samples per person.

Hierarchical clustering analysis of the arrays based on similarity of expression profiles was performed using the normalized and log-transformed data. Clustering was done using Gene Cluster version 3.0, using the “centered” Pearson correlation similarity metric and complete linkage clustering method, and visualized using Java TreeView.

Validation of microarray data by Q-RT PCR

The microarray results were validated by quantitative real time PCR (Q-RT PCR). All Q-RT PCR reactions utilized SYBR green based chemistry. For validation, six of the most differentially expressed genes: 3 up-regulated (ANKRD22, ANXA3, ARG1) and 3 down-regulated (FCER1A, GRAMD1C, and MS4A1) by microarray were chosen. Validation was done in a randomly selected subset of the original samples (submitted for microarray analysis) that included nine healthy controls and twelve PC patients. The fold-change in gene expression was determined by the 2^(-ΔΔCt) method using the same human reference RNA as that employed in the microarray. To determine the correlation between the microarray and Q-RT PCR results, we calculated the median fold change in expression (for a given gene) for PC vs. healthy controls, and compared it to the fold change seen by microarray to determine whether the gene was still differentially expressed in the same direction.

Correlation of gene signatures with clinicopathologic characteristics in PC

To determine if there is the differential expression of genes in PC patients correlates with patient characteristics, a mixed effects model was applied to the PC samples that were grouped according to the following criteria: surgical status (pre- vs. post-surgery), chemotherapy status (pre- vs. post-chemotherapy), history of type-II diabetes mellitus prior to the diagnosis of PC (present vs. absent), location of the tumor (head vs. body/tail), and stage of PC (localized vs. non-localized, and metastatic vs. non-metastatic). Significant genes were chosen based on an allowable false discovery rate of less than 10%. Stage 1a and 2a PC were
considered localized, while stages 2b, 3, and 4 PCs were considered non-localized, and stage 4 tumors were considered metastatic. For two patients recruited into the study, information on tumor stage, tumor location, and history of type-II diabetes mellitus could not be obtained.

Identification of a gene predictor set that distinguishes PC from healthy individuals

BRB-ArrayTools Version 3.8.0 was used to analyze all possible combinations of the 21,671 valid genes identified by microarray to determine whether a genetic signature could be identified that would distinguish PC patients from healthy controls with the optimum combination of sensitivity and specificity. The microarray data for 24 randomly chosen PC samples and 20 healthy controls was entered into the analysis. Genes to be selected for the predictor set were required to be significantly different between the PC and healthy control groups with a significance level of $p \leq 0.0001$ and with a fold difference expression between the two groups $\geq 1.5$. Cross validation of the gene predictor set was repeated 1 times K-fold ($K = 10$). The gene predictor set arrived at through these methods was analyzed by various methods, including Compound Covariate Predictor, Diagonal Linear Discriminant Analysis, 1-Nearest Neighbor, 3-Nearest Neighbors, Nearest Centroid, Support Vector Machines, and Bayesian Compound Covariate Predictor. Of these, the Compound Covariate Predictor gave the best predictive capabilities using the gene predictor set and consequently used.

Validation of the gene predictor set

Once the predictor set was established, it was validated in a second set of randomly selected PC and healthy samples. The statistician was blinded to the identity of the samples. Applying the cut-off obtained through the Compound Covariate Prediction method, the samples were classified as either “PC” or “non-PC”. The analyzer (M.B.) was then unblinded and the accuracy of the prediction determined by comparison with the actual diagnosis. We also applied the same equation to a subset of pre-chemotherapy pre-surgical PC patients to determine the ability of the predictor set to correctly classify patients into PC vs. non-PC. This is important as the influence of chemotherapy and/or surgery on the gene expression profile of PBMCs cannot be ruled out. Further, the latter group of patients represents the ideal patient population in whom the test, if validated would be applied in a clinical setting.

### Table 1. Demographics of PC Patients and Controls used in the study.

| Characteristic                          | PC            | Healthy Controls |
|----------------------------------------|---------------|------------------|
| **Samples**                            |               |                  |
| Number of patients                     | 26            | 33               |
| Number of PBMC samples                 | 35            | 33               |
| **Gender**                             |               |                  |
| Male                                    | 13 (50%)      | 6 (18.2%)        |
| Female                                  | 13 (50%)      | 22 (66.7%)       |
| Unknown                                 | 0 (0%)        | 5 (15.2%)        |
| **Age**                                |               |                  |
| Mean (±SD)                             | 64.4 (±9.0)   | 55.7 (±6.1)      |
| Unknown                                | 0 (0%)        | 5 (15.2%)        |
| **Race**                               |               |                  |
| Caucasian                              | 21 (80.8%)    | 25 (75.8%)       |
| Non-Caucasian                          | 3 (11.5%)     | 3 (9.1%)         |
| Unknown                                | 2 (7.7%)      | 5 (15.2%)        |
| **Stage at Diagnosis**                 |               |                  |
| Resectable (stage 1-2a)-patients recruited into study | 4 (15.4%) | N/A              |
| Resectable (stage 1-2a)-blood samples drawn | 6 (17.1%) | N/A              |
| Non-resectable (stage 2b or higher)-patients recruited into study | 20 (76.9%) | N/A              |
| Non-resectable (stage 2b or higher)-blood samples drawn | 27 (77.1%) | N/A              |
| Stage unknown-patients recruited into study | 2 (7.7%) | N/A              |
| Stage unknown-blood samples drawn      | 2 (5.7%)      | N/A              |
| **Surgical Status**                    |               |                  |
| Pre-surgical                           | 23 (65.7%)    | N/A              |
| Post-surgical                          | 12 (34.3%)    | N/A              |
| **Chemotherapy Status**                |               |                  |
| Pre-chemotherapy                       | 15 (42.9%)    | N/A              |
| Post-chemotherapy                      | 20 (57.1%)    | N/A              |
| Undergoing Chemotherapy at time of Sample Collection | 10 (28.6%) | N/A              |

Abbreviations: PC, pancreatic cancer; PBMC, peripheral blood mononuclear cells. doi:10.1371/journal.pone.0017014.t001
Results

After normalization and filtering of the microarray data, 21,671 genes remained for analysis. Of these, 383 genes were found to have a significant differential expression between PC patients and healthy controls (Table S1). Of these, 65 genes were observed to have a differential expression ≥1.5-fold between the two groups (Tables 2–3).

A hierarchical clustering of the microarray data identified two clusters of samples, shown in Figure 1 and in dendrogram form in Figure 2, a PC group and a healthy control group. Two PC samples however clustered with the healthy controls, while one healthy control fell into the cluster containing the majority (32/35) of the PC samples. Additionally, the gene expression profile of one PC sample did not cluster with either the healthy controls or the other PC samples.

Q-RT PCR Validation

Six of the most differentially expressed genes (ANKRD22, ANXA3, ARG1, FCER1A, GRAMD1C, and MS4A1) were chosen for validation by Q-RT PCR in a randomly selected subset of 21 PBMC samples (comprised of 12 PC samples and 9 healthy control samples from the original 68 used in the microarray). The median fold expression for five of them was in the same direction as that in the microarray, giving us a validation rate of 83%.

Correlation of PBMC expression profile with clinicopathologic characteristics

To determine whether a correlation existed between the PBMC gene expression profile in PC patients and clinically relevant patient characteristics, we divided the PC samples based on surgical status (23 pre-surgery vs. 12 post-surgery), history of chemotherapy (15 pre-chemotherapy vs. 20 post-chemotherapy), diagnosis of type-II diabetes mellitus prior to the diagnosis of PC (14 with a positive history vs. 19 with a negative history), location of the primary tumor (25 head vs. 8 body/tail), and stage of the PC at diagnosis (6 localized (Stage 1/2A) vs. 12 non-localized non-metastatic (Stage 2B/3) vs. 15 metastatic (Stage 4) PC). However, we did not observe any significant difference in gene expression between any of these patient groups applying the criterion of an FDR <10%.

Gene Predictor Set

We next investigated whether we could identify a minimal gene-predictor set that would accurately discriminate PC cases from healthy controls. To do this, 44/68 samples comprising 24 PC and 20 healthy control samples were randomly chosen. All 21,671

Table 2. Genes Shown to be at Least 1.5 Fold Upregulated in PBMCs of PC Patients.

| Gene symbol | Unique id | FDR   | Fold-change (PC/HC) | Gene Information and Normal Gene Function |
|-------------|-----------|-------|--------------------|------------------------------------------|
| TMEM22      | PH_hs_0038059 | 0.069 | 4.812              | Transmembrane protein 22, Function unknown |
| MMP8        | PH_hs_0024515 | 0.076 | 2.351              | Neutrophil protein used to degrade type I, II and III collagens |
| ARG1        | PH_hs_0025817 | 0.021 | 2.106              | Catalyzes the hydrolysis of arginine to ornithine and urea |
| DEFA4       | PH_hs_0000344 | 0.086 | 2.098              | A neutrophil protein thought to be involved in host defense |
| SLC27A3     | PH_hs_0025689 | 0.039 | 2.024              | Protein with acyl-CoA ligase activity for LCFA and VLCA |
| USH1C       | PH_hs_0023496 | 0.086 | 2.022              | May be involved in protein-protein interaction |
| FBXW12      | PH_hs_0035757 | 0.067 | 1.893              | Substrate-recognition component of SCF-type E3 ubiquitin ligase |
| CRISP3      | PH_hs_0024631 | 0.027 | 1.891              | A secreted protein found in the salivary gland, pancreas and prostate |
| USP30       | PH_hs_0026074 | 0.084 | 1.819              | Responsible for c-terminal deubiquitination |
| ANXA3       | PH_hs_0021146 | 0.039 | 1.793              | Important in cell growth/signaling and possibly anti-coagulation |
| HIST1H4I    | PH_hs_0029514 | 0.071 | 1.786              | A member of the histone H4 family |
| PROS1       | PH_hs_0003988 | 0.054 | 1.751              | Helps to prevent coagulation and stimulates fibrinolysis |
| GYG1        | PH_hs_0010438 | 0.015 | 1.722              | Involved in glycogen ababolism |
| ANKRD22     | PH_hs_0032205 | 0.063 | 1.676              | Ankyrin repeat domain-containing protein 22 |
| GADD45A     | PH_hs_0004630 | 0.025 | 1.596              | Responds to environmental stresses through activation of p38/JNK |
| F5          | PH_hs_0002589 | 0.010 | 1.587              | Coagulation factor V which circulates in plasma |
| KIF15       | PH_hs_0023756 | 0.062 | 1.577              | A member of the kinesin-like protein family |
| ST14        | PH_hs_0003679 | 0.031 | 1.551              | Degrades extracellular matrix and may play a role in cancer invasion |
| HIST1H2BG   | PH_hs_0034684 | 0.072 | 1.546              | A member of the histone H2B family |
|CLU          | PH_hs_0025525 | 0.038 | 1.545              | A secreted protein of unknown function |
|C19orf59     | PH_hs_0010615 | 0.093 | 1.523              | Speculated to be involved in regulating mast cell differentiation |
|ATP9A        | PH_hs_0019278 | 0.053 | 1.512              | Catalyzes ATP+H2O+phospholipid(lipid) = ADP+phosphate+phospholipid |
|FKBP5        | PH_hs_0000782 | 0.046 | 1.511              | Plays a role in immunoregulation, protein folding, and trafficking |
|ASGR2        | PH_hs_0000166 | 0.092 | 1.510              | Mediates endocytosis of plasma glycoproteins |
|SLC37A3      | PH_hs_0025758 | 0.025 | 1.500              | Sugar phosphate exchanger 3 (Solute carrier family 37 member 3) |

Abbreviations: PC, pancreatic cancer; HC, healthy controls; PBMC, peripheral blood mononuclear cells; FDR, false discovery rate.

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FCER1A was the only gene for which a positive correlation was not obtained. The results are depicted in Table 4.
genes for each of the samples were entered into the analysis. An eight-gene predictor set was obtained and comprised of SSBP2, Ube2b-rs1, CA5B, F5, TBC1D8, ANXA3, ARG1, and ADAMTS20. Using the Compound Covariate Prediction Method (CCPM), this predictor set gave a correct classification of PC vs. non-PC with an accuracy of 73%, providing a sensitivity of 71% and a specificity of 75%. The weights given to each gene using CCPM were –4.97, –4.83, –4.38, 4.43, 4.44, 4.53, 4.84, and 4.96 respectively with a threshold value of 38.98 such that if \( \sum w_i x_i \) was > threshold for a sample it was predicted as being from a PC patient (where \( w_i = \) gene weight, \( x_i = \log_2 \) gene expression intensity).

**Validation of Gene Predictor Set**

Using this eight-gene predictor set, classification of a sample as being either PC or a healthy control was attempted in a blinded manner using a sample set consisting only of the samples that were

| Table 3. Genes Shown to be at Least 1.5 Fold Downregulated in PBMCs of PC Patients. |
|-----------------|--------|--------|------------------------------------------------|
| **Gene symbol** | **Unique id** | **FDR** | **Fold-change (PC/HC)** | **Gene Information and Normal Gene Function** |
| CCR5            | PH_hs_0031237 | 0.021  | 0.66 | A chemokine receptor expressed by T cells and macrophages |
| MTAC2D1         | PH_hs_003705  | 0.058  | 0.66 | Tandem C2 domains |
| UBA5H5A         | PH_hs_0010839 | 0.052  | 0.66 | Promotes accrual of activated TCRs, EGFR and PDGFRB on cell surface |
| AKT3            | PH_hs_0023601 | 0.013  | 0.66 | An AKT serine/threonine kinase stimulated by PDGF, insulin, and IGF1 |
| PRF1            | PH_hs_0000291 | 0.097  | 0.65 | Perforin, non-specifically lyse target cells |
| PKI6A           | PH_hs_0020144 | 0.014  | 0.65 | Potent competitive inhibitor of cAMP-dependent protein kinase activity |
| PLEKH1A         | PH_hs_0014922 | 0.058  | 0.65 | Binds specifically to PtdIns3,4P2, highly expressed in the pancreas |
| AQF3            | PH_hs_0012796 | 0.003  | 0.65 | A water channel protein that also transports of nonionic small solutes |
| CD1C            | PH_hs_0000177 | 0.027  | 0.65 | Mediates the presentation lipid and glycolipid antigens to T cells |
| LRRB3C          | PH_hs_002135  | 0.020  | 0.64 | Leucine-rich repeat-containing protein 8C |
| GZMA            | PH_hs_0005055 | 0.027  | 0.64 | A T cell and NK cell serine protease, possibly needed for target cell lysis |
| SH2D1A          | PH_hs_0009133 | 0.008  | 0.64 | An inhibitor of SLAM self-association |
| PTPN4           | PH_hs_0023771 | 0.010  | 0.63 | Responsible for protein tyrosine dephosphorylation |
| CD5             | PH_hs_0003778 | 0.028  | 0.63 | May act as a receptor in regulating T-cell proliferation |
| PTPRCAP/ CORO1B | PH_hs_0009399 | 0.071  | 0.63 | PTPRCAP is a regulator of T- and B-lymphocyte activation. CORO1B regulates leading edge dynamics and cell motility in fibroblasts |
| FOSB            | PH_hs_0002354 | 0.001  | 0.63 | Dimerizes with JUN proteins to form the AP-1 complex |
| LCK             | PH_hs_0000240 | 0.009  | 0.63 | Essential to TCR-linked signal transduction and T-cell proliferation |
| LY9             | PH_hs_0024619 | 0.015  | 0.63 | May participate in adhesion between T lymphocytes and accessory cells |
| CD3G            | PH_hs_0000306 | 0.095  | 0.63 | CD3-gamma, important in T-cell response to antigen recognition |
| LEF1            | PH_hs_0003252 | 0.027  | 0.62 | Regulates T-cell receptor alpha enhancer function |
| CA5B            | PH_hs_0039389 | 0.001  | 0.62 | A zinc metalloenzyme that catalyzes the hydration of carbon dioxide |
| CD3D            | PH_hs_0005206 | 0.020  | 0.62 | CD3-delta, important in T-cell response to antigen recognition |
| TRAT1           | PH_hs_0031781 | 0.069  | 0.61 | Stabilizes the TCR/CD3 complex at the surface of T-cells |
| LAT             | PH_hs_0026523 | 0.019  | 0.61 | Recruits downstream proteins near the site of TCR engagement |
| KIAA0748        | PH_hs_0002788 | 0.085  | 0.61 | Hypothetical protein LOC9840 |
| CD3G            | PH_hs_0030882 | 0.004  | 0.60 | CD3-gamma, important in T-cell response to antigen recognition |
| GPR15           | PH_hs_0022672 | 0.059  | 0.60 | An orphan G-protein coupled receptor 2 |
| VSG9            | PH_hs_0033271 | 0.017  | 0.59 | Thought to assist in regulating T-cell dependent B-cell responses |
| TRAV20/ TRDV2   | PH_hs_0036583 | 0.068  | 0.58 | T cell receptor alpha variable 20 T cell receptor delta variable 2 |
| CD160           | PH_hs_0004672 | 0.005  | 0.58 | Associates with NK cell and CD8 T-cell cytolytic activity, found on T cells |
| DYSTK           | PH_hs_0010380 | 0.004  | 0.57 | Activates TP53 to induce apoptosis in response to DNA damage |
| EB2             | PH_hs_0040414 | 0.043  | 0.57 | Predicted to encode a GPCR related to the thrombin receptor, found on B cells |
| MS4A1           | PH_hs_0025653 | 0.015  | 0.56 | Helps in the development and differentiation of B-cells into plasma cells |
| CCR3            | PH_hs_0026576 | 0.061  | 0.55 | A receptor for C-C type chemokines |
| EDG1            | PH_hs_0009283 | 0.026  | 0.54 | Possibly involved in regulating endothelial cell differentiation |
| FCRER1A         | PH_hs_0000108 | 0.058  | 0.53 | An IgE receptor found on Mast cells central to allergic disease |
| EB2             | PH_hs_0000092 | 0.001  | 0.52 | Predicted to encode a GPCR closely related to the thrombin receptor |
| GRAM1C          | PH_hs_0037695 | 0.009  | 0.52 | GRAM domain containing 1C, Function unknown |

Abbreviations: PC, pancreatic cancer; HC, healthy controls; PBMC, peripheral blood mononuclear cells; FDR, false discovery rate.

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not used to create the predictor set (i.e. 24/68). In this blinded validation, using the equation derived above, the gene predictor set accurately predicted the diagnosis of PC with 73% accuracy, giving a sensitivity of 83% and a specificity of 64%.

In an attempt to further test the potential diagnostic utility of this gene predictor set, a new subset of samples, comprising of 12 PC samples obtained from patients who were both pre-chemotherapy and pre-surgery, along with an equal number of randomly selected healthy controls, were again blinded and analyzed to predict their classification. This time the eight-gene predictor was able to correctly classify these samples 79% of the time, giving an 83% sensitivity and 75% specificity of diagnosis.

Discussion

In recent years it has been repeatedly demonstrated that genetic expression in PBMCs is altered in the context of malignancy [13,14,21,22]. This observation of an altered PBMC genetic expression profile in cancer patients was first reported in diffuse large B-cell lymphoma and chronic lymphocytic leukemia and later extended beyond hematological malignancies through the analysis of PBMC expression profiling in patients with advanced renal cell carcinoma (RCC) [13–14]. In both hematologic malignancies and in RCC, it was reported that the variation in gene expression between patients with disease and healthy controls was much greater than the inter-sample variation observed for the healthy patients alone, suggesting that PBMCs could be useful surrogate markers with potential diagnostic and prognostic applications in cancer. Further, in RCC, it was shown that an 8-gene classifier set developed from the differentially expressed genes could predict the diagnosis of malignancy with 100% accuracy [14].

Recently, Huang et al. have reported that a differential gene expression profile does exist in PBMCs of PC patients [22]. While this study also used microarray and Q-RT PCR validation to establish differential genetic expression in the peripheral blood of PC patients, its purpose was to establish potential biomarkers that could differentiate newly diagnosed diabetic patients with PC from diabetics without PC. While the study authors reported that 48 genes were differentially expressed between PC patients and healthy controls by microarray, only 8 samples were used in each of the two groups and they provided no further information regarding these genes. The smaller sample size and a lack of blinded validation further contrast this study with the present report. Additionally, we did not find any significantly differentially expressed genes based on history of either prior surgery or chemotherapy, history of type-II diabetes mellitus, or stage of PC in our study. Importantly, the study by Huang et al. utilized GAPDH as the housekeeping gene against which the expression of every gene was normalized. In our study, however, we noted that GAPDH was one of the most significantly overexpressed genes in PBMCs of PC patients. Upregulation of GAPDH has also been reported in several malignancies including ovarian, thyroid, hepatocellular and pancreatic cancers [23–26]. The choice of the ideal internal reference gene in studies investigating potential clinical biomarkers by microarray remains an important question that will need to be addressed in future studies.

This present study represents the first in-depth analysis of the transcriptome of PBMCs from patients with PC compared to healthy controls, and only the third instance of such profiling for solid tumors in general. Establishment of such differential expression has the potential to yield a rich compendium of potential genes for further pursuit as novel diagnostic or therapeutic targets. Further, the gene networks identified in our study offer novel insights into the deregulation of the immune system in PC (Figure 3). With the fact that only 15–20% of PC patients are diagnosed with resectable disease and given the stubborn resistance of the malignancy to chemo and radiotherapy, early detection of the disease offers the greatest hope for an immediate impact on improving patient prognosis.

The potential for PBMC differential gene expression profiling, or of a pre-determined gene predictor set established from it, to be useful for early diagnosis of PC is theoretically quite high; especially when it is considered that the two most likely mechanisms underlying this differential expression are the immune system’s recognition of the cancer and the evasion of the immune system by the cancer. While other biomarkers, such as CA19-9, are released from the cancer cells and thus rise with...
increasing tumor burden, differential expression in PBMCs may begin, at least partially, as soon as cancer immunogenicity or immune evasion is established. Immune system evasion has been shown to be initiated as early as pre-malignant disease in PC, thus supporting the premise that analysis of differential gene expression in immune cells may offer the ability to detect a neoplastic lesion even before it gains invasive capabilities [27].

While this study itself does not attempt to look at the early diagnostic capabilities of PBMCs, the results obtained from the work are a necessary first step towards a multiplexed assay based on alteration of gene expression in PC for potential application in high-risk groups [28]. The fact that an 8-gene predictor set was able to establish a sensitivity of 83% with a specificity of between 64 and 75% in a blinded set of samples is promising and will need to be validated in a large sample set. While the number of samples is too small to perform any further detailed analysis, the fact that the sensitivity for the gene predictor set did not decrease when applied to PC patients prior to chemotherapy or surgery points toward the potential utility of this 8-gene predictor set in a diagnostic setting, the main area in which CA19-9 is lacking [4–8]. Additionally, PBMC gene expression analysis is no more invasive of a test than CA19-9, both being amenable to a simple venopuncture, and the overall analysis need not be substantially more expensive than current clinical methods for testing CA19-9.

### Table 4. Median fold change (PDAC/Normal) of selected genes chosen for validation by Q-RT PCR.

| Gene Name | Microarray | Q-RT PCR |
|-----------|------------|----------|
| ANKRD22   | 1.68       | 1.16     |
| ANXA3     | 1.79       | 1.71     |
|ARG1       | 2.11       | 1.95     |
|FCER1A     | 0.53       | 1.24     |
|GRAMD1C    | 0.52       | 0.49     |
|MS4A1      | 0.56       | 0.62     |

Microarray results were validated by quantitative real time PCR (Q-RT PCR) using SYBR green based chemistry. 3 genes shown to be up-regulated by microarray (ANKRD22, ANXA3, ARG1) and 3 down-regulated (FCER1A, GRAMD1C, and MS4A1) genes were validated in a randomly selected subset of samples that included 9 healthy controls and 12 PC patients. The fold-change in gene expression was determined by the 2^(-ΔΔCT) method using the same human reference RNA as the standard employed in the microarray. Correlation between microarray and Q-RT PCR results were determined through calculation of the median fold change for the PDAC and healthy samples by Q-RT PCR and comparing it to the fold change seen by microarray to determine whether the gene was differentially expressed in the same direction using both methods. doi:10.1371/journal.pone.0017014.t004

![Figure 3. Potential effect of the differential genetic expression of PBMCs.](image)

All genes shown were found to be down-regulated greater than 1.5 fold. The respective amount of differential expression per gene as well as the stated function can be found in Table 3. The differential expression of these genes indicate that there is a global decrease in cell number, activation, and effectiveness of the adaptive immune system in patients with PDAC that may have a significant effect on both their morbidity and mortality. Dashed lines indicate the association of cells while solid lines indicate the differentiation or proliferation of a particular cell type. Numbers represent individual points of interaction between the genes and the immune differentiation and response pathway: 1, Presentation of antigen to Th0 cells; 2, Differentiation of Th0 cells down the Th1 or Th2 pathway; 3, Immune cell proliferation; 4, Stimulation of cytotoxic T-cell activity by Th1 cells; 5, Stimulation of humoral immunity by Th2 cells; 6, Recognition and response to target cells by cytotoxic T lymphocytes (CTL); 7, Differentiation of naive B-cells; 8, Lysis of target cells by CTLs. Letters represent individual cell populations: a, Cytotoxic T-lymphocytes (CTL); b, B-cells. Decrease in genes associated with points a and b may represent a decrease in their respective associated cell’s population. doi:10.1371/journal.pone.0017014.g003
If only the 8-gene classifier set is used for analysis, PBMC testing could be accomplished through the use of mini-cDNA microarray chips or through multiplex PCR reactions, both of which are clinically viable and would be fairly simple to add to the repertoire of tests provided by a standard clinical lab. Beyond the diagnostic potential of this PBMC differential expression profile, the normal functions and direction of differential expression of each of the genes, especially the 65 that were ≥1.5 fold differentially expressed, hints at potential pathophysiologic mechanisms. 18/65 genes have the potential to directly decrease T-cell proliferation, T-cell receptor signaling, or cytotoxic T-lymphocyte (CTL) cytoxicity while four can directly modulate a decrease in B-cell activation/differentiation or signal a decrease in the number of circulating B-cells. Three of the genes can directly decrease the cytotoxicity of NK cells, and two can decrease macrophage response. Taken together, the results of our study suggest that PC is characterized by a significant decrease in the ability of the immune system to respond to non-self antigens, including tumor associated antigens, as summarized in Figure 3. A partial hint about the mechanisms underlying this immune compromise may come from the observed upregulation of ARG1, observed to be upregulated more than 2-fold in PBMCs of PC patients. An expression of ARG1 is closely associated with an increase in the presence of myeloid derived suppressor cells (MDSCs) [29]. MDSCs are classically known to decrease CTL response, mostly through destabilization of T-cell receptors and decreased expression of certain CD3 subtypes, ultimately leading to CTL apoptosis. However, MDSCs are known to specifically cause the down regulation of CD3Z, which was not shown to be differentially expressed in PBMCs analyzed in this study [29]. Additionally, MDSCs are known to cause a funneling of the immune system away from cellular immunity and toward humoral and allergic-response immunity, a property that is not clearly represented in the PBMC differential expression data. Conversely, it appears (from the alteration in gene expression) that the number of circulating B-cells is decreasing while both FCER1A, a receptor central to allergic response, and MS4A1, which plays a role in B-cell to plasma cell differentiation, are down regulated. Thus, while MDSCs may play a role in modulating the differential expression seen in PBMCs of PC patients, they likely operate in concert with other mechanisms to affect a down-regulation of both the body’s cellular and humoral immune response machinery.

A comparison of the gene expression profile observed in our study with that reported in other diseases revealed little similarity with other benign (pre-ecplasia, rheumatoid arthritis (RA), and chronic pancreatitis (CP)) and malignant diseases (RCC). In total, 6 genes (CD160, GOLGA3B, RABGAP1L, MMP9, CRISP5, and ARG1) that were shown to be differentially expressed in PBMCs of patients with pre-ecplasia were also differentially expressed in PBMCs of PC patients, with 4 (CD160, MMP9, CRISP5, and ARG1) being differentially expressed in the same direction (1% commonality) [10]. Twelve genes (BTG2, CCND3, CD151, CD7, CLU, CTSB, KLK1, SPN, GSTO1, PCMT1, PRDX6, and PRFI) that were shown to be differentially expressed in PBMCs of patients with RA were also differentially expressed in PBMCs of PC patients, 8 of which (CCND3, CD151, CLU, CTSB, GSTO1, PCMT1, PRDX6, and PRFI) were in the same direction (2% commonality) [11]. Two genes (PDE3B and GADD45a) were also differentially expressed in PBMCs of PC patients, neither of which being differentially expressed in the same direction (0% commonality) [12]. However, there was no similarity in the list of significantly differentially expressed genes between PC and RCC [14]. These results strongly suggest that the gene expression observed in the present study is highly specific to PC, and thus increases the potential applicability of differential expression profiling of PBMCs as a diagnostic tool in PC.

To investigate the possibility that the gene expression profile in PBMCs is a reflection of the genes expressed in the PC tissue itself, we also investigated if there was a similarity between the differentially expressed genes identified in our study and those identified by microarray analysis in pancreatic primary tumors. In total it was found that, of the 383 differentially expressed genes found in PBMCs of PC patients, only 4 (ADAM9, IMP3, BTG2, and G6PD) were also shown to be differentially expressed in PC primary tissues, with 2 (ADAM9 and BTG2) being differentially expressed in the same direction (0.5% commonality) [30]. Thus it appears that, in general, the gene expression in PBMCs does not mimic that in the primary tumor.

Further, if circulating tumor cells from the pancreas were the cause of the differential expression profile seen in this study, it would be expected that genes normally expressed in pancreatic cells but not peripheral blood cells would be identified by microarray. While this was the case for USH1C, CRISP3, and USP90, all genes that are expressed at low to moderate levels in the pancreas but not expressed normally in the peripheral blood, PLEKHAI1, a gene that is normally highly expressed in the pancreas but only expressed at very low levels in the peripheral blood, was shown to be down regulated in our samples, adding to the evidence that the differential expression we report truly is from PBMCs.

In conclusion, we have shown that a differential gene expression profile exists in PBMCs of patients with PC. Further, an 8 gene classifier set has been established which provides, in a blinded subset of our samples, an improved sensitivity over CA19-9 with a similar specificity [3,4,6,28]. Significantly, there was no decrease in sensitivity when employing samples from patients prior to any form of chemotherapy or surgery. Comparison with other studies points toward this differential expression profile as being specific to PBMCs and particularly to PC. Additionally, the differential gene expression seems to represent a systemic compromise of both cellular and humoral immunity, although it does not point toward one particular underlying mechanism.

Based on these results, future research is needed to establish the various mechanisms behind PC-induced differential PBMC genetic expression and how much effect this differential expression actually has on the body’s immunologic capabilities. Further, the 8 gene classifier set must be tested in an expanded set of both healthy controls and PC patients as well as in a set of non-PC patients with benign/malignant disease to clarify its sensitivity and specificity. PC sample selection for such a study should be biased toward early stage patients to elicit the diagnostic capabilities of PBMC differential expression in the patient population in which it has the greatest likelihood of having a positive impact on patient outcome. Due to the difficulty of attaining ample specimens from early stage patients, preliminary study of early stage PC diagnosis through PBMC expression analysis may be first carried out in a spontaneous PC murine model which recapitulates the preneoplastic and early neoplastic processes seen in human PC as a proof of concept [31,32]. Upon conclusion of such a murine study, resources could then be expended in the recruitment and testing of enough early stage human PC subjects for ample analysis to be conducted. Once PBMC expression has been diagnostically validated in an expanded sample set, the gene set could also be used to characterize potential prognostic abilities of the PBMC differential expression in PC.

Though further studies are necessary to contingently state the diagnostic and prognostic potential of PBMC gene expression profiling in general and of the 8 gene PC classifier set in particular,
our current results are promising and point toward the potential for peripheral blood mononuclear cells to be highly efficacious tools for improving the prognosis of one of the world’s deadliest cancers.

**Supporting Information**

**Table S1** Genes Shown to be Statistically Differentially Expressed in PBMCs of PDAC Patients (FDR < 0.10). Global expression profiles of peripheral blood mononuclear cells from 26 pancreatic ductal adenocarcinoma (PC) patients and 33 age, race, and gender matched healthy controls were compared by whole genome microarray. After normalization, filtering, and statistical analysis, 383 genes were found to be significantly differentially expressed (FDR < 0.10) between the two groups.

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**Author Contributions**

Conceived and designed the experiments: MJB, SC, SKB. Performed the experiments: MJB, SC, KM. Analyzed the data: MJB, SC, LS. Contributed reagents/materials/analysis tools: SKB, AS. Wrote the paper: MJB, SC, KM, LS, AS, RB, SKB. Provided clinical insight and information: ARS REB.

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