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Immunodeficiency in Pancreatic Adenocarcinoma with Diabetes Revealed by Comparative Genomics

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Abstract

Purpose—Pancreatic adenocarcinomas (PAAD) often are not diagnosed until their late stages leaving no effective treatments. Currently immunotherapy provides a promising treatment option against this malignancy. However, a set of immunotherapy agents benefit patients with many types of cancer, but not PAAD. Sharing the origin in the same organ, diabetes and PAAD tend to occur concurrently. We aimed to identify the impact of diabetes on immunotherapy of PAAD by conducting a comparative genomics analysis.

Experimental Design—We analyzed levels 3 PAAD genomics data (RNAseq, miRNAseq, DNA methylation, somatic copy number and somatic mutation) from TCGA and Firehose. The differential molecular profiles in PAAD with/out diabetes were performed by the differential gene expression, pathway analysis, epigenetic regulation, somatic copy number alteration and somatic gene mutation.

Results—Differential gene expression analysis revealed a strong enrichment of immunogenic signature genes in diabetic individuals including PD-1 and CTLA4 that were currently targetable for immunotherapy. Pathway analysis further implied that diabetic individuals were defective in immune modulation genes. Somatic copy number aberration (SCNA) analysis showed a higher frequency of amplification and deletion occurred in the cohort without diabetes. Integrative analysis revealed strong association between differential gene expression and epigenetic regulations, however seemed not affected by SCNAs. Importantly, our somatic mutation analysis showed that the occurrence of diabetes in PAAD was associated with a large set of gene mutations encoding genes participating in immune modulation.

Conclusions—Our analysis reveals the impact of diabetes on immunodeficiency in PAAD patients and provides novel insights into new therapeutic opportunities.

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Keywords

Pancreatic cancer; Gene expression; DNA methylation/epigenetics; Cancer vaccines; Immune response to cancer; Immunomodulation

Introduction

Pancreatic cancer is the 4th leading cause of cancer-related death in USA. In 2017, an estimate of 53,670 patients will be diagnosed with pancreatic cancer in United State(1). Early detection of pancreatic cancer is difficult since cancer-specific symptoms occur only in the advanced stage resulting in a low 5-year survival rate of 8%(1). Of all types of pancreatic cancer, pancreatic adenocarcinoma (PAAD) is the most common. Treatment of PAAD is still a major challenge and surgery is the only curative therapy. However, only 15~20% patients are suitable for resection and up to 80% of the individuals that undergo surgery will suffer relapse(2). Radiotherapy and chemotherapy have been shown to benefit individuals with PAAD and increase the overall survival rate; however survival is still very low(2). No therapeutic agent has provided long term benefit for patients who are not surgical candidates(2). Immunotherapy represents an exciting new anticancer therapy that recruits and activates the immune system to recognize tumor-specific antigens(3). Clinical trials of immunotherapy against PAAD have shown promising outcomes by increasing survival rate(4). Moreover, individuals with drug resistance were suitable for immunotherapy(5).

Diabetes is an endocrine disease ranking the 7th leading cause of death in USA. There is a significant association between diabetes and pancreatic cancer, although it is still under debate whether diabetes is a cause or a result of the malignancy(6). Current thinking regards diabetes as a risk factor for pancreatic cancer. Evidence to support this notion includes a large cohort study of 109,581 individuals hospitalized in Denmark showing that incidence ratio of diabetics developing pancreatic cancer is 2.1(7). The risk of developing pancreatic cancer is relatively higher for new onset diabetes, especially for older subjects(8). A meta-analysis conducted in 1995 including 20 case-control and cohort studies between 1975 and 1994 reported that the pooled relative risk of pancreatic cancer for diabetics to non-diabetics is 2.1(9). Another meta-analysis including 36 studies between 1996 and 2005 also demonstrates that diabetes is a risk factor for pancreatic cancer with the overall odds ratio of 1.8(10).

In contrast, other studies do not show that diabetes is a risk factor, but rather is a consequence of pancreatic cancer. This comes from the observation that in a majority of subjects with pancreatic cancer (56.1%), diabetes is diagnosed concomitantly or 2 years before the diagnosis of cancer(11). Insulin sensitivity and diabetes metabolic control is improved in pancreatic cancer patients 3 months following surgery(12). The pancreatic cancer cell line MIA PaCa2 could induce hyperglycemia in immunodeficient mice and the diabetogenic agent was identified as a 14 amino acid peptides from N-terminal of S100A8(13, 14).

Genomics-scale technologies foster advances in the identification of a molecular profile in PAAD subjects. Exome sequencing and copy number analysis reveals a list of mutations

aggregating into multiple molecular pathways(15, 16). In the mutational landscape, KARS (Lysyl-tRNA synthetase), TP53 (tumor protein p53), SMAD4 (SMAD family member 4) and CDKN2A (cyclim-dependent kinase inhibitor 2A) are the four most common mutated genes, of which KARS mutation has the highest frequency and is almost ubiquitous(15, 17). Molecular mechanisms involved in the mutational profiles include activating mutations of KRAS, TGF-β signaling, WNT signaling, SWI-SNF complex, NOTCH signaling, disruption of G1/S transition, ROBO/SLIT signaling, histone modification, DNA damage repair and RNA processing(16). DNA copy number variation resulted in the genetic loss of tumor suppressor gene and increase copy number of oncogenes (MYC (c-Myc), KRAS and EGFR (epidermal growth factor receptor))(18). Explicit studies of genomics profiles have attempted to uncover the molecular aberrations in PAAD, however, despite the well-known association of diabetes in PAAD, the differential molecular profiles in PAAD with/out diabetes remain unknown. In this study, we examine the molecular signatures and find that PAAD with diabetes is accompanied by immunodeficiency indicating potential challenges for immunotherapy in this specific subgroup.

Materials and methods

Data resource

The Cancer Genome Atlas (TCGA) sponsored by National Cancer Institute is publicly available resource depositing multi-dimensional cancer genomics and clinical data set. We downloaded PAAD clinical information, level 3 genomics data (RNAseqV2, miRNASeq, DNA Methylation and somatic mutation data) from TCGA data portal (https://tcga-data.nci.nih.gov/tcga/). SCNA data was downloaded from Firehose (http://gdac.broadinstitute.org/). The data used in this study was the updated as of 8/15/2016. Clinical information and DNA methylation data were downloaded by TCGA Assembler(19). miRNASeq were downloaded by tcga2stat(20). Individuals with PAAD that had a history of diabetes were regarded as PAAD with diabetes while individuals with PAAD but without a history of diabetes were considered PAAD without diabetes. An independent RNAseq dataset with accession number of GSE79668 was downloaded from GEO database. GSE79668 dataset was originally used to conduct the association study between gene expression and long-term survival in pancreatic adenocarcinoma patients(21). We also download another independent microarray dataset (GSE15932) from GEO database to further validate our result. This microarray dataset was originally used to study the blood biomarkers of pancreatic cancer associated with diabetes(22).

Clinical information analysis

Clinical information was analyzed in R (version 3.2.0) and SAS 9.3 (SAS institute). Fisher's exact test was used to test significance in categorical data and logistic regression for continuous variable. Survival curves for PAAD with/out diabetes were plotted by Kaplan-Meier method. The comparison of the survival curves was conducted by log rank test. A total of 38 PAAD with diabetes and 111 PAAD without diabetes patients deposited in TCGA data portal were used for the clinical information analysis.

Analysis of differential gene expression of RNAseq data and microarray data

Raw counts of gene expression from RNAseq deposited in TCGA data portal were used for the differential gene expression. The analysis was performed through edgeR package in R(23). edgeR examined the differential gene expression by accounting variability through an overdispersed Poisson model and moderating the degree of overdispersion by Empirical Bayes methods(23). In this study, CPM (count per million) was calculated through the program and only genes with CPM larger than 1 across at least 15 samples (10% of all samples) were considered. A generalized linear model plus likelihood ratio test were used to calculate the significance as well as the fold change (FC). The genes were considered as statistically significant when the adjusted p value was less than 0.05 and the absolute FC larger than 1.5. A total of 147 patients were analyzed, including 38 PAAD with diabetes and 109 PAAD without diabetes. We performed the same strategy to analyze the differential gene expression in GSE79668 dataset. For GSE15932 microarray dataset, we used Wilcoxon rank-sum test to conduct the differential expression of each probe.

Gene set enrichment analysis(GSEA) of RNAseq data

To investigate potential biological pathways in subjects of PAAD with/without diabetes, we downloaded normalized gene expression data from TCGA data portal with RSEM (RNA-Seq by Expectation-Maximization) values provided. The dataset for canonical pathways were downloaded from msigdb(24). The enrichment score as well as the significance were evaluated by GSEA 1.0(24). In this analysis, a total of 1320 gene sets were included. Only the gene sets with size not less than 15 genes were considered. A total number of 5000 random permutations were performed to calculate p value. The pathways with false discovery rate (FDR) q-value less than 0.05 were considered as statistically significant. A total of 147 patients were analyzed, including 38 PAAD with diabetes and 109 PAAD without diabetes.

Analysis of somatic copy number aberration between PAAD with/out diabetes

SCNA data was downloaded from Firehose and split into the sets of PAAD with/out diabetes respectively. GISTIC 2.0 was used to conduct SCNA analysis(25). GISTIC 2.0 was a revised computational program to identify somatic copy number alteration by investigating the frequency and amplitude of observed events(25). GISTIC 2.0 investigated the significance of the amplification or deletion of the regions of the genome. In this study, the genes within the significant genomic regions were further analyzed to examine the overlay with those significantly differentially expressed as identified from RNAseq.

Integration of gene expression and epigenetic change

miRNASeq data deposited in TCGA data portal provided the miRNA stem-loop expression level as rpmmm (reads per million miRNA mapped). In this study, a total of 147 patients were analyzed for miRNA stem loop expression, including 38 PAAD with diabetes and 109 PAAD without diabetes. To investigate the potential gene regulation by miRNA, we focused on miRNA (n_{miRNA}=44) with largest difference in PAAD with/out diabetes (here we selected absolute fold change larger than 1.2) and the significant differential gene selected from RNAseq. Since miRNASeq only provided the expression level of the stem loop, the

stem loop's expression level was considered as the mature miRNA. The relationship between miRNA and gene was analyzed by microRNA Target Filter module in Ingenuity Pathway Analysis (IPA). Pairs selected for further analysis showed i) the miRNA and gene in a negative relationship; ii) high prediction accuracy or iii) experimental evidence based on laboratory studies. The relationship between miRNA and gene was illustrated by network.

DNA methylation profile change could affect gene expression and this regulation was mostly modulated by methylation of CpG sites near the promoters. DNA methylation data was downloaded from TCGA data portal and average beta value of TSS200 (within 200 bp from transcription start sites) was calculated by TCGA Assembler(19). To integrate gene expression and DNA methylation profiles, we only focused on significantly differentially expressed genes as identified from RNAseq and then the gene's corresponding methylation profile. We conducted the differential correlation analysis by the transformed Pearson's correlation coefficient and permutation test(26). The details of the analysis were as follows: a) Pearson's correlation coefficients between gene and its corresponding methylation probe were calculated in PAAD with diabetes (R_{wi_dia}) and without diabetes (R_{wo_dia}) respectively; b) the Pearson's correlation coefficients were subjected to Fisher's z-transformation as

$$Z_{wi\_dia} = \frac{1}{2} \ln \left[\frac{1 + R_{wi\_dia}}{1 - R_{wi\_dia}}\right] \text{ and } Z_{wo\_dia} = \frac{1}{2} \ln \left[\frac{1 + R_{wo\_dia}}{1 - R_{wo\_dia}}\right] \quad (1)$$

c) differential correlation between PAAD with diabetes and without diabetes was calculated as

$$R_{diff} = \sqrt{\frac{N_{wi\_dia} - 3}{2}} * Z_{wi\_dia} - \sqrt{\frac{N_{wo\_dia} - 3}{2}} * Z_{wo\_dia} \quad (2)$$

Here, N_{wi_dia} was the samples size of PAAD with diabetes and N_{wo_dia} denoted samples size of PAAD without diabetes; Z_{wi_dia} and Z_{wo_dia} was the transformed z values for PAAD with or without diabetes derived from equation (1). The statistical significance of differential correlation was assessed by 5000 permutation test by randomly shuffling the samples and P value less than 0.05 was considered as statistically significant.

Analysis of somatic mutation data

Mutation annotation format (MAF) files deposited in TCGA data portal for somatic mutation were downloaded for the analysis. In this study, the mutation occurred in only one or two samples was regarded as rare mutation and filtered out for further analysis. The number of mutant samples for each gene in each group was counted. Fisher's Exact test was used to analyze the association between mutation status and the occurrence of diabetes in PAAD. The log odds ratio was calculated to assess the risk of having diabetes if mutation was present. To avoid the zero or infinity issue in odd ratio, we added 0.5 to each cell of the

table having zero cell count. Benjamini-Hochberg method was used to adjust the multiple hypothesis testing (27). The mutations with adjusted p value less than 0.2, indicating that the result is likely to be valid 4 out of 5 times, were selected for further analysis. A total of 145 patients were available for somatic mutation analysis, including 37 PAAD with diabetes and 108 PAAD without diabetes.

Results

Patient characteristics

To characterize PAAD patients with/out diabetes, we first analyzed the clinical indices of these two groups of patients including age at initial pathologic diagnosis, maximum tumor dimension, gender, race, ethnicity, history of other malignancy, pathologic stage, smoking and alcohol history (Table 1). Within this 147 patient cohort, we did not find significant associations between occurrences of diabetes with any of the factors. Interestingly, we found that there was a trend toward being significant between smoking status and the occurrence of diabetes in PAAD (Table 1, p value=0.058). Consistent with what is reported by other groups, we did not observe a significant difference in the survival between diabetic and non-diabetic patients (Fig. S1, p value=0.738)(28).

Differential gene expression profile in PAAD with/out diabetes

To investigate the differential gene expression pattern in PAAD with/out diabetes, we analyzed RNASeq data deposited in TCGA data portal by edgeR program(23). By setting the adjusted p value cutoff of 0.05 and the absolute fold change of 1.5, a total of 408 genes were significantly different (Fig. 1A, table S1). The number of genes over-expressed in diabetic subjects was almost two folds larger than that of down-regulated genes (Fig. 1A). The most significant gene was Thyroglobulin (TG) which was highly expressed in diabetic patients, showing a fold-change larger than 14. Diabetes is associated with various degrees of deterioration of thyroid function and the up-regulation of TG suggested thyroid dysfunction in PAAD with diabetes(29). We also observed that a large set of genes were strongly associated with immune modulation and some of them were the key genes targeted for immunotherapy. PD-1 (programmed death 1), an immunoinhibititory receptor expressed on various immune cells, including T cells, B cells, natural killer cells and tumor-infiltrating lymphocytes(30), was one of the immunotherapy targeted genes. In our study, we observed a highly up-regulation of this gene in PAAD with diabetes with FDR of 0.023 and fold change of 1.84 (Fig. 1B). Cytotoxic T-lymphocyte associated protein-4 (CTLA4), functioning as an immune checkpoint and another promising cancer immunotherapy target(31), was over-expressed in PAAD with diabetes with FDR of 0.045(fold change: 1.71) (Fig. 1C). Chemokine (C-X-C motif) ligand 12 (CXCL12), restricting immune cells migration and the recognition of cancer antigens by creating a network of dense stroma (32), was up-regulated in diabetic subjects (FDR: 0.045; FC: 1.70; Fig. 1D). Indoleamine 2,3-dioxygenase (IDO), whose expression was up-regulated in PAAD with diabetes (FDR: 0.045; FC: 2.03; Fig. 1E), is an enzyme to catabolize tryptophan into kynurenine which inhibits T cell activation and stimulates regulatory T cell differentiation(33).

The PANC-1 cell line was examined in vitro used conditions to mimic several aspects of PAAD with diabetes, the addition of 25mM glucose and10 nM insulin plus 0.4 mM palmitate bound to BSA. We found that this short 6 hr metabolic treatment in PANC-1 increased gene expression of PD-1, CTLA4, CXCL12 and IDO, key genes identified from TCGA cohort. However, while there was an increase, statistical significance was not achieved (Fig. S2).

In addition to TCGA cohort, two independent datasets (GSE79668 and GSE15932) with diabetes were also identified to provide additional pancreatic adenocarcinoma samples. From GSE79668 RNAseq dataset, PD-1, CTLA4, CXCL12 and IDO were all highly expressed in PAAD with diabetes, validating our observations from the TCGA cohort (Fig. 1B-1E). Even in the nucleated blood cells of individuals with pancreatic adenocarcinoma, the expression level of CTLA4 was significantly increased in diabetics (Fig. S3). IDO was also increased in diabetics, although it did not reach significant level (Fig. S3).

In addition to individual genes, the potential biological pathways affected by diabetes in PAAD were conducted by GSEA. Among 1320 canonical pathways, we observed a much larger number of pathways highly enriched in subjects of PAAD with diabetes (Fig. 1F). By setting significant FDR q value at 0.05, a total of 5 pathways were selected: hematopoietic cell lineage; primary immunodeficiency; T cell receptor signaling pathway; CD8 TCR pathway and NKT pathway (Fig. 1G). These 5 highly up-regulated pathways regulated immune cell development and function supporting the notion of dysfunction of immune system in subjects with PAAD and diabetes. Extending the number of pathways by setting FDR q value at 0.10, we found that 32 pathways were involved and these pathways were all up-regulated in diabetic subjects (Table 2).

Differential somatic copy number aberration in PAAD with/out diabetes

To explore the SCNA in PAAD with/out diabetes, we used GISTIC 2.0 to analyze the alteration of chromosome regions. A larger number of cytobands were significantly amplified in non-diabetic subjects, but only 8q24.13 and 18q11.2 were observed in the diabetic subgroup (Fig. 2A). Among the 22 chromosomes, significant copy number amplification was only observed in Chr1, 7, 8, 9, 12, 17, 18 and 19 (Fig. 2A). A total of 85 genes were within the chromosome regions with significant copy number amplification in the non-diabetic subjects; however, the number of genes for PAAD with diabetes was 4 (Fig. 2B). When these genes were overlaid with the significantly differentially expressed genes identified by RNAseq, 4 out of 85 genes (SPAG17, PGAP3, ERBB2, RMRP) within the amplification regions in non-diabetic subgroup showed the concordant expression pattern in RNAseq. This implied that the differential expression of these genes may be partially due to the copy number amplification (Fig. 2B). In PAAD without diabetes, a total of 18 chromosome regions were identified as deletions (Fig. 2D). Two of them (9p21.3 and 18q21.2) reached statistical significance in diabetic subjects (Fig. 2C). The chromosomes with significant deletion were Chr1, 4, 5, 6, 9, 12, 13, 16, 17, 18, 19 and 22 (Fig. 2C). The numbers of genes within the deletion in PAAD with/out diabetes were 5 and 2137 respectively, with 3 of them occurred in both groups (Fig. 2D). Thirty-four genes in the non-diabetic subgroup were also identified as statistically down-regulated, implying the gene

expression level may be partially due to the copy number variation (Fig. 2D). Although a small set of genes showed concordance between RNAseq and SCNA, regardless of amplification and deletion, the majority of the differentially expressed genes identified from RNAseq were not affected by SCNA, indicating the independence of gene expression and SCNA in PAAD with/out diabetes.

Integration of epigenetic and gene expression in PAAD with/out diabetes

Gene expression could be regulated by the expression of microRNAs (miRNAs). MicroRNAs are 19~24nt small RNA that could bind to 3′UTR of the gene to induce the degradation of mRNA. Various diseases have been reported to be associated with the dys-regulation of miRNA expression. In this study, we examined the potential regulation of gene expression by miRNA. The relationship between miRNA and its target gene was evaluated by microRNA Target Filter module in IPA. A total of 64 pairs were identified with 41 pairs having gene expression highly up-regulated in PAAD with diabetes (Fig. 3A). Among these pairs, hsa-miR-135-5p negatively regulated 7 genes. Each gene was negatively regulated by one or two miRNAs. There were nine genes of IGSF1, DCX, CACNA1A, CALN1, FAM23A, ZNF831, TRHDE, PPP1R16B and NR4A3 which were negatively regulated by two miRNAs (Fig. 3A). A set of genes responsible for immune modulation, such as CXCL12, TNFRSF13B (Tumor Necrosis Factor Receptor Superfamily Member 13B), IL16 (Interleukin 16) and CXCR5 (C-X-C Motif Chemokine Receptor 5), were potentially regulated by miRNA expression. These pairs of miRNAs and genes from IPA were all based on prediction and further laboratory experimental validation will be needed.

Another epigenetic factor affecting gene expression is DNA methylation. DNA methylation occurred in the CpG island of promoters would suppress gene expression. Similar to miRNA, abnormal DNA methylation can lead to disease development and progression. To examine the gene regulation by DNA methylation in PAAD with/out diabetes, the differential correlation analysis was performed by transforming Pearson's correlation coefficients and permutation test. The majority of the correlation between DNA methylation and gene expression in either diabetic or non-diabetic subjects was negative (Fig. 3B). A permutation test identified 13 genes having differential correlation with the corresponding methylation status (Fig. 3B). One of the genes was ITGB7 (Integrin Subunit Beta 7), which showed no correlation in non-diabetic subjects (r^2= -0.007), but a strong negative correlation in PAAD with diabetes (r^2= -0.631) (Fig. 3C). The other interesting gene identified was SPAG6 (Sperm Associated Antigen 6) which is regarded as a novel target for cancer immunotherapy(34). We observed a weak negative correlation (r^2= -0.287) in diabetic subjects, but a stronger correlation in non-diabetic subjects (r^2= -0.615) (Fig. 3D). The differential correlation pattern implies that diabetes status in PAAD affects the regulation of gene expression by DNA methylation, suggesting that the diabetes status should be carefully considered when considering the treatment option for PAAD.

Somatic mutation analysis in PAAD with/out diabetes

Gene function is affected not only by its expression level and epigenetic regulation, but also by mutation status. Analysis of somatic mutation showed that there were considerably more mutational genes in diabetic subgroup compared to non-diabetic subjects (Fig.4A). By

setting the cutoff of false discovery rate less than 0.2, we identified a list of 28 mutational genes (Fig. 4B). These mutations all occurred in diabetic subjects indicating diabetes status in PAAD was associated with frequent gene mutations (Fig. 4B). Of these mutational genes, one gene (MAP2K4: Mitogen-Activated Protein Kinase Kinase 4) had the highest frequency which occurred in 4 out of 37 diabetic patients (Fig. 4B). MAP2K4, a tumor suppressor, is a member of the mitogen-activated protein kinase family and responsible for signal transduction to regulate various cellular process including proliferation, differentiation and development(35). Genetic inactivation of MAP2K4 in pancreatic cancer has been observed(35); here we found that the mutation of this gene exclusively occurred in the diabetes subgroup (Fig. 4B). A total of four missense and one splice mutation were observed in this gene (Fig. 4C). Importantly, we also observed that several genes encoding receptors were mutated in diabetic subjects (Fig. 4B). IL4R (Interleukin 4 Receptor), a type I cytokine receptor, could bind to IL4 and/or IL13 to stimulate immune response by antibody production and macrophages activation(36). The critical role of IL4R in tumor biology, tumor immunology and immunosurveillance rendered this gene an effective target for cancer therapy, including immunotherapy(36). We observed that this gene was mutated in diabetic subjects implying the toughness of this therapy in PAAD with diabetes (Fig. 4B). There were three mutations occurred for this molecule with two missense and one deletion (Fig. 4C).

Discussion

Individuals suffering from diabetes are more likely to develop cancers of the liver, pancreas, endometrium, colon, rectum, breast and bladder(37, 38). Previously, studies have focused on the link between pancreatic cancer and diabetes, since these two diseases share their origin in the same organ. Although which of these two disease comes first when they are found in the same individual is still under active debate, it is clear that diabetes status impacts the clinical outcome by increasing tumor size and worsening the histological grade of the tumor(6, 39). Until this study, to our knowledge, the impact of genomics profiling in pancreatic cancer with/out diabetes was incomplete. Our study depicts these differential molecular alterations and provides a better understand for this complex disease.

Similar to other types of cancer, PAAD develops a set of mechanisms to avoid the recognition of the immune system(40). One of the mechanisms is over-expression of ligands to evade immunological checkpoints that may interrupt effector T cell responses(40). CTLA4 and PD-1 pathways are the two negative co-stimulatory pathways mediating immunosuppression in a diversity of cancer types, including melanoma, ovarian and lung cancers(41). In pancreatic cancer, over-expression of PDL-1 results in lymphocyte exhaustion, down-regulation of most MHC class I members, and is associated with shorter disease-free survival and overall survival(42). Moreover, PAAD is a heterogeneous disease involving types of molecular and cellular pathways. Bailey *et. al.* recently showed in a study of 456 PAAD subjects that the gene expression profiles fall into 4 subtypes: squamous, pancreatic progenitor, immunogenic and aberrantly differentiated endocrine exocrine(16). The immunogenic class was associated with an activated immune system including up-regulation of B cells, $CD4^+$ T cells, antigen presentation and $CD8^+$ T cells (16). From our differential gene expression and pathway analysis, we found that PAAD with diabetes was

strongly associated with immunomodulation by up-regulating numerous immune pathways and stimulating cytokine production. The up-regulation of the immune system pattern we observed was consisted with that found by Bailey et al(16). Since Bailey *et. al.* did not include diabetes as one of the factors in the study, we suspected that the immunogenic class was derived from the subgroup of PAAD subjects with diabetes. Thus, we put forth that it may be more biologically meaningful to separate diabetic subjects for optimization of medical management especially for immunotherapy.

Another mechanism that could contribute to the immunosuppression observed in this cohort is abnormality in the antigen presentation. Continuous generation of tumor variants and/or alteration of antigen processing machinery by increased frequency of mutations can result in an escape of the tumor cells from recognition by the immune system(43). In addition, cancer microenvironment, a complex tissue consists of tumor cells as well as stromal cells, extracellular matrices, vasculature and inflammatory cells, results in immunosuppression by preventing effective lymphocyte priming and suppressing infiltrating effector cells. Over-expression of CXCL12 stimulates fibroblast migration and proliferation and creates a dense network in cancer microenvironment to restrict immune cells migration to recognize cancer antigens (32). Up-regulation of IDO impairs immune clearance by creating a cancer microenvironment rich in immunosuppressive regulatory T cells but devoid of effector T cells (44). Besides CXCL12 and IDO, a large set of genes as well as mutations relevant to the antigen processing pathway and/or immune cell functions were observed in PAAD with diabetes. This provides additional insight into possible mechanisms of immune deficiency in this disease and proves the different biological behavior in diabetic subjects.

Discovery of immunosuppression in cancer has prompted the novel therapeutic approach of invoking the immune system to attack the tumor. Numerous immunotherapy treatments, including checkpoint inhibitors, cancer vaccine, adoptive therapy and monoclonal antibodies, have been proposed to cure pancreatic cancer(3). Some of these approaches have even been applied in clinical trials. However, these studies did not, or to a lesser extent, consider the co-occurrence of diabetes and the potential impact of diabetes for treatment(45-48). Based on our results, the immune deficiency caused by diabetes may influence the clinical outcomes. Two immunotherapy clinical trials against pancreatic cancer showed a partial response for anti-CTLA4 treatments(45, 48). Another phase I clinical trial of BMS-936559 using anti PDL-1 conducted in 2012 recruited 207 patients including 14 pancreatic cancers. This study showed an objective response for melanoma, renal-cell cancer, non-small-cell lung cancer and ovarian cancer, but not pancreatic cancer(46). These clinical outcomes might be improved if the study took diabetes occurrence into consideration, since our data has shown an abnormally higher expression of both CTLA4 (FDR: 0.045; fold change: 1.71) and PD-1(FDR: 0.023; fold change: 1.84) compared to the non-diabetic subjects. In addition, trials using vaccine immunotherapy and various monoclonal antibody treatments similarly resulted in a low percentage of response(49, 50). We could expect a high response rate to be observed if future trials could consider the presence of diabetes as one of the influential factors for immunotherapy.

In summary, for the first time to our knowledge, we conducted the differential molecular profile analysis in PAAD with/out diabetes and found that the disease features diverse modes

of genomic alteration, but not a single genetics component. Our findings through gene expression and pathway analysis showed a large set of up-regulated genes in PAAD with diabetes and the occurrence of diabetes was associated with immunodeficiency. Integration of gene expression and epigenetic changes reveal that regulation is at the levels of miRNA expression and DNA methylation. We analyzed the somatic mutation and found a high number of gene mutations in diabetic subjects and a large set of these mutated genes involved immune responses supporting the mechanism of immune deficiency in this subgroup.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Pancreatic adenocarcinomas (PAAD) and diabetes share their origin in the same organ and often occur concurrently. A list of immunotherapy agents deliver benefits for individuals with many types of cancer, but not PAAD. The reasons for this remain unclear. Here, we conduct the differential molecular profile analysis in PAAD with/out diabetes from the TCGA cohort, which reveals the impact of diabetes on immunodeficiency. To the best of our knowledge, this is the first comparative genomics study that provides novel molecular insight on the impact of immunotherapy for PAAD, which will help identify individuals who are most likely to benefit from treatment as well as facilitate in identification of optimal patient populations for immunotherapy clinical trials.

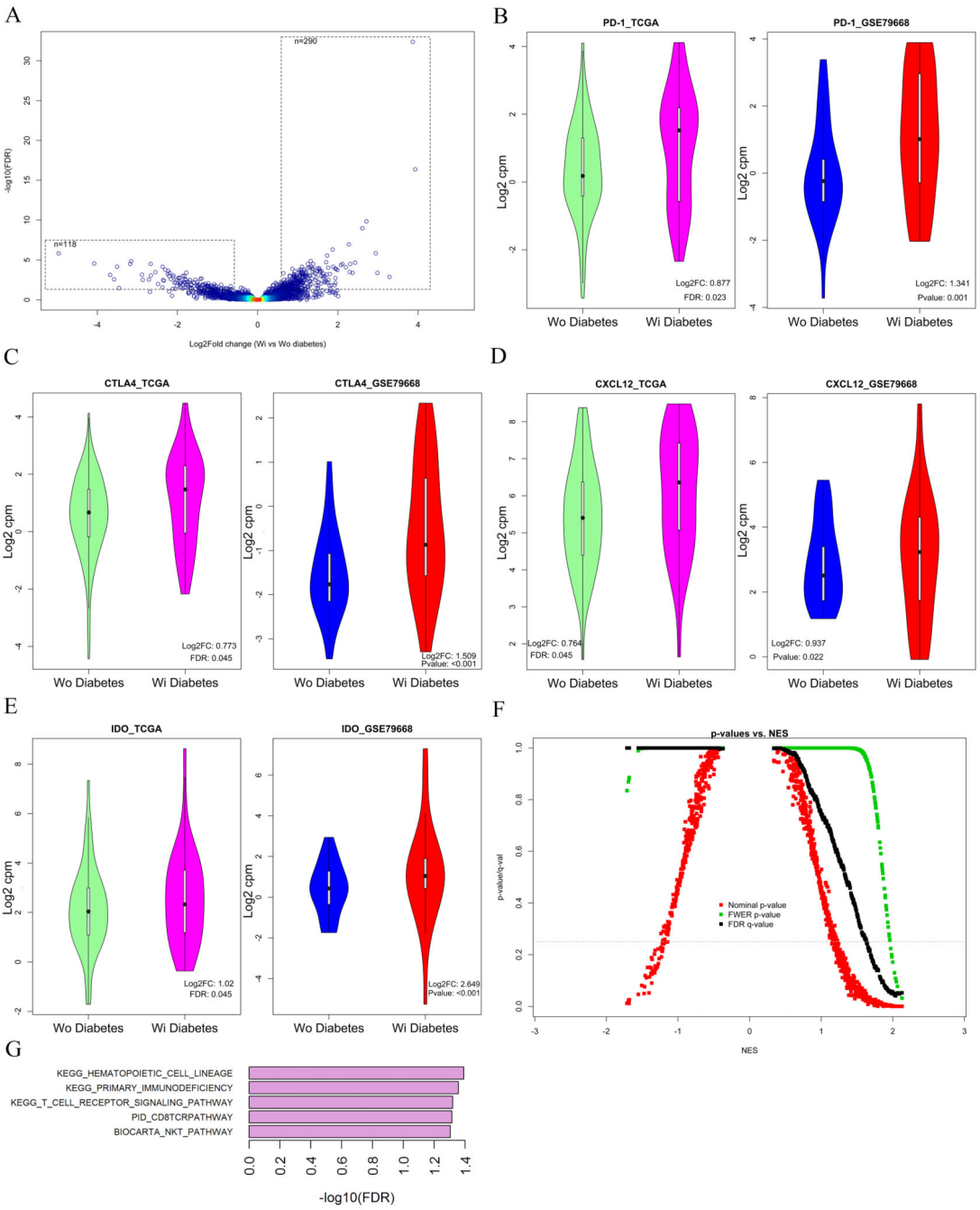

Figure 1.

Differential gene expression and pathway regulation in subjects with PAAD with/out diabetes. Overall pattern of differential gene expression in PAAD with/out diabetes is shown by volcano plot (A). Each point in the plot represents a gene and the gene over-expressed in PAAD with diabetes has log2 fold change larger than 0 which lies in the right-hand side of the plot (A). Genes in the top right corner are up-regulated with a small p value in PAAD with diabetes vs. without diabetes (A). Expression levels of PD-1 (B), CTLA4 (C), CXCL12 (D) and IDO (E) in PAAD with/out diabetes are provided in violin plot. In the violin plot,

the black dot represents the median; the thick white bar in the center represents the interquartile range and the black line represents the 95% confidence interval. A wider section of the violin plot means a higher density of points. Scatter plot of normalized enrichment score (NES) and nominal p-value (red), family wise-error rate p-value (FWER p-value; green), false discovery rate q-value (FDR q-value; black) is used to show differential pathway regulation in diabetes (F). Each point in the plot (F) represents a pathway from GSEA analysis. The pathway having a small p-value and large enrichment score in PAAD with diabetes lies in the right-bottom of the plot (F). Bar plot for significant pathways with FDR q-values less than 0.05 (G).

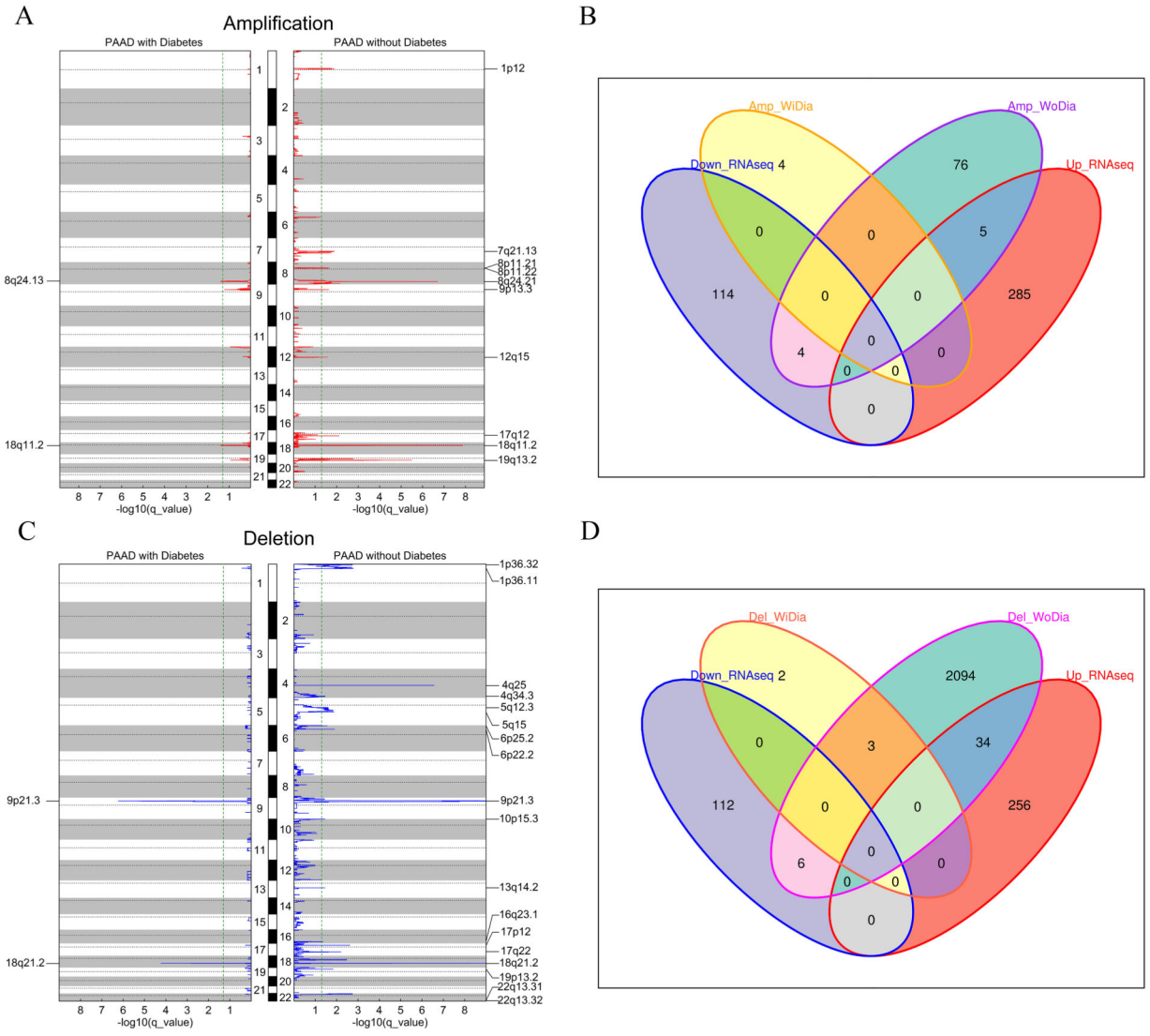

Figure 2.

SCNA analysis in PAAD with/out diabetes. Significance of SCNA was tested by GISTIC 2.0 and the genomic regions showing significant amplification are provided (A). The dash green line indicates the q-value less than 0.05 (A). A venn diagram is used to show the number of genes within genomic regions showing significant amplification as well as the overlay with significant genes identified from RNAseq (B). Each circle in the venn diagram represents one set and the number in the overlaid area represents the common genes between the sets (B). There are 4 genes (pink area) overlaid between amplification regions in non-diabetic subgroup of SCNA analysis and down-regulation in diabetic subgroup of RNAseq analysis (B). The genomic regions with significant deletion in PAAD with/out diabetes (C). Number of genes within the significant deletion and the overlay with differentially expressed genes from RNAseq (D). The number of genes within the deletion regions in the diabetic subgroup is 5 (the sum of number in “Del_WiDia” set) and in non-diabetic subgroup is 2137 (the sum of numbers in “Del_WoDia” set) (D). There are 3 genes within the significant deletion

regions which are common in both diabetic and non-diabetic subgroups (orange area) (D). There are 34 genes (blue area) overlaid between the deletion regions in non-diabetic subgroup of SCNA analysis and up-regulation in diabetic subgroup of RNAseq analysis (D).

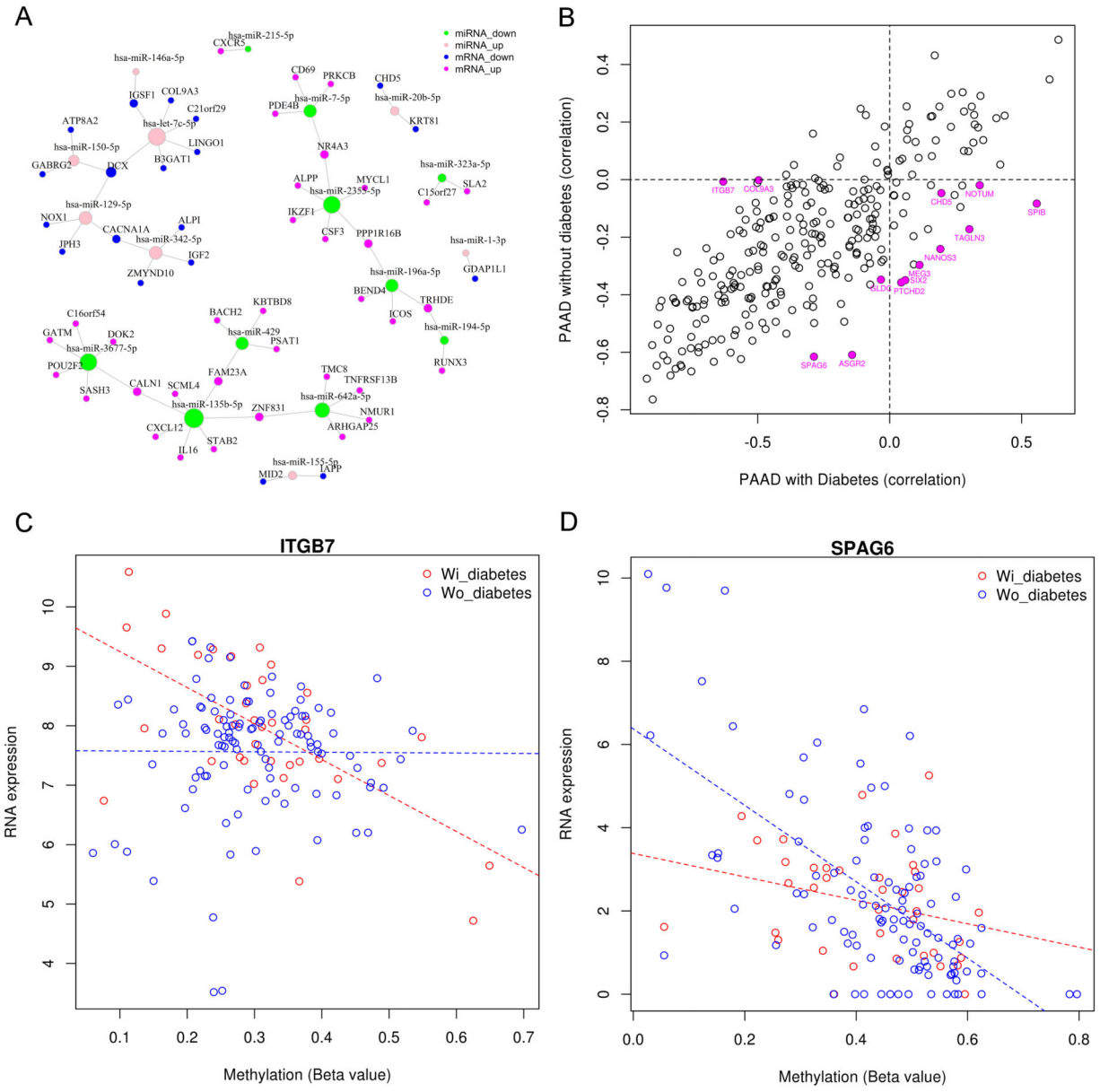

Figure 3.

Integration of epigenetic change and gene expression in PAAD with/out diabetes. Regulation of gene expression by miRNA is analyzed by microRNA target filter in IPA and plot as network in R (version 3.2.0) (A). miRNA is denoted in green (down-regulated in PAAD with diabetes) and pink (up-regulated in PAAD with diabetes) nodes. Up-regulated gene in PAAD with diabetes identified from RNAseq is colored in red and the down-regulated one is in blue (A). The differential correlation profile between gene and its DNA methylation in PAAD with/out diabetes is provided (B). Each point represents a gene and the ones in purple are significantly different from permutation test (B). The correlation coefficient value between gene expression and beta value of DNA methylation in PAAD with diabetes is plotted in the X-axis (B). The dash lines represent zero correlation coefficients (B). The

differential correlation of ITGB7 and its DNA methylation in PAAD with/out diabetes is shown in scatter plot with each point representing a sample(C). The differential correlation of SPAG6 and its DNA methylation in PAAD with/out diabetes is shown in scatter plot (D).

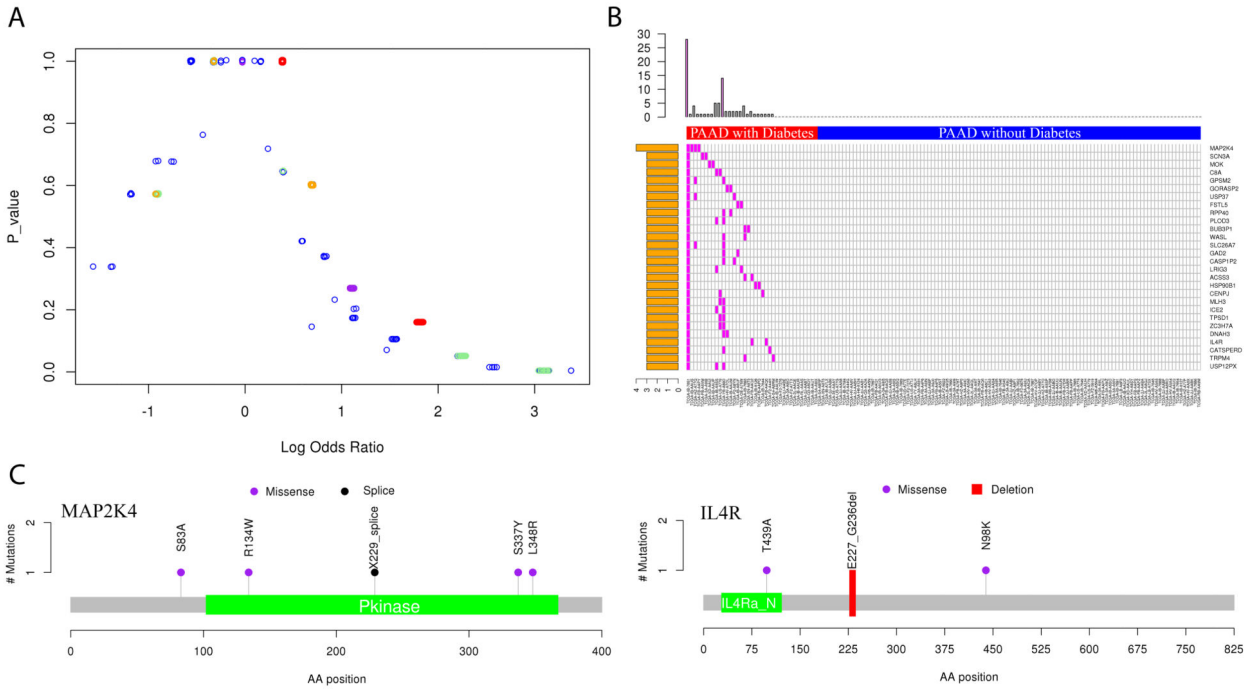

Figure 4.

Somatic mutation analysis in PAAD with/out diabetes. Log odds ratio of diabetes having mutation as well as p values derived from Fisher's Exact test is shown in scatter plot (A). Each point in the plot represents a mutant gene (A). To prevent over-plotting, the points are jittered and use of color to denote extent of over-plotting (Blue for lightest and Red for heaviest). A heatmap is to show the mutation pattern of the selected genes (B). Magenta color in heatmap means the mutation is detected (B). Bar plot above heatmap denotes the number of mutations occurring for each subject and left side bar plot is to show number of subjects having a mutation for each gene. The details of mutation occurred in MAP2K4 and IL4R are provided (C).

Table 1
Baseline characteristics of participants in the PAAD with/out diabetes

	PAAD without Diabetes (N=111)	PAAD with Diabetes (N=38)	Pvalue
Age_at_initial_pathologic_diagnosis, mean(sd)	63.66(11.92)	65.95(9)	0.275
Maximum_tumor_dimension, mean(sd)	3.8(1.57)	3.73(1.23)	0.816
Gender, N(%)			
Female	54(80.6%)	13(19.4%)	
Male	57(69.51%)	25(30.49%)	0.135
Race, N(%)			
White	92(71.32%)	37(28.68%)	
Asian	10(90.91%)	1(9.09)	
Black or African American	5(100%)	0(0%)	0.217
Unknown or Not Evaluated(Not used for statistical testing)	*4(100%)*	*0(0%)*	
Ethnicity, N(%)			
Hispanic or Latino	2(66.67%)	1(33.33%)	
Not Hispanic or Latino	76(71.03%)	31(28.97%)	1
Unknown or Not Evaluated or Not Evaluated(Not used for statistical testing)	*33(84.62%)*	*6(15.38%)*	
History_other_malignancy, N(%)			
Yes	13(81.25%)	3(18.75%)	
No	98(73.68%)	35(26.32%)	0.762
Pathologic_stage, N(%)			
Stage I	14(77.78%)	4(22.22%)	
Stage II	94(75.2%)	(31(24.8%)	
Stage III	2(66.67%)	1(33.33%)	
Stage IV	0(0%)	1(100%)	0.443
Not Available or Discrepancy(Not used for statistical testing)	*1(50%)*	*1(50%)*	
Smoking, N(%)			
Current smoker	51(66.23%)	26(33.77%)	
Lifelong Non-smoker	51(80.95%)	12(19.05%)	0.058
Unknown(Not used for statistical testing)	*9(100%)*	*0(0%)*	
Alcohol_history_documented, N(%)			
Yes	71(71.72%)	28(28.28%)	
No	36(78.26%)	10(21.74%)	0.543
Unknown(Not used for statistical testing)	*4(100%)*	*0(0%)*	

Table 2
Pathways with FDR q value less than 0.10

Gene Set	Size	NES	NOM.p.val	FDR.q.val
KEGG_HEMATOPOIETIC_CELL_LINEAGE	81	2.037	0.0009	0.0406
KEGG_PRIMARY_IMMUNODEFICIENCY	35	2.023	<0.0001	0.0442
KEGG_T_CELL_RECEPTOR_SIGNALING_PATHWAY	108	2.091	<0.0001	0.048
PID_CD8TCRPATHWAY	52	2.038	0.0004	0.0485
BIOCARTA_NKT_PATHWAY	29	1.983	0.0008	0.0498
PID_CD8TCRDOWNSTREAMPATHWAY	64	2.049	0.0005	0.0503
KEGG_TYPE_I_DIABETES_MELLITUS	23	2.063	<0.0001	0.0517
PID_IL12_2PATHWAY	60	2.127	<0.0001	0.0525
PID_IL12_STAT4PATHWAY	31	1.97	0.002	0.0539
SIG_BCR_SIGNALING_PATHWAY	46	1.983	0.0028	0.0546
REACTOME_COSTIMULATION_BY_THE_CD28 _FAMILY	55	1.998	0.0024	0.055
PID_TCR_PATHWAY	64	1.947	0.002	0.0562
KEGG_CELL_ADHESION_MOLECULES_CAMS	113	1.956	0.0027	0.0562
KEGG_GRAFT_VERSUS_HOST_DISEASE	19	1.986	<0.0001	0.0574
KEGG_CHEMOKINE_SIGNALING_PATHWAY	188	1.949	0.0017	0.0576
KEGG_INTESTINAL_IMMUNE_NETWORK_FOR_IGA _PRODUCTION	34	1.957	0.0055	0.0598
KEGG_NATURAL_KILLER_CELL_MEDIATED _CYTOTOXICITY	127	1.936	0.0027	0.0613
KEGG_AUTOIMMUNE_THYROID_DISEASE	32	1.929	0.0016	0.0637
KEGG_CYTOKINE_CYTOKINE_RECEPTOR _INTERACTION	264	1.919	0.0019	0.0686
KEGG_ALLOGRAFT_REJECTION	17	1.905	0.0029	0.0702
REACTOME_CHEMOKINE_RECEPTORS_BIND _CHEMOKINES	55	1.911	0.0056	0.0715
BIOCARTA_IL12_PATHWAY	21	1.906	0.0013	0.0722
REACTOME_CD28_DEPENDENT_PI3K_AKT _SIGNALING	21	1.892	0.0028	0.0752
ST_T_CELL_SIGNAL_TRANSDUCTION	44	1.895	0.0086	0.076
BIOCARTA_TH1TH2_PATHWAY	17	1.881	0.0029	0.0817
REACTOME_GENERATION_OF_SECOND _MESSENGER_MOLECULES	20	1.866	0.0004	0.0924
PID_CD40_PATHWAY	31	1.847	0.0114	0.0925
PID_IL2_1PATHWAY	55	1.847	0.0096	0.0952
BIOCARTA_DC_PATHWAY	22	1.859	0.0063	0.096
REACTOME_TCR_SIGNALING	44	1.854	0.0085	0.098
PID_FCER1PATHWAY	61	1.851	0.0087	0.0983
BIOCARTA_LAIR_PATHWAY	17	1.847	0.0055	0.0984