Original Article

Genetic alterations in the neuronal development genes are associated with changes of the tumor immune microenvironment in pancreatic cancer

Kaiyi Mu[1,2,3,4], Juan Fu[1,2,3,4], Jessica Gai[1,2,3,4], Harshitha Ravichandran[1,2,3,4], Lei Zheng[1,2,3,4,5], Wei-Chih Sun[1,2,3,4]^

[1]Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD, USA; [2]Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, MD, USA; [3]Pancreatic Cancer Precision Medicine Center of Excellence Program, Johns Hopkins University School of Medicine, Baltimore, MD, USA; [4]Bloomberg-Kimmel Institute for Cancer Immunotherapy, Johns Hopkins University School of Medicine, Baltimore, MD, USA; [5]Department of Surgery, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Contributions: (I) Conception and design: J Fu, L Zheng; (II) Administrative support: J Fu, L Zheng; (III) Provision of study materials or patients: L Zheng; (IV) Collection and assembly of data: J Gai, H Ravichandran; (V) Data analysis and interpretation: K Mu, WC Sun; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Wei-Chih Sun. Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, 1650 Orleans Street, CRB1, Baltimore, MD 21287, USA; Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, MD, USA; Pancreatic Cancer Precision Medicine Center of Excellence Program, Johns Hopkins University School of Medicine, Baltimore, MD, USA; Bloomberg-Kimmel Institute for Cancer Immunotherapy, Johns Hopkins University School of Medicine, Baltimore, MD, USA. Email: wsun40@jhmi.edu.

Background: Pancreatic ductal adenocarcinoma (PDAC) has a poor prognosis and is highly metastatic. Our prior studies have demonstrated the critical role of axon guidance pathway genes in PDAC and the connection between neuronal development and the tumor microenvironment. A recent study newly identified 20 neuronal development genes [disks large homolog 2 (*DLG2*), neuron-glial-related cell adhesion molecule (*NRCAM*), neurexin3 (*NRXN3*), mitogen-activated protein kinase 10 (*MAPK10*), platelet-derived growth factor D (*PDGFD*), protein kinase C epsilon (*PRKCE*), potassium calcium-activated channel subfamily M alpha 1 (*KCNMA1*), polycystic kidney and hepatic disease 1 (*PKHD1*), neural cell adhesion molecule 1 (*NCAM1*), neuregulin-1 (*NRG1*), zinc finger protein 667 (*ZNF667*), cystic fibrosis transmembrane conductance regulator (*CFTR*), acyl-CoA medium-chain synthetase-3 (*ACSM3*), complement 6 (*C6*), protein tyrosine phosphatase receptor type M (*PTPRM*), hypoxia-inducible factor 1 alpha (*HIF1A*), adenylyl cyclase 5 (*ADCY5*), adherens junctions-associated protein 1 (*AJAP1*), neurobeachin (*NBEA*), sodium voltage-gated channel alpha subunit 9 (*SCN9A*)] that are associated with perineural invasion and poor prognosis of PDAC. The relationship between genetic alterations in these 20 genes and tumor immune microenvironment (TME) has not previously been investigated.

Methods: We hence applied the sequential multiplex immunohistochemistry results of biopsy specimens from 63 PDAC patients to investigate this relationship.

Results: We found that, except for *PTPRM* and *NBEA*, genetic alterations involving these 20 genes are associated with significant changes in the densities of major immune cell subtypes. Except for *AJAP1*, the copy number loss involving this panel of neuronal development genes is significantly associated with changes in immune cell infiltrates. In contrast, the copy number gain in fewer genes, including *NRXN3*, *ZNF667*, *ACSM3*, *C6*, *ADCY5*, *SCN9A*, and *PRKCE*, is significantly associated with changes in immune cell infiltrates.

Conclusions: Our study suggested that neuronal development genes play a role in modulating TME in a pancreatic cancer setting.

Keywords: Pancreatic ductal adenocarcinoma (PDAC); neuronal development genes; tumor immune microenvironment (TME)

^ ORCID: 0000-0001-8117-0359.

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Introduction

The annual incidence of pancreatic cancer is increasing worldwide and is projected to become the second-leading cause of cancer death by 2040 in the US (1). Pancreatic ductal adenocarcinoma (PDAC), accounting for approximately 90% of pancreatic cancer, is an aggressive disease characterized by a dismal outcome with a 5-year survival rate of 12% (2). The poor prognosis can be attributed to delayed diagnosis, invasive tumor nature, frequent metastasis, and high resistance against all conventional therapies (3). The tumor immune microenvironment (TME) surrounding PDAC cells significantly determines tumor growth, metastatic ability, and treatment resistance (4). Additionally, accumulating evidence indicates the important role of neural signaling, neural regulation, and neurotransmitters in the TME and development of PDAC (5-7). In 2012, the International Cancer Genomics Consortium (ICGC) found that PDAC was enriched with axon guidance gene family genetic alterations. This suggests the potential involvement of the nervous system in PDAC carcinogenesis and has led to a rising interest in this aspect of neuronal mechanism (8).

Axon guidance family molecules have been studied for their roles in angiogenesis, tumorigenesis, and immune regulation (9-12). Semaphorins (SEMA) are a large family of axon guidance molecules and have been recognized as critical contributors to neural development, the immune response, and tumor progression (13). In our previous studies, PDAC cells secreted Sema3D, which is regulated by Annexin A2 (*ANXA2*) and binds Plexin D1 (*PLXND1*) and neuropilin-1 through an autocrine signaling mechanism. This binding interaction was also shown to promote invasion and metastasis of PDAC (14,15). In addition, we also proved that the SEMA3D-PLXND1 axon guidance pathway mediates paracrine signaling between tumor cells and nerves to enhance innervation and perineural invasion (PNI) in PDAC (16). Our group found that nerve-derived Sema3D and potentially other SEMA promote tumor progression and metastasis by reprograming tumor-associated macrophages toward M2 polarization. In a recent single-nucleus and spatial transcriptome profiling study of PDAC (17), 20 neuronal development genes [disks large homolog 2 (*DLG2*), neuron-glial-related cell adhesion molecule (*NRCAM*), neurexin3 (*NRXN3*), mitogen-activated protein kinase 10 (*MAPK10*), platelet-derived growth factor D (*PDGFD*), *PRKCE*, potassium calcium-activated channel subfamily M alpha 1 (*KCNMA1*), polycystic kidney and hepatic disease 1 (*PKHD1*), neural cell adhesion molecule 1 (*NCAM1*), neuregulin-1 (*NRG1*), zinc finger protein 667 (*ZNF667*), cystic fibrosis transmembrane conductance regulator (*CFTR*), acyl-CoA medium-chain synthetase-3 (*ACSM3*), complement 6 (*C6*), protein tyrosine phosphatase receptor type M (*PTPRM*), hypoxia-inducible factor 1 alpha (*HIF1A*), adenylyl cyclase 5 (*ADCY5*), adherens junctions-associated protein 1 (*AJAP1*), neurobeachin (*NBEA*), sodium voltage-gated channel alpha subunit 9 (*SCN9A*)] were found to be associated with PNI and poor prognosis in their independent cohorts. We aim to further investigate the findings of Hwang *et al.* (17) by examining the relationships between specific genetic alterations in neuronal development genes and the TME.

Highlight box

Key findings

- The role of neuronal development genes in the tumor immune microenvironment (TME) of pancreatic cancer.

What is known and what is new?

- Neuronal development genes are associated with perineural invasion and poor prognosis of pancreatic ductal adenocarcinoma (PDAC).
- Alterations of neuronal development genes are associated with changes in the densities of major immune cells in TME of PDAC.

What is the implication, and what should change now?

- It remains to be further investigated how neuronal development genes modulate the TME of PDAC.

Methods

Patient specimens

Specimens from 89 pancreatic cancer patients treated at the Johns Hopkins Hospital, comprised of 41 surgically resectable and 48 locally advanced pancreatic cancer (LAPC) patients, were included in the study (available at https://cdn.amegroups.cn/static/public/10.21037apc-23-13-1.xlsx). The

study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the institutional review board of Johns Hopkins Medicine (J1568). These specimens were archived from two clinical trials (NCT02451982; NCT02648282), and written informed consent to allow the usage of archived specimens for other studies (the Johns Hopkins Medical Institution Institutional Review Board approved protocols at the Johns Hopkins Pancreatic Cancer Precision Medicine Center of Excellence Program) including this study was obtained from all the patients. Specimens were either biopsy specimens collected via endoscopic ultrasound-guided fine needle core biopsies or surgically resected tumors. The demographics of the human cohort were described in the studies by Li *et al.* (18).

Sequential multiplex immunohistochemistry (mIHC)

The sequential mIHC was performed on biopsy specimens before the patients received any experimental therapy, the method to analyze the percentage of immune cell infiltration, and markers to identify immune cells, as described in the study by Li *et al.* (18). The published quantitative results were used without any modification for the analysis in this study. Sixty-three out of 89 patients have mIHC quantitative results available for this study.

Statistical analysis

Welch's *t*-test was performed to compare the two groups. We assume the sample means being compared for two normally distributed populations, and the populations have equal variances. A P value <0.05 was considered statistically significant. Statistical analyses and graphs were generated using GraphPad Prism v.9.5.1.

Results

A decrease in intratumoral CD8⁺ T cells is associated with the genetic alteration of neuronal development genes

Following our previous studies suggesting axon guidance molecules such as the class 3 SEMA modulate the TME, we attempted to understand how the genetic alteration of axon guidance genes correlates with the infiltration of immune cell subtypes in PDACs. In addition to those axon guidance genes reported to be associated with PDACs by our group and others, Hwang *et al.* (17) recently identified 20 PDAC-associated neuronal development genes through a comprehensive approach. We, therefore, analyzed the genetic alterations in these 20 neuronal development genes in our published PDAC patient cohorts whose intratumoral immune infiltrates have been characterized. The whole exome sequencing (WES) and whole genomic sequencing (WGS) data were obtained from the Precision Medicine Application Platform at Johns Hopkins, where the WES and WGS data are deposited. Due to the lack of functional assays to determine the significance and functions of the missense mutations, we focused on the copy number gain or loss in this study. Fifty-two of 89 PDACs have both WGS and WES data; for these cases, we determined the copy number gain (3 or more copies) or loss (0 or 1 copy) by using the WGS data (available at https://cdn.amegroups.cn/static/public/10.21037apc-23-13-1.xlsx). The remaining 37 PDACs have only WES data; we determined the copy number gain or loss using the WES data for these cases. We performed Welch's *t*-test to compare the subgroup with the copy number gain or loss with the subset without either gain or loss (Tables S1,S2).

In many of these 20 genes, either there is a gain or loss, and $CD8^+$ T cells were significantly decreased compared to PDAC without gain or loss of these genes (*Figure 1*). Nevertheless, the loss was more likely and significantly associated with decreased intratumor $CD8^+$ T cells. The only exception is that loss of *ZNF667* is associated with an increase in $CD8^+$ T cells.

An increase in intratumoral B cells and a decrease in intratumoral natural killer (NK) cells is associated with the loss of neuronal development genes

An increase in intratumoral B cells is associated with the loss of *CFTR*, *ACSM3*, and *HIF1A*. In contrast, gain in any of these 20 genes is not associated with a significant change in B cell infiltration (*Figure 2A*). The only exception is that *CFTR* gain is associated with decreased B cell infiltration. NK cells vary in association with axon guidance genes (*Figure 2B*). However, a significant reduction of NK cells is associated with the gain of *NRCAM*, *KCNMA1*, *HIF1A*, and *CFTR* genes. In contrast, a significant decrease in NK cells is associated with the loss of *ZNF667* and *C6*.

Genetic alterations in neuronal development genes are correlated with changes in CD4⁺ T cell infiltration, including their subtypes

A significant decrease in intratumoral $CD4^+$ T cell

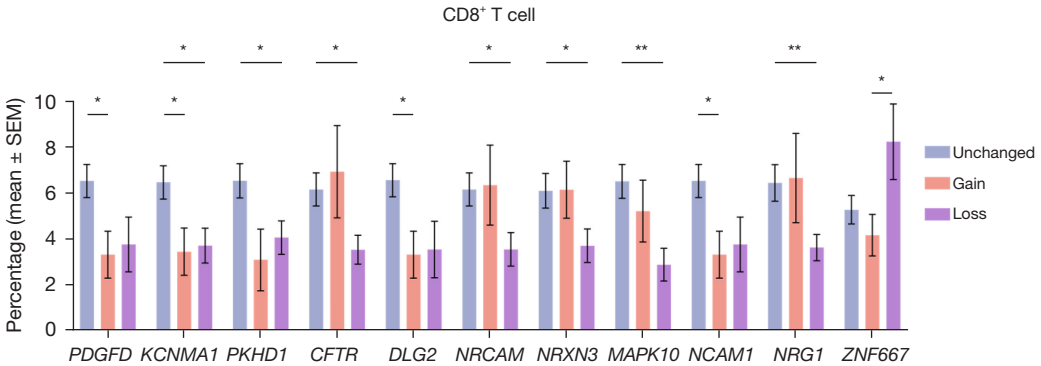

Figure 1 Comparison of the percentages of CD8+ T cells on all cells in the tumor area of 89 PDACs between different types of gene alteration: 'Unchanged' (no genetic alteration), 'Gain' (gain and amplification), and 'Loss' (including loss of heterogeneity and deletion). Data are shown as mean ± SEM. P values determined by unpaired *t*-test. *, P<0.05; **, P<0.01. SEM, standard error of the mean; PDAC, pancreatic ductal adenocarcinoma.

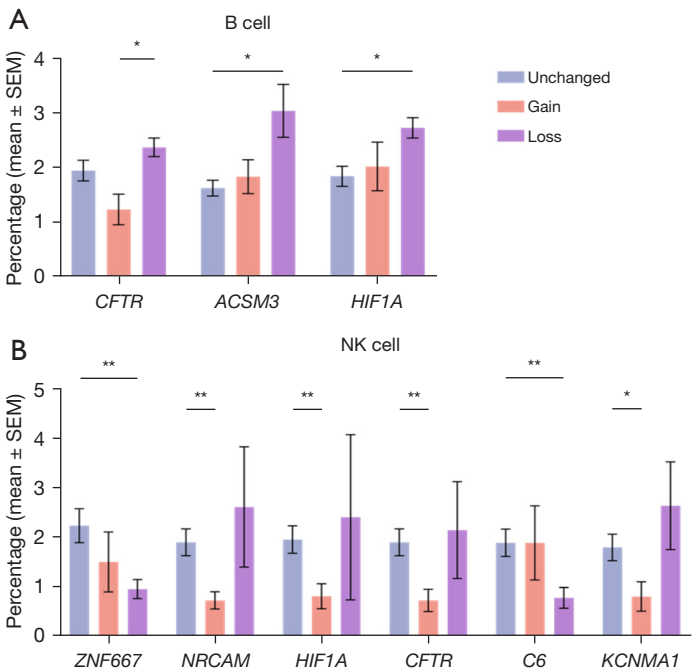

Figure 2 Comparison of the percentages of B cell (A) and NK cell (B) on all cells in the tumor area of 89 PDACs between different types of gene alteration: 'Unchanged' (no genetic alteration), 'Gain' (gain and amplification), and 'Loss' (including loss of heterogeneity and deletion). Data are shown as mean ± SEM. P values determined by unpaired *t*-test. *, P<0.05; **, P<0.01. SEM, standard error of the mean; NK, natural killer; PDAC, pancreatic ductal adenocarcinoma.

infiltration is associated with the loss of the *MAPK10*, *NRG1*, and *C6* genes (*Figure 3*). In contrast, a significant increase in the T helper 1 (Th1) subtype is associated with the loss of the *ZNF667*, *KCNMA1*, and *PKHD1* gene, and a significant decrease in the Th2 subtype is associated with the gain of the *NRCAM* and *PKHD1* genes. A significant decrease in the Th17 subtype is associated with the loss of *DLG2*, *PDGFD*, protein kinase C epsilon (*PRKCE*), *ADCY5* and *NCAM1*. The only significant increase in the regulatory T (Treg) subtype is associated with the loss of *PRKCE*. Interestingly, a considerable decrease in Th1, Th2, and Th17 infiltrations is related to the loss of *ADCY15*.

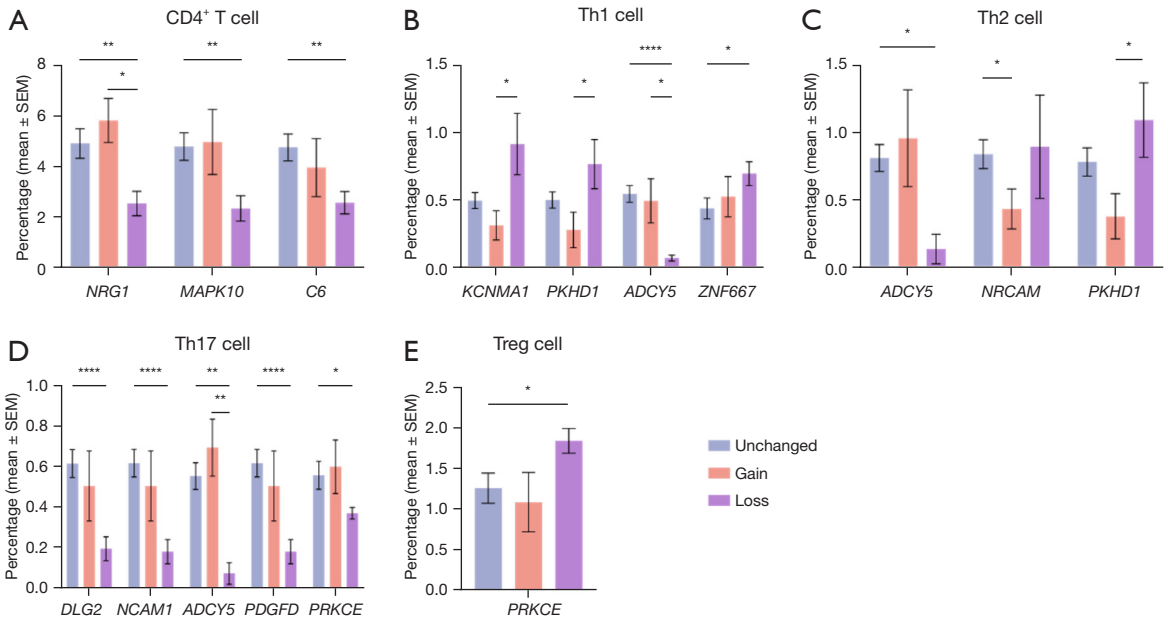

Figure 3 Comparison of the percentages of CD4⁺ T cell (A), Th1 (B), Th2 (C), Th17 (D), and Treg (E) on all cells in the tumor area of 89 PDACs between different types of gene alteration: 'Unchanged' (no genetic alteration), 'Gain' (gain and amplification), and 'Loss' (including loss of heterogeneity and deletion). Data are shown as mean ± SEM. P values determined by unpaired *t*-test. *, P<0.05; **, P<0.01; ****, P<0.0001. SEM, standard error of the mean; Th, T helper; Treg, regulatory T; PDAC, pancreatic ductal adenocarcinoma.

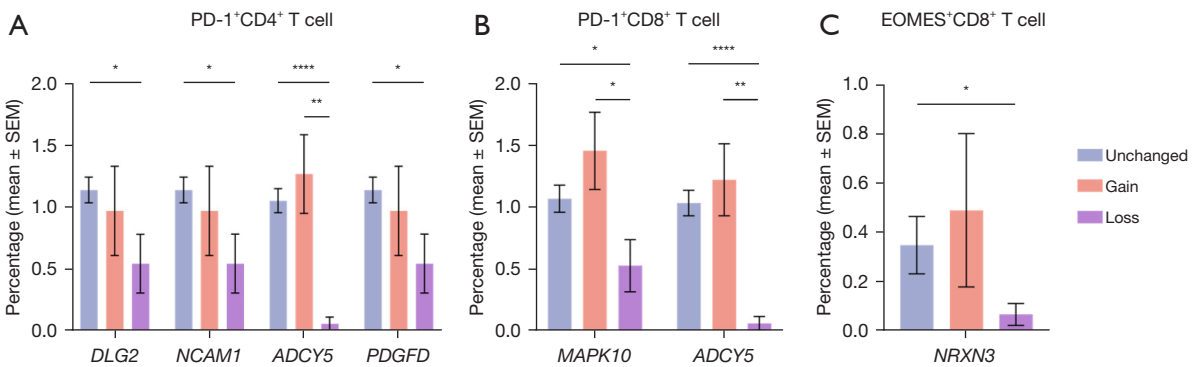

Figure 4 Comparison of the percentage of PD-1⁺CD4⁺ T cell (A), PD-1⁺CD8⁺ T cell (B), and EOMES⁺CD8⁺ T cell (C) on all cells in the tumor area of 89 PDACs between different types of gene alteration: 'Unchanged' (no genetic alteration), 'Gain' (gain and amplification), and 'Loss' (including loss of heterogeneity and deletion). Data are shown as mean ± SEM. P values determined by unpaired *t*-test. *, P<0.05; **, P<0.01; ****, P<0.0001. SEM, standard error of the mean; PDAC, pancreatic ductal adenocarcinoma.

Genetic alterations in neuronal development genes are associated with T cell exhaustion

A significant decrease in the PD-1⁺CD4⁺ T cells is associated with the loss of *DLG2*, *PDGFD*, *ADCY15*, and *NCAM1*, while a significant decrease in the PD-1⁺CD8⁺ T cells is associated with the loss of *ADCY15* and *MAPK10* (*Figure 4*). A significant decrease in the EOMES⁺CD8⁺ T cells is associated with the loss of *NRXN3*.

A significant decrease in M1-like tumor-associated macrophages (TAM), M2-like TAM, and tumor-associated neutrophils (TAN) is associated with the loss of neuronal development genes

A significant decrease in M1-like TAM infiltration is

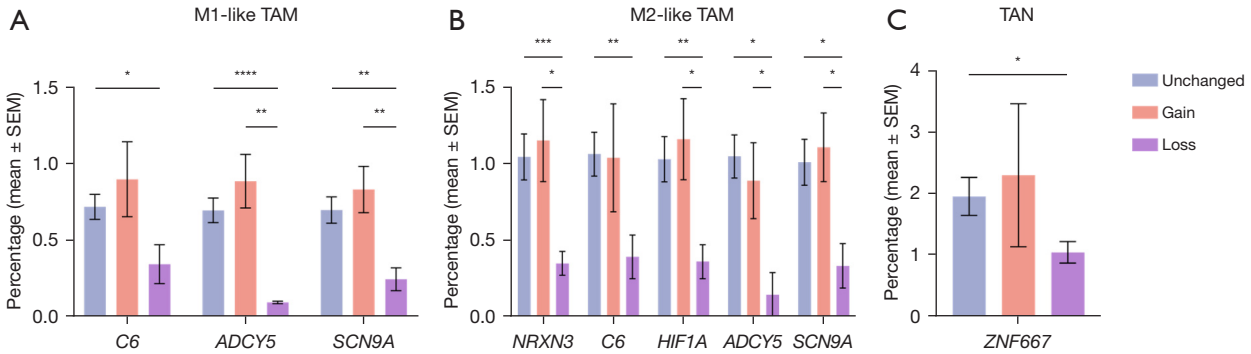

Figure 5 Comparison of the percentage of M1-like TAM (A), M2-like TAM (B), and TAN (C) on all cells in the tumor area of 89 PDACs between different types of gene alteration: 'Unchanged' (no genetic alteration), 'Gain' (gain and amplification), and 'Loss' (including loss of heterogeneity and deletion). Data are shown as mean ± SEM. P values determined by unpaired *t*-test. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. SEM, standard error of the mean; TAM, tumor-associated macrophages; TAN, tumor-associated neutrophils; PDAC, pancreatic ductal adenocarcinoma.

associated with the loss of *C6*, *ADCY5*, and *SCN9A*. A significant decrease in the infiltration of M2-like TAM is associated with the loss of *NRXN3*, *C6*, *HIF1A*, *ADCY5*, and *SCN9A* (*Figure 5*). Thus, a decrease in both M1-like and M2-like TAMs is associated with the loss of the same genes. It should be noted that a significant reduction in the CD66b$^+$ TAN is associated with the loss of *ZNF667*.

Changes in dendritic cell (DC) count are linked to genetic alterations in neuronal development genes

A significant decrease in the immature DCs is associated with the gain of *DLG2*, *PDGFD*, *NCAM1*, NRG1, *NCAM1*, and *PKHD1* genes, and a significant decrease in the mature DCs is associated with the gain of *MAPK10*, *PRKCE*, *PKHD1*, *NRG1*, and *AJAP1* genes (*Figure 6*). It should be noted that a significant decrease in the immature DCs is associated with the loss of *ADCY5* and that a significant increase in the mature DCs is associated with the loss of *PRKCE*.

Discussion

In this study, we performed a comprehensive analysis of the association of the PDAC-related neuronal development genes with the modulation of major immune cell subtypes in the tumor microenvironment of PDAC. Except for *PTPRM* and *NBEA*, genetic alterations involving this 20-gene panel are associated with significant changes in specific immune cell subtypes. Except for *AJAP1*, the loss involving this panel of neuronal development genes is significantly associated with changes in immune cells. In contrast, gain in specific genes, including *NRXN3*, *ZNF667*, *ACSM3 C6*, *ADCY5*, *SCN9A*, and *PRKCE*, is significantly associated with changes in immune cells. Where there is a loss involving this panel of neuronal development genes, a decrease was noted in most immune cell subtypes examined. Among the immune cell subtypes, CD8$^+$ T cells were most commonly decreased due to the loss in the neuronal development genes, followed by Th17, M2-like TAM, and PD-1$^+$CD4$^+$ T cells. Interestingly, where there is a gain in these neuronal development genes, only a decrease was observed as a statistically significant change in the immune infiltrates across all the immune cell subtypes examined. Additionally, immature DCs are the most commonly decreased subtype, followed by mature DC, CD8$^+$ T cells, and NK cells. Our results suggest that genetic alterations in the neuronal development genes in PDAC lead to a decrease in adaptive and effector cell immune response. Although some of these neuronal development genes were implicated in the immune modulation in literature as described below- for most of these genes, it is the first time that their potential roles in the TME of PDACs have been demonstrated.

The *DLG2* gene encodes an excitatory postsynaptic scaffold protein, which is a member of the membrane-associated guanylate kinase (MAGUK) family and is abundantly expressed in brain tissues (19). In the transcriptomic profiling studies, researchers found the expression of *DLG2* in mast cells, splenic red pulp

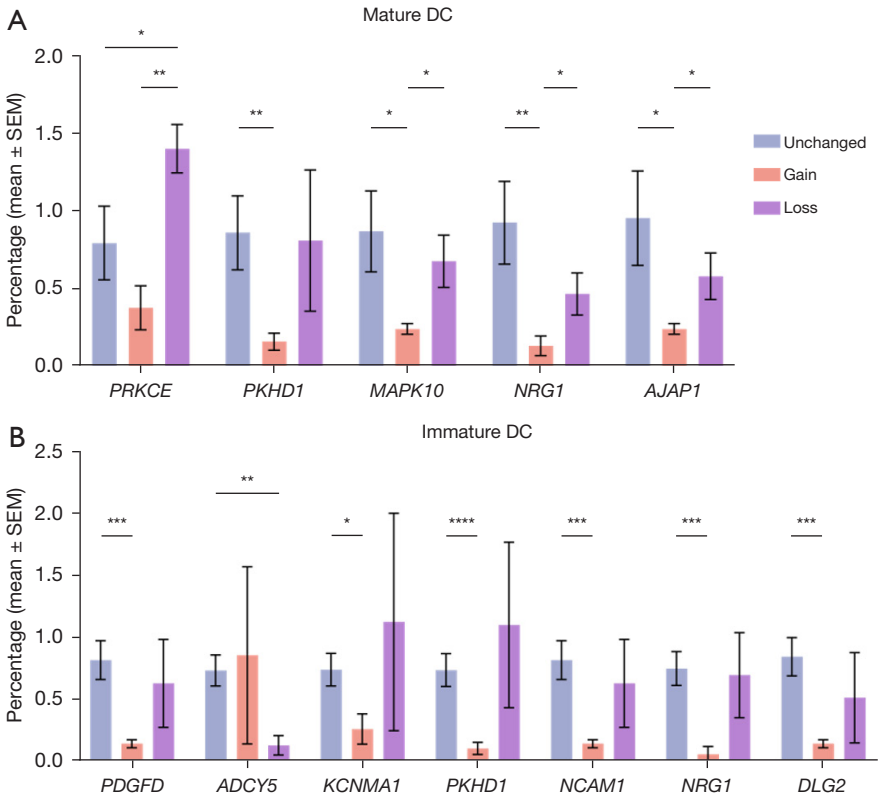

Figure 6 Comparison of the percentage of mature DC (A) and immature DC (B) on all cells in the tumor area of 89 PDACs between different types of gene alteration: 'Unchanged' (no genetic alteration), 'Gain' (gain and amplification), and 'Loss' (including loss of heterogeneity and deletion). Data are shown as mean ± SEM. P values determined by unpaired *t*-test. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. DC, dendritic cell; SEM, standard error of the mean; PDAC, pancreatic ductal adenocarcinoma.

macrophages, and plasmacytoid DCs that produce interferons-β (20). However, there was a lack of data supporting the role of the *DLG2* expression in the TME, except for one study showing that *DLG2* stimulated inflammasome formation and increased apoptosis in macrophage-like cells (21). Our results show that loss of *DLG2 was* significantly associated with a decrease of intratumoral PD-1$^+$CD4$^+$ T cells, Th17, and CD8$^+$ T cells in PDAC, and gain of *DLG2* was significantly associated with an increase of immature DCs and CD8$^+$ T cells.

The *NRCAM* gene encodes a transmembrane protein composed of multiple immunoglobulin-like C2 domains and a fibronectin type III domain. *NRCAM* plays an essential role in neuronal development, including neuron-neuron adhesion, axon growth, signal transduction, synapse formation, and formation of myelinated nerve structures (22,23). Only one study reported that the differentiation of Th1 cells was affected by regulating *NRCAM* gene transcription in Graves' disease (24). Our results showed that loss of *NRCAM* was significantly associated with decreased CD8$^+$ T cells in PDAC; gain of *NRCAM* was significantly associated with reduced NK cells and Th2 cells.

NRXN3 gene, a member of the neurexin gene family, is a synaptic modulator and encodes a protein that functions in the vertebrate nervous system as a cell adhesion molecule during synaptogenesis and intercellular signaling (25-27). The role of *NRXN3* in the TME has not been previously studied. Our results show that loss of *NRXN3* was significantly associated with a decrease of CD8$^+$ T cells, EOMES$^+$CD8$^+$ T cells, and M2-like TAMs in PDAC, and gain of *NRXN3* was significantly associated with an increase of M2-like TAMs.

MAPK10 involves various processes such as neuronal proliferation, differentiation, migration, and programmed cell death. In a recently published study of ovarian cancer, expression of *MAPK10* has been found to be positively correlated with the infiltration of eosinophils and naive B cells and negatively correlated with the infiltration of NK

cells and memory B cells (28). Another study of breast cancer showed that mutations in *MAPK10* were associated with a decreased infiltration of activated $CD4^+$ T cells (29). Consistently, our results showed that loss of *MAPK10* was significantly associated with a decrease of $CD4^+$ T cells, PD-1^+CD8^+ T cells, and mature DCs in PDAC; gain of *MAPK10* was significantly associated with an increase of PD-1^+CD8^+ T cells and mature DCs.

NCAM1 is a synaptic adhesion molecule that supports synaptic connections by trans-homophilic binding. NCAM1 has been implicated in several brain-related biological processes, including neuronal migration, axonal branching, fasciculation, and synaptogenesis, with a pivotal role in synaptic plasticity. In human hematopoiesis, *NCAM1* (also called CD56) is highly expressed in NK cells (30,31). In addition, *NCAM1* expression was found in rare subsets of T and B lymphocytes, DCs, and neural or mesenchymal stem cells (32). However, no study evaluates the immune-modulating effect of *NCAM1*. Our results showed that loss of *NCAM1* was significantly associated with a decrease of Th17 and PD-1^+CD4^+ T cells in PDAC; gain of *NCAM1* was significantly associated with an increase of $CD8^+$ T cells and immature DCs.

NRG1 belongs to the epidermal growth factor (EGF) family, contains an EGF-like domain, and is a HER3 (ERBB3) ligand. *NRG1* is primarily known for its essential role in Schwann cell and oligodendrocyte differentiation, maintenance, and myelination in the central and peripheral nervous systems (33). Furthermore, through the NRG1-HER3 signaling axis, *NRG1* contributes to malignant tumor development in several cancer types, including gastric, pancreatic, breast cancer, squamous cell carcinoma, and non-small-cell lung cancer. Additionally, its overexpression is closely associated with poor prognosis (34-36). A recent study indicated that *NRG1* augments regulatory populations of macrophages, T cells, and B cells peripherally and in injured spinal cord tissue (37). In a study of colorectal cancer, *NRG1* expression was positively correlated with activated DCs, neutrophils, plasma cells, and resting $CD4^+$ memory T cells and negatively with memory B cells and macrophage M1 (38). In addition, a study of oral squamous cell carcinoma also showed that *NRG1* expression was negatively correlated with the infiltration of B cells and $CD8^+$ T cells (39). Our results show that loss of the *NRG1* gene was significantly associated with a decrease of $CD4^+$ T cells in PDAC; gain of *NRG1* was significantly associated with a decrease of mature DCs and immature DCs.

ZNF667 is a novel C_2H_2 zinc finger protein that is found to be significantly upregulated during myocardial and cerebral ischemic preconditioning (40,41). A recent study showed that *ZNF667* exhibited anti-inflammatory effects in LPS-induced macrophages by suppressing the mTOR-dependent expression of glycolytic genes and glycolysis (42). Our results show that loss of *ZNF667* is significantly associated with a decrease of NK cells and TAN and an increase of $CD8^+$ T cells in PDAC.

HIF1A is involved in cellular metabolism, cell death, and proliferation of neuronal progenitors in the sympathetic system (43,44). *HIF1A* is a crucial regulator of immune cell function in health and disease (45). *HIF1A* regulates M1 macrophage polarization, DC maturation and migration, and neutrophil NET formation and survival (46,47). Our results showed that loss of *HIF1A* was significantly associated with a decrease of M2-like TAMs and an increase of B cells in PDAC. In contrast, a gain of *HIF1A* was significantly associated with reduced NK cells.

PDGFD gene belongs to the PDGF family of proteins, which can regulate neurogenesis and diverse functions in the brain. A recent study found that exogenous administration of PDGFB, which shares the same binding receptor with PDGFD, promotes the proliferation of neural progenitor cells (48). Another study found that *PDGFD* significantly correlates with plasma cells, CD4 memory T cells, follicular helper T cells, monocytes, macrophage M0 and M1, DCs, and mast cells in gastric cancer (49). Our results showed that loss of *PDGFD* was significantly associated with a decrease of Th17 and PD-1^+CD4^+ T cells in PDAC; gain of *PDGFD* was significantly associated with a decrease of $CD8^+$ T cells and immature DCs.

ACSM3 is a member of the acyl-CoA synthase gene family, which regulates cellular phospholipid acyl-chain diversity in the brain (50). A study of melanoma found that *ACSM3* was positively correlated with central memory and naïve $CD8^+$ cells, regulatory T cells, macrophages, and DCs (51). Our study found that loss of *ACSM3* was significantly associated with an increase of B cells in PDAC.

AJAP1 is an integral transmembrane protein that interacts with the E-cadherin-catenin complex at adherens junctions, which mediate adhesion between pre-and postsynaptic membranes (52). The role of the *AJAP1* expression in the TME was not previously reported. Our study found that the gain of *AJAP1* was significantly associated with a decrease of mature DCs and Th1 cells in PDAC.

KCNMA1 gene encodes a protein that forms large-conductance calcium-activated potassium channels in cells.

These channels play a crucial role in regulating the flow of potassium ions across cell membranes, an essential step in various physiological processes, including neurotransmitter release (53). Mutations of the *KCNMA1* gene can lead to channel dysfunction and are associated with neurological conditions, including seizures, movement disorders, developmental delay, and intellectual disability (54). The role of *KCNMA1* in the TME was not reported. Our results showed that loss of *KCNMA1* was significantly associated with a decrease of $CD8^+$ T cells and an increase of Th1 cells in the tumor microenvironment of PDAC; gain of *KCNMA1* was significantly associated with a decrease of $CD8^+$ T cells, NK cells, and immature DCs.

PKHD1 gene encodes a protein known as fibrocystin/polyductin, which is primarily found in the primary cilia of renal epithelial cells. Mutations of the *PKHD1* gene are associated with autosomal recessive polycystic kidney disease (ARPKD), a rare genetic disorder characterized by the formation of cysts in the kidneys and liver (55,56). During embryogenesis, *PKHD1* is widely expressed in epithelial derivatives, including neural tubules (57). The role of *PKHD1* in the TME has not been previously studied. Our results found that loss of *PKHD1* was significantly associated with a decrease of $CD8^+$ T cells and an increase of Th1 and Th2 cells in PDAC; gain of *PKHD1* was significantly associated with a decrease of mature DCs, immature DCs, and Th2 cells.

CFTR gene encodes channel proteins that belong to the ATP-binding cassette transporter superfamily. CFTR binds ATP and promotes substrate transport across membranes. Mutations in the *CFTR* gene are the cause of cystic fibrosis. *CFTR* has been reported to be involved in modulating neuronal excitability through chloride transporters in both the peripheral and central nervous systems (58). One study on cystic fibrosis showed that *CFTR* regulated B cell activation and lymphoid follicle development. However, the role of *CFTR* in the TME was not studied. Our results showed that loss of *CFTR* was significantly associated with a decrease of $CD8^+$ T cells in PDAC; gain of *CFTR* was significantly associated with a decrease of NK cells.

The *C6* gene encodes a component of the complement cascade as a part of the membrane attack complex that can be incorporated into the cell membrane and cause cell lysis. C6 is part of the immune system and is crucial in defending the body against infections and pathogens. In addition to being a component of innate and adaptive immunity, complement proteins regulate several physiologic processes, including synaptic pruning during brain development (59-62). However, the role of the *C6* expression in the TME was not well studied. Our results showed that loss of *C6* was significantly associated with a decrease of NK cells, $CD4^+$ T cells, M1-like TAM, and M2-like TAMs in PDAC.

ADCY5 is a member of membrane-bound adenylyl cyclase enzymes that regulate cellular activities by mediating G protein-coupled receptor signaling through synthesizing the second messenger cAMP (63). *ADCY5* is highly expressed in the brain and myocardium (64). Mutations in *ADCY5* have mainly been linked to various complex movement disorders often associated with neurodevelopmental phenotypes (65). However, the role of *ADCY5* in the TME was not reported. Our results showed that loss of *ADCY5* was significantly associated with a decrease of immature DCs, Th1, Th2, Th17, PD-1^+CD4^+ T cells, PD-1^+CD8^+ T cells, M1-like TAMs, M2-like TAMs, and immature DCs in PDAC.

SCN9A, known as $Na_v1.7$, are voltage-gated sodium channels that are preferentially expressed in the dorsal root ganglia and sympathetic neurons (66-68). *SCN9A* mediates cellular excitability and plays a crucial role in gating pain transmission from the periphery to the central nervous system (69,70). The role of *SCN9A* in the TME was not reported. Our results showed that loss of *SCN9A* was significantly associated with a decrease of M1-like TAMs and M2-like TAMs in PDAC.

PRKCE belongs to a family of serine- and threonine-specific protein kinases that can be activated by calcium and the second messenger, diacylglycerol. The gene product of *PRKCE* plays an essential role in regulating multiple cellular processes, including neuron growth and immune response (71,72). Our results show that loss of *PRKCE* was significantly associated with a decrease of Th17 cells and an increase of Treg cells and mature DCs in PDAC.

PTPRM is a member of the protein tyrosine phosphatase (PTP) family (73). *PTPRM* is a key regulator of neurite outgrowth and synapse formation in cortical neurons (74,75). *NBEA* is a kinase anchor protein that contributes to the regulation of protein trafficking and recycling of ionotropic glutamate and GABA receptors (76,77). *NBEA* has also been implicated in vesicular traffic at the synapse and has been shown to be required for normal development of the synapses (78). The role of *PTPRM* or *NBEA* in the TME was not reported. Our results showed that neither the genetic alterations of *PTPRM* nor *NBEA* were significantly associated with a change in immune cells in PDAC.

The limitation of this study is that the study only

compared the percentage of different immune cells corresponding to various gene alterations. It is unclear how and why the immune cells are regulated by those genes.

Conclusions

It remains to be further investigated how these neuronal development genes and other axon guidance genes coordinate to modulate the TME. Different neuronal development genes may regulate the trafficking of various immune cells. Similar to axon guidance, these genes may guide the trafficking of immune cells. Tumor cells have acquired the ectopic expression of neuronal development genes and axon guidance genes; therefore, they have acquired the function of axon guidance genes ectopically. Similar to axon guidance molecules (which can function as nerve repellants or attractants depending on the subtype), genetic alterations in neuronal development genes in tumor cells allow the tumor cells to repel specific immune cells while attracting other immune cells. Such a hypothesis would need further testing with functional assays to measure the repellent and attractant function against tumors.

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Footnote

Data Sharing Statement: Available at https://apc.amegroups.com/article/view/10.21037/apc-23-13/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://apc.amegroups.com/article/view/10.21037/apc-23-13/coif). L.Z. was supported by NIH grant R01 CA169702, NIH grant R01 CA197296, and Sidney Kimmel Comprehensive Cancer Center Support Grant P30 CA006973. L.Z. serves as the Editor-in-Chief of *Annals of Pancreatic Cancer*. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the institutional review board of Johns Hopkins Medicine (J1568). These specimens were archived from two clinical trials (NCT02451982; NCT02648282), and written informed consent to allow the usage of archived specimens for other studies (the Johns Hopkins Medical Institution Institutional Review Board approved protocols at the Johns Hopkins Pancreatic Cancer Precision Medicine Center of Excellence Program) including this study was obtained from all the patients.

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Supplementary

Table S1 Change of tumor-infiltrating immune cells in pancreatic adenocarcinoma with loss of neuronal development gene

Gene	Cell															
	$CD4^+$	$CD8^+$	B	NK	Th1	Th2	Th17	Treg	PD-1^+ $CD4^+$	PD-1^+ $CD8^+$	$EOMES^+CD8^+$	M1-like TAM	M2-like TAM	TAN	Mature DC	Immature DC
DLG2							▼****		▼*							
NRCAM		▼*														
NRXN3		▼*									▼*		▼***			
MAPK10	▼**	▼**								▼*						
NCAM1							▼****		▼*							
NRG1	▼**	▼**														
ZNF667		△*		▼**	△*									▼*		
HIF1A			△**										▼**			
PDGFD							▼****		▼*							
ACSM3			△*													
AJAP1																
KCNMA1		▼*			△*											
PKHD1		▼*			△*	△*										
CFTR		▼*														
C6	▼**			▼**								▼*	▼**			
ADCY5					▼****	▼*	▼**		▼****	▼****		▼****	▼*			▼**
SCN9A												▼**	▼*			
PRKCE							▼*	△*							△*	
PTPRM																
NBEA																

▼, decrease; △, increase. *, 0.01≤P<0.05. **, 0.001≤P<0.01; ***, 0.0001≤P<0.001; ****, P<0.0001. NK, natural killer; Th, T helper; Treg, regulatory T; TAM, tumor-associated macrophages; TAN, tumor-associated neutrophils; DC, dendritic cell.

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Table S2 Change of tumor-infiltrating immune cells in pancreatic adenocarcinoma with gain of neuronal development gene

Gene	Cell															
	$CD4^+$	$CD8^+$	B	NK	Th1	Th2	Th17	Treg	PD-1^+ $CD4^+$	PD-1^+ $CD8^+$	$EOMES^+CD8^+$	M1-like TAM	M2-like TAM	TAN	Mature DC	Immature DC
DLG2		▼*														▼***
NRCAM				▼**		▼*										
NRXN3																
MAPK10															▼*	
NCAM1		▼*														▼***
NRG1															▼**	▼***
ZNF667																
HIF1A				▼**												
PDGFD		▼*														▼***
ACSM3																
AJAP1															▼*	
KCNMA1		▼*		▼*												▼*
PKHD1						▼*									▼**	▼****
CFTR				▼**												
C6																
ADCY5																
SCN9A																
PRKCE															▼**	
PTPRM																
NBEA																

▼, decrease; △, increase. *, 0.01≤P<0.05. **, 0.001≤P<0.01; ***, 0.0001≤P<0.001; ****, P<0.0001. NK, natural killer; Th, T helper; Treg, regulatory T; TAM, tumor-associated macrophages; TAN, tumor-associated neutrophils; DC, dendritic cell.

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