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NK cell and IFN signatures are positive prognostic biomarkers for resectable pancreatic cancer

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ABSTRACT

To establish prognostic biomarkers and to identify potential novel therapeutic targets, we performed integrative immunomonitoring of blood and tumor in patients with resectable pancreatic cancer. Flow cytometry (FC) was employed for phenotyping immune cells, multiplex bead assays for plasma cytokine and chemokine determination, and RNA-Seq for the analysis of gene expression in the tumor. Nineteen pancreatic cancer patients were stratified into those with longer or shorter than median recurrence-free survival after surgery (median, 426 days). There were no significant differences between the two groups for clinical parameters including age, sex, surgical procedure, stage, or postoperative adjuvant therapy. However, we found that the percentages of NK cells as assessed by FC in peripheral blood mononuclear cells were higher in patients with late recurrence ($P = .037$). RNA-Seq data indicated no differences in the amount of immune cells or stromal cells between the two groups, although NK cells in the tumor did tend to be higher in patients with late recurrence ($P = .058$). Type I and II IFN signatures were enriched in late-recurring tumors (FDR q-value <0.001), while genes related to KRAS signaling and the epithelial mesenchymal transition (EMT) were enriched in early recurrence. We conclude that tumor-intrinsic properties of metastasis and recurrence influence prognosis, whereas NK cells that might contribute to prevent metastasis are associated with longer recurrence-free survival. Therefore, enhancement of NK cell activity and inhibition of the EMT and KRAS signaling might represent appropriate therapeutic targets following surgical resection of pancreatic cancer.

1. Introduction

The outlook for pancreatic cancer patients after surgery remains highly unsatisfactory; approximately 23,000 Japanese die of this disease per year, making it the 4th leading cause of cancer-related deaths in the country. Recent studies have documented that adjuvant chemotherapy using gemcitabine (GEM) or S-1 can improve recurrence-free and overall survival [1–3]. However, the recurrence rate is >80% even after potentially curative surgery. Therefore, development of more effective therapies is urgently needed.

Recently, immune checkpoint inhibitors have shown major clinical benefit, especially in melanoma and lung cancer. However, pancreatic cancer remains poorly responsive to checkpoint blockade [4]. Marked infiltration of immunosuppressive cells into the dense desmoplastic tumor stroma is observed in most

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pancreatic cancer patients, and intratumoral infiltration of effector cells is commonly very poor. It has been shown that prognosis is better for resectable pancreatic cancer patients whose tumors have more T-cell infiltration. However, the immunological profile and determinants of immune activation in pancreatic cancers are still poorly understood. Novel immunotherapeutic approaches could emerge from a better understanding of these issues.

Here, we performed integrative immunomonitoring of peripheral blood and tumor from resectable pancreatic cancer patients. We sought immunological factors for predicting the prognosis after surgery, which might reveal novel potential targets for pancreatic cancer immunotherapy.

2. Methods

2.1. Patient selection

From August 2012 to December 2013, 45 patients with pancreatic cancers underwent surgical resections at the Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery at the University of Tokyo Hospital. Of these, patients with R0 or R1 resections were selected. Patients who were receiving systemic steroid therapy which affects host immune system were excluded. Double cancer cases were also excluded from the study.

2.2. Sample preparation, RNA sequencing and data processing

Pancreatic tumors and peripheral blood from the same patients were collected by the University of Tokyo Hospital following the approval of the institutional review board and patients' written informed consent (ID: G3545). Tumor samples were obtained immediately after surgical resection and stored in RNAlater RNA Stabilization Reagent (Qiagen, Hilden, Germany). Total RNA samples were prepared using either AllPrep DNA/RNA Mini Kit or AllPrep DNA/RNA/miRNA Universal Kits (Qiagen) according to the manufacturer's instructions. Poly-A-selected RNA libraries were prepared and sequenced at 50-bp single-read mode (PK001, PK004, PK005) and at 100-bp pair-end mode (PK009, PK011, PK013, PK015, PK017, PK018, PK022, PK024, PK025, PK029, PK030, PK045, PK055, PK065) on HiSeq 1500 or HiSeq 4000 (Illumina, San Diego, CA) at Kazusa DNA Research Institute (Kisarazu, Japan) and BGI Japan K.K. (Kobe, Japan). RNA sequencing reads were aligned using Tophat (v2.1.1) [5]. Averages of total mapped reads were 99.9M 50-bp single-end reads and 60.1M 100-bp pair-end reads. Data are expressed as fragments per kilobase of transcript per million fragments mapped (FPKM) using Cufflinks (v2.2.1) [6].

2.3. PBMC isolation and flow cytometry

Peripheral blood (20 mL) was obtained twice, one week before and 4–6 weeks after pancreatectomy but before initiating adjuvant therapy with gemcitabine (GEM), GEM plus γδ T cell therapy, or TS-1. PBMCs and plasma were obtained by density gradient centrifugation using Lymphoprep™ (Axis-Shield Poc AS, Oslo, Norway), and cryopreserved in Bambanker™ freezing medium (NIPPON Genetics, Tokyo, Japan) until use. Cryopreserved PBMCs were thawed in RPMI supplemented with 50 IU/ml Benzonase™ Nuclease (Sigma-Aldrich, St Louis, MO), and then stained with the following antibodies: FITC-labeled anti-CD3 (clone UCTH1) and anti-TCR Vγ9 (clone IMMU 360), PE-labeled anti-CD19 (clone J3-119), anti-CD56 (clone NKH1) and anti-TCR Pan alpha/beta (clone IP26A), and ECD-labeled anti-CD8 (clone SFCI21Thy2D3) and anti-CD45RA (clone 2H4LDH11LDB9) (all from Beckman Coulter, Immunotech, Marseille, France). FITC-labeled anti-CD4 (clone RPA-T4) was from BD Biosciences (San Jose, CA). FITC-labeled anti-PD-1 (clone EH12.2H7), PE-labeled $IgG1_{\kappa}$, anti-CD25 (clone BC96), anti-PD-1 (clone EH12.2H7) and anti-Tim-3 (clone F38-2E2), PerCP Cy5.5-labeled anti-CD3 (clone HIT3a), APC-labeled anti-CD4 (clone RPA-T4) and anti-CD45 (clone HI30), Alexa Fluor® 488-labeled $IgG1_{\kappa}$ (clone MOPC-21), and anti-FoxP3 (clone 259D), Pacific Blue-labeled anti-CD4 (clone RPA-T4), anti-CD8 (clone HIT8a), anti-CD14 (clone M5E2) and anti-CD45 (clone HI30), Alexa Fluor® 647-labeled anti-CD3 (clone HIT3a), were all from BioLegend (San Diego CA). Alexa Fluor® 488-labeled anti-Tim-3 (clone 344823), PE-labeled polyclonal goat anti-LAG3 were purchased from R&D Systems Inc. (Minneapolis, MN). FITC-labeled anti-LAG3 (clone 17B4) was purchased from Enzo Life Sciences (Farmingdale, NY). For excluding dead cells from the analysis, samples were stained with 7-AAD Viability Dye (Beckman Coulter) or Fixable Viability Dye eFluor® 780 (eBioscience, San Diego, CA). Stained cells were analyzed on a Gallios (Beckman Coulter) and data analyzed by Kaluza software (Beckman Coulter).

2.4. Cytokine measurement

The presence of the cytokines and chemokines, interleukin (IL)-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, bFGF, eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1 (MCAF), MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF in the plasma was measured using the Bio-Plex Pro Human Cytokine 27-plex Assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Briefly, four-fold diluted plasma was incubated with microbeads labeled with specific antibodies to one of the cytokines/chemokines for 60 min. Following a washing step, the beads were incubated with the detection antibody cocktail, with each antibody specific for a single cytokine, for 30 min. After another washing step, the beads were incubated with streptavidin-phycoerythrin for 10 min, washed again and the concentration of each cytokine determined using the MAGPIX reader system (Merck Millipore, Darmstadt, Germany).

2.5. In silico quantification of tumor-infiltrating lymphocytes

We applied R package software MCP-counter for the quantification and comparison of tumor-infiltrating lymphocytes [7]. In brief, abundance scores for $CD3^+$ T cells, $CD8^+$ T cells, cytotoxic lymphocytes, NK cells, B lymphocytes, monocytic lineage cells, myeloid dendritic cells and neutrophils, as well as endothelial cells and fibroblasts, were generated from a gene expression matrix for each sample. The scores were then used for comparisons of the abundance of the corresponding cell type across the samples.

2.6. Gene set enrichment analysis (GSEA)

GSEA is a computational method to determine whether particular sets of genes show expression patterns significantly different between two groups of samples. GSEA was performed using the GSEA GenePattern module (v17) with the default settings, except for permutation type parameter (permutation type = gene_set) [8,9]. In brief, gene expression matrix files and a phenotype label file which defines the phenotype label of each sample (i.e. Early Recurrence Group-vs-Late Recurrence Group) were submitted. GSEA first ranked the genes according to the significance of differential gene expression levels between the Early and Late Recurrence Groups. An enrichment score for each gene set was then calculated using the entire ranked list, which reflects how the genes

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