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# Host *IDO2* gene status influences tumor progression and radiotherapy response in *KRAS*-driven sporadic pancreatic cancers

**Avinoam Nevler**[1,5,#,*], **Alexander J. Muller**[2,5,6,*], **Erika Sutanto-Ward**[6], **James B. DuHadaway**[6], **Kei Nagatomo**[6], **Eric Londin**[3], **Kevin O'Hayer**[1], **Joseph A. Cozzitorto**[1], **Harish Lavu**[1,5], **Theresa P. Yeo**[1,5], **Mark Curtis**[3], **Tatiana Villatoro**[3], **Benjamin E. Leiby**[4,5], **Laura Mandik-Nayak**[6], **Jordan M. Winter**[1,5], **Charles J. Yeo**[1,5], **George C. Prendergast**[3,5,6,†], and **Jonathan R. Brody**[1,5]

[1]Department of Surgery and the Jefferson Pancreas, Biliary and Related Cancer Center, Thomas Jefferson University, Philadelphia PA USA

[2]Department Microbiology and Immunology, Thomas Jefferson University, Philadelphia PA USA

[3]Department Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia PA USA

[4]Division of Biostatistics, Thomas Jefferson University, Philadelphia PA USA

[5]Sidney Kimmel Medical College and Sidney Kimmel Cancer Center, Thomas Jefferson University, Philadelphia PA USA

[6]Lankenau Institute for Medical Research, Wynnewood PA USA

[*]Co-first authors

[#]The Dr. P. Borenstein Talpiot Medical Leadership Program (2012), Chaim Sheba Medical Center, Israel.

## Abstract

**Purpose:** Heritable genetic variations can affect the inflammatory tumor microenvironment, which can ultimately impact cancer susceptibility and clinical outcomes. Recent evidence indicates that IDO2, a positive modifier in inflammatory disease models, is frequently upregulated in pancreatic ductal adenocarcinoma (PDAC). A unique feature of *IDO2* in humans is the high prevalence of two inactivating single nucleotide polymorphisms (SNPs) which affords the opportunity to carry out loss-of-function studies directly in humans. In this study we sought to address whether genetic loss of IDO2 may influence PDAC development and responsiveness to treatment.

[†] Corresponding author: Lankenau Institute for Medical Research, 100 Lancaster Avenue, Wynnewood PA 19096 USA. Tel. 484-476-8475; prendergast@limr.org.

COI Statement: G.C.P. is an inventor on U.S. Patent No. 8,058,416 claiming IDO2 gene sequences. The other authors declare no potential conflicts of interest.

**Experimental Design:** Transgenic *Ido2*$^{+/+}$ and *Ido2*$^{-/-}$ mice in which oncogenic KRAS is activated in pancreatic epithelial cells were evaluated for PDAC. Two patient datasets (N=200) were evaluated for the two IDO2-inactivating SNPs together with histologic, RNA expression and clinical survival data.

**Results:** PDAC development was notably decreased in the *Ido2*$^{-/-}$ mice (30% vs 10%, P<0.05), with a female predominance similar to the association observed for one of the human SNPs. In patients, the biallelic occurrence of either of the two IDO2-inactivating SNPs was significantly associated with markedly improved disease-free survival in response to adjuvant radiotherapy (P<0.01), a treatment modality that has been highly debated due to its variable efficacy.

**Conclusions:** The results of this study provide genetic support for IDO2 as a contributing factor in PDAC development and argue that *IDO2* genotype analysis has the immediate potential to influence the PDAC care decision-making process through stratification of those patients who stand to benefit from adjuvant radiotherapy.

### Keywords

indoleamine 2,3-dioxygenase; cancer immunology; inflammation; pancreas; radiation

## Introduction

Immune checkpoint therapeutics, such as the CTLA-4, PD1 and PD-L1 directed antibodies that have revolutionized the treatment of melanoma and non-small cell lung cancer, have generated enormous interest for their prospects to restore T cell-mediated immune surveillance in other types of cancer but several, including pancreatic ductal adenocarcinoma (PDAC), have thus far proven refractory to such agents (1), with the exception of cases with microsatellite instability (MSI-H). The prospect of improving immune checkpoint therapy responsiveness has intensified the search for other host factors involved in licensing immune escape. The tryptophan catabolic enzyme, indoleamine 2,3 dioxygenase-1 (IDO1), has attracted attention in this regard as a mediator of tumor promoting inflammation and the locoregional suppression of antitumor T cells (2,3). IDO1 overexpression occurs in >50% of human cancers where it has been generally associated with a poor prognosis (4). A large body of preclinical work has established that small molecule IDO inhibitors (IDOi) can reprogram the inflammatory microenvironment and overcome immune barriers erected by tumors against antitumor T cells, including those involving T regulatory cells (Treg), tumor-associated macrophages (TAM) and myeloid-derived suppressor cells (MDSC) (2). Preclinical studies have argued that IDOi will be particularly effective as part of a combined therapy including DNA-damaging chemotherapy, radiotherapy and immune checkpoint therapy (6–8), although the absence of demonstrable benefit in a recent Phase 3 trial indicates that a deeper mechanistic understanding is essential for the effective therapeutic use of these agents. An important genetic paralog, IDO2, has been relatively less investigated, but it has been recently implicated in B cell-mediated autoimmunity (9,10). Intriguing preclinical studies in mice also suggest genetic and biochemical interactions between IDO1 and IDO2 which may influence IDO1-induced Treg activation (11,12), a possibility supported by studies of human dendritic cells where expression of both of these distinct *IDO* genes has been noted (13).

In tumors, overexpression of IDO2 appears to be much less common than IDO1, but PDAC displays the unusual feature of overexpressing both IDO1 and IDO2 (14,15). Two single nucleotide polymorphisms (SNPs) in the coding region of human *IDO2* have been described (rs4503083 and rs10109853), which decrease enzymatic activity through amino acid substitution at the active site (R248W) or by truncation of the enzyme (Y359X), respectively (16). Notably, while both of these SNPs are quite prevalent in human populations, their clinical significance has remained undefined (16). In this study, we have obtained genetic evidence supporting IDO2's relevance to PDAC tumorigenesis using a *Kras*-driven PDAC mouse model (17) in which *Ido2* was genetically targeted for deletion, in conjunction with an analysis of the prevalence of the two *IDO2* inactivating SNPs in PDAC patients. Based these findings, we performed retrospective analyses of treatment outcomes for surgically resected PDAC patients based on their IDO2 genotype status. For the subset of patients who had received adjuvant radiotherapy during the course of treatment, these analyses uncovered a significant association between *IDO2* deficient status and improved disease free survival, a finding with potential ramifications for informing future treatment decisions for this intractable disease.

## Materials and Methods

### Mouse husbandry and histopathology.

*IDO2* nullizygous mice and the genetically defined KC mouse model of KRAS-induced PDAC on a common C57BL/6J background strain have been described (11,17). *PDX-1-cre;LSL-Kras*$^{G12D}$ transgenic mice (KC mice) develop pancreatic intraepithelial neoplasias (PanINs) with complete penetrance along with sporadic focal pancreatic carcinomas with reduced penetrance due to PDX1-*cre*-mediated activation of the latent oncogenic *Kras*$^{G12D}$ allele in pancreatic progenitor cells (17). These KRAS-induced lesions elicit a robust inflammatory response including B cell contributions (18) where IDO2 may act to influence the tissue microenvironment (10). To investigate this hypothesis in an autochthonous pancreatic tumor setting we introduced *Ido2*$^{-/-}$ (*Ido2*-nullizygous) alleles (11) into the *LSL-Kras*$^{G12D}$ mouse strain. Mice were interbred to generate *Ido2*$^{+/+}$ and *Ido2*$^{-/-}$ KC siblings in which KRAS is activated with similar kinetics for longitudinal comparisons of disease initiation and progression for 11–13 months duration. Two independent cohorts were generated and analyzed. Histological analysis of pancreatic lesions was conducted by standard methods as previously described (17,19).

### Flow cytometry analysis of infiltrating immune cells in mouse pancreata.

Single cell suspensions were prepared from resected pancreata using a gentleMACS Octo Dissociator with the Tumor Dissociation Kit as per the manufacturers' instructions. Levels of the following cell surface markers were directly measured by flow cytometry on a BD FACSCanto (BD Biosciences) in two separate groups as noted and analyzed using FlowJo Software (Tree Star). Group 1: CD45 (APC; BioLegend), CD11b (PE/Cy7; BioLegend), Gr1 (PerCP; BioLegend), CD11c (PE; BioLegend), F4/80 (Alexa Fluor® 488; BioLegend), Fixable Viability Dye (eFluor™ 780; eBioscience) Group 2: CD45 (APC; BioLegend), Th1.2 (Alexa Fluor® 488; BioLegend), IgMa (PE; BD Pharmingen), CD4 (PE/Cy7;

BioLegend), CD8a (PerCP; BD Pharmingen), Fixable Viability Dye (eFluor™ 780; eBioscience).

### Preparation and genotyping of TJUH patient tissue specimens.

The study was conducted in accordance with the ethical guidelines of the Belmont Report with a statement of informed written consent obtained from each subject as appropriate. From the IRB-approved Thomas Jefferson University Hospital dataset (TJUH dataset, all patients in the cohort have given their informed consent) genomic DNA from surgically resected pancreatic tissue specimens (normal and tumor tissues) was extracted using the DNAeasy® Blood and Tissue Kit genomic DNA purification kit (Qiagen Inc., Valencia, CA). DNA fragments containing the IDO2 coding region polymorphisms rs4503083 (Exon 11) and rs10109853 (Exon 9) were amplified by PCR as described previously (IDO2 oligonucleotide primers R248W FWD and R248W REV; Y359X FWD and Y359X REV) (14), as detailed in Supplemental Table SI. PCR reactions were performed in 25 μL using 100 ng of gDNA, 0.5 μg/μL of Taq polymerase (Affymetrix, Santa Clara CA), 1 μl of 10 μM oligonucleotide primers (forward and reverse), 2.5 μL of 10X PCR buffer (Affymetrix, Santa Clara CA), and 0.5 μL 10 mM dNTP Mix (Affymetrix, Santa Clara CA). PCR reaction products were purified using a commercial PCR purification kit (Qiagen Inc., Valencia CA). Each PCR reaction was examined by gel electrophoresis on a 0.75% DNA agarose gel before Sanger sequencing by a commercial provider (Genscript Inc.) using the DNA oligonucleotide primers mentioned above (14). Genotyping steps were blinded to clinical data and familial-sporadic patient status. IDO2 genotype in patients was determined by chromatogram (14).

### Patient tissue specimens.

Two pancreatic cancer patient sets were employed in this study as described below. Demographic and histological data are summarized in Table I.

**TCGA-PAAD dataset:** The cohort used for this dataset included 123 patients from The Cancer Genome Atlas (TCGA) research network database (20). All patients included in the analysis were diagnosed with histologically confirmed PDAC. The set included demographic data, operative findings, histologic features including percentages of lymphocyte and neutrophil infiltrates, RNA expression data, complete variant genotyping as well as survival and recurrence data.

**TJUH resected pancreatic cancer dataset:** The cohort used for this dataset included 77 patients who underwent surgical resection with curative intent at the Thomas Jefferson University Hospital, and we had available tissue for DNA analysis (TJUH, IRB Consented). Patient specimens analyzed included PDAC cases that were familial (n=14, 18%) or sporadic (n=63, 82%). Familial cases were defined as two 1$^{st}$ degree family members with PDAC as established in previous reports (21). Medical history, pre-operative laboratory tests, surgical and histological findings, and oncologic follow up data were recorded from the patients' medical records. Familial data were extracted from medical records and from the Jefferson Pancreatic Tumor Registry (JPTR).

**Creation of a pooled PDA dataset:** TJUH and TCGA datasets were pooled into a single large dataset and screened for duplicates. A unified set containing only PDA patients which underwent primary resection was composed (N=200).

### Human tissue histology and neutrophil-lymphocyte ratio (NLR) analysis.

In the TJUH dataset, slides were reviewed by Thomas Jefferson University pathologists (MC, TV). For slide quantification, hematoxylin and eosin stained sections of formalin-fixed paraffin-embedded tumor from the selected patients were reviewed for pathologic confirmation and the adequacy of tissue quantity and preservation for NLR determination. NLR was derived from the quotient of the absolute neutrophil count and the absolute lymphocyte count. For each sample, NLR was determined in three areas each measuring 0.785 $mm^2$. Final NLRs for each specimen were calculated as the average value of the three areas analyzed. Cases in which no immune cell infiltrates and no tumor cells were found, were excluded from the analysis as well as cases with active billio-pancreatic sepsis (e.g, cholangitis, acute pancreatitis, peripancreatic abscess, etc.). NLR data of 43 TJUH patients (56%) were available for analysis. In the TCGA dataset, hematoxylin and eosin (H&E) stained sections of snap-frozen OCT embedded tissues were reviewed by TCGA participating histopathologists for validation as PDAC and analysis of histologic features. Tumor, normal and stromal components were quantified (in percentages) as well as proportion of immune cells (neutrophils, monocytes, lymphocytes) (22). Slides with no or sparse immune cell infiltrates (Lymphocytes% + Neutrophils% ≤ 10%) were excluded from the analysis. Neutrophil/lymphocyte ratio (NLR) was calculated for each slide and averaged per patient in cases of multiple patient slides. Cases with no visible neutrophilic infiltration were recorded as 0.01% infiltration to allow subsequent $\log_{10}$ transform and Z-score calculation. NLR data of 56 patients (46%) were available for analysis. Overall, 99 patients were included from the pooled dataset.

### Statistical Analysis.

Categorical data were expressed as percentages and continuous data were expressed as mean ± standard deviation. For normally distributed continuous variables, a Student's T-test was used. Variables were assessed for normality of distribution with the Kolmogorov-Smirnov test. Comparisons between genotype groups were performed using Mann-Whitney and Jonckheere-Terpstra (J-T) tests for non-parametric distributions and t-tests for normal distributions (23). Categorical data were compared by $\chi^2$ test or Fisher's exact test. P-values of ≤0.05 were defined as significant.

**Genotype distribution analysis:** Genotype distribution was quantified for all sets as well as separately for familial PDAC cases and sporadic PDAC cases. Genotype distribution of each polymorphism was analyzed for Hardy-Weinberg deviation using $\chi^2$ test and Fisher's exact test. A genotype distribution set of Utah residents (CEPH) with northern and western ancestry was available from the 1000 Genomes Project to be used as a control for comparison of the PDAC patient sets. Patients were grouped into two categories dependent on *IDO2* genotype: homozygous alleles of either R248W (rs4503083) or the Y359X (rs10109853) polymorphism and patients with a double heterozygous genotype (i.e. WT:R248W or WT:Y359X) were considered IDO2 deficient genotypes, whereas all other

combinations were considered as active or partially active IDO2 genotypes. For correlation studies, a three-tier scale (as detailed in Supplementary Table S2) was used to rate the possible combined R248W/Y359X genotypes in terms of probable IDO2 functionality.

***IDO2* genotype correlation with inflammation and neutrophil to lymphocyte ratio (NLR):** TJUH and TCGA specimens in which histopathological examination defined a high tumor cellularity (≥50% cellularity) and evidence of inflammatory infiltrate (Lymphocyte%+Neutrophil% > 10%) were included in the analysis. Bivariate nonparametric Spearman's test was used to assess correlations between IDO2 functionality grade and histologic NLR scores. In-between group comparisons were performed for the pooled dataset with sub-analyses for both datasets. Slides were reviewed by TJUH pathologists (MC, TV).

***IDO2* genotype correlation with immune expression profile:** RNA expression data were collected from the TCGA database using cBioPortal (24,25). Tissue immune cell counts were imputed from GAPDH normalized gene expression levels (based on the Affymetrix 133Plus2 gene expression set) using the validated *MCP-Counts* algorithm described previously (26,27). Neutrophil to lymphocyte ratio scores were imputed from the cell count estimates. Estimates and ratios were compared across *IDO2* gene functionality grades (fully active genotype, partially active genotype, deficient genotype).

**Survival analysis:** The primary hypothesis was that *IDO2* deficient genotypes conferred a favorable prognostic effect. Kaplan-Meier survival analysis stratified by lymph node metastasis was used with log-rank tests to compare survival according to tumor grade, tumor size, perineural invasion and *IDO2* genotype (deficient vs. active/partially active). Survival data from the TCGA-PAAD cohort was limited due to 40% and 63% censorship rates for overall survival (OS) and disease-free survival (DFS), respectively. Survival data from the TJUH cohort revealed censorship rates of 18% and 19% for OS and DFS which were suitable for subsequent survival analysis. In order to utilize the full extent of the survival data, we performed the survival analyses on the pooled dataset as well as in the separate subsets. To prevent possible misclassification biases from patients who were operated upon expecting early-stage disease but actually having advanced disease, analyses of DFS excluded cases in which recurrence of the disease was diagnosed before two months had elapsed from surgery (i.e. DFS ≤2 months). Factors with $P<0.2$ were subsequently included in a Cox multivariate hazard model and were used to assess the impact of IDO2 deficient genotypes on OS time and DFS time. The model was further optimized by sequential inclusion of statistically relevant factors ($P\geq0.2$) until achievement of a final optimal model fit ($P\leq0.05$). P values ≤0.05 were considered statistically significant. Statistical analysis was performed using the Statistical Package for Social Sciences (IBM SPSS, Ver.20, SPSS Inc., Chicago, IL, USA).

## Results

### IDO2 deficiency is associated with reduced PDAC tumor development in mutant *Kras* transgenic female mice and in later onset patients.

Based on evidence of frequent overexpression of *IDO2* in human PDAC tumors (14), we hypothesized that *IDO2* inactivation (i.e., loss of function *IDO2* alleles) might limit the development of pancreatic cancer. *KRAS* mutations occur in over 90% of invasive PDAC and are considered to be an early oncogenic event (28). The transgenic mouse model *Pdx-1-cre;LSL-Kras*$^{G12D}$ (KC), with an inducible oncogenic *Kras* allele that is activated in pancreatic progenitor cells, spontaneously develops pancreatic intraepithelial neoplasia (PanIN) with complete penetrance and PDAC with reduced penetrance (17). To investigate our hypothesis that IDO2 contributes to pancreatic tumor development in this autochthonous tumor setting, we introduced *Ido2*$^{-/-}$ (*Ido2*-nullizygous) alleles into the KC strain through interbreeding (11). Cre-mediated expression of the mutant *Kras* allele resulted in small duct proliferation in the pancreas regardless of the *Ido2* status, along with a decreased frequency of macrophages (CD45$^{+}$ CD11b$^{+}$ Gr1$^{+}$ CD11c$^{-}$ F4/80$^{+}$) and an increased frequency of dendritic cells (CD45$^{+}$ CD11b$^{+}$ Gr1$^{-}$ CD11c$^{+}$) (Fig. 1A and Supplemental Fig. S1). Increased frequencies of neutrophils/MDSCs (CD45$^{+}$ CD11b$^{+}$ Gr1$^{+}$ CD11c$^{-}$), and helper T cells (CD45$^{+}$ Th1$^{+}$ IgM$^{-}$ CD4$^{+}$ CD8$^{-}$) were also associated with mutant *Kras* expression, but were only significant in the mice that also lacked IDO2. No significant differences associated with either *Kras* or *Ido2* status were observed in the frequency of cytotoxic T cells (CD45$^{+}$ Th1$^{+}$ IgM$^{-}$ CD8$^{+}$ CD4$^{-}$) while the overall number of mature B cells (CD45$^{+}$ Th1$^{-}$ IgM$^{+}$) was consistently too low to produce meaningful comparisons. Ductal adenocarcinomas were identified in *Ido2*$^{+/+}$ KC mice with an overall lifetime incidence of 30% compared to 10% in the *Ido2*$^{-/-}$ KC mice (P<0.05, Figure 1B,C). Unexpectedly, the impact of *Ido2* loss on PDAC development in this model was predominantly associated with females, with no ductal adenocarcinomas identified in the 22 female *Ido2*$^{-/-}$ KC mice while the incidence in *Ido2*$^{+/+}$ KC females remained at 30% (P<0.01, Figure 1C). These observations provide genetic evidence that IDO2 can contribute to the progression of early-stage pancreatic precursor lesions to malignant carcinoma and suggest that IDO2's involvement in this process may be subject to some degree of sexual dimorphism. Similar findings of dimorphism in KC mice have been reported by Chang, et al (29) showing that female KC mice on a high fat-high calorie diet were less likely to develop PDAC compared to male KC mice (0% vs. 44% and 33% vs. 50%, at 6 and 9 months, respectively). Similar to these findings in the KC experiment, sequencing of the R248W SNP in the TJHU cohort revealed a significant paucity of R248W homozygous cases (*IDO2* deficient status) in females with sporadic PDAC compared with the CEU control population (OR 0.19, CI95% 0.05–0.65,P<0.01), correlating with the lack of *Ido2*$^{-/-}$ murine females with PDAC. A pooled analysis combining the TJUH dataset with the TCGA dataset (n=200), looking at all PDAC cases yielded similar results, showing a significant absence of females harboring a homozygous R248W (*IDO2* deficient) genotype (OR 0.35, CI95% 0.17–0.74,P<0.01).

### *IDO2* SNPs and patient PDAC outcomes.

Having obtained genetic evidence implicating IDO2 in pancreatic cancer development, we next examined how IDO2 functional status might influence the responses of PDAC patients

to treatment based on the occurrence of the two functionally disruptive SNPs within the coding region of the human *IDO2* gene (16). For the purpose of this study two independent datasets were analyzed, a publically available dataset from the Cancer Genome Atlas Research Network (TCGA-PAAD) and an internally generated dataset collected under an IRB-approved study at the Thomas Jefferson University Hospital in Philadelphia (TJUH) (14,30). Distributions of both *IDO2* coding region SNPs rs4503083 (Y359X) and rs10109853 (R248W) in the TCGA-PAAD set were within the normal range of predicted Hardy-Weinberg equilibrium frequencies ($\chi^2$ test, P=NS), consistent with previously published data (14). In the TJUH patient dataset, a statistically significant 2-fold increase in prevalence of the Y359X homozygous genotype was noted compared to the predicted Hardy-Weinberg frequencies (P<0.05), with additional analysis using Fisher's exact comparison supporting some deviation from equilibrium (P<0.05). However, in a second comparison with the 1000 Genome CEU dataset (Utah residents with Northern and Western European ancestry) as a normal control, we observed no significant deviation in distribution (30).

The TCGA dataset includes histological information on neutrophil to lymphocyte ratio (NLR) for many of the resected tumors, which is categorized as a basic indicator of a pro-tumorigenic inflammatory state. As IDO2 is implicated in immune regulation, the association of IDO2 SNP status with NLR status was interrogated. Pooled analysis of histologic immune data from the TCGA cohort and slides collected from the TJUH cohort (N=99) showed that IDO2 deficient genetic status significantly correlated with decreased neutrophil infiltration and improved (lower) NLR scores (P=0.047 and P=0.036, respectively. Figure 3). RNA expression data available from the TCGA cohort (N=123) further corroborate these findings. *IDO2* genetic status correlated with decreases in the imputed neutrophil to overall-lymphocytes ratio, neutrophil to T-cell ratio and neutrophil to B-cell ratio, and an increase in expression of cytotoxic lymphocytes (P=0.018, P=0.037, P=0.082 and P=0.018, respectively. See Supplementary Figure S2). These results suggest that the loss of IDO2 function is associated with an improved immune signature.

Given the genetic and histological indications of IDO2 involvement in PDAC development, we explored whether an association could also be drawn between *IDO2* genetic status and patient treatment outcomes (31). Due to the high censorship rate (71%) of the disease-free survival (DFS) data in the TCGA-PAAD cohort, we pooled these data with our TJUH cohort to perform survival analyses. In the overall PDAC patient pool, Kaplan-Meier analysis suggested a trend toward increased DFS in patients with *IDO2* deficient status that did not reach a level of significance (median survival 20.3±3.5 vs. 32.4±9.9 months, Figure 2A). This observation suggested the possibility of a wider differential among a specific subgroup of patients that is masked in the overall patient pool. Indeed, a sub-analysis of microscopic tumor involvement (R1 resection margin cases, N=61) revealed that *IDO2* deficient status was significantly associated with a favorable prognosis (Figure 2B, P=0.004) with the *IDO2* deficient group not reaching its median survival in 32 months of follow-up. No significant association was seen in related sub-analyses based on nodal metastasis, tumor grade or tumor size (≤3 cm or >3 cm) in this cohort (Supplementary Figure S3), however, a similar favorable trend was observed in IDO2 deficient patients with large tumors (Size>3 cm, P=0.099). A multivariate Cox regression identified only resection margin status as a

statistically significant independent risk factor (HR 1.81, CI95% 1.10–2.97, P=0.02) for disease free survival. IDO2 deficient status, also showed a strong trend towards positive long-term disease free survival (HR 0.64, CI95% 0.37–1.10, P=0.10). Tumor size, nodal involvement and tumor grade were not found to be significant. These observations encouraged further analysis of whether specific treatment regimens received by patients with positive R1 resection margins might be influenced by *IDO2* genetic status.

### *IDO2* deficient genotype correlates with improved survival of PDAC patients who received adjuvant radiotherapy.

In considering the possible mechanisms through which IDO2 status modifies survival in in patients with positive resection margins, we investigated the use of adjuvant radiotherapy as a potential mediating factor (32). Reinforcing this exploratory logic was the finding of an association between *IDO2* deficient status and reduced Neutrophil to Lymphocyte Ratio (NLR), the latter of which has been identified by several studies as a positive prognostic factor for patients receiving radiotherapy for various cancers including PDAC (33). Thus, an aggregate evaluation of the patient data suggested the possibility that *IDO2* status might be of particular relevance to patients who had received adjuvant radiotherapy during their course of treatment.

A total of 54 PDAC resection cases with DFS ≥2 months and including documentation of administered radiotherapy were available for evaluation in the pooled TCGA-PAAD and TJUH cohorts, together with a corresponding pooled cohort of 77 patients who did not receive radiotherapy. In comparing these two groups, there was no demonstrable improvement in DFS attributable to adjuvant radiotherapy (Figure 4A), consistent with other studies that have reported a lack of benefit. However, inclusion of the *IDO2* status in the survival analysis of the radiotherapy treatment cohort revealed a significant association of the *IDO2* deficient genotype with mean DFS almost doubling in duration mean survival (39.0±6.3 vs. 74.1±6.4 months, Figure 4B, P=0.023) and over 50% survival during the follow up time (median survival was not reached). Importantly, *IDO2* SNP-based stratification of the cohort that did not receive radiotherapy showed no significant evidence of a DFS benefit associated with *IDO2* deficient status (Figure 3C). Elaborating further on the association of *IDO2* host genetic status with radiotherapeutic impact, a Cox multivariate hazard analysis of DFS defined *IDO2* deficient genotype to be an independent positive factor (HR 0.39, 95% CI 0.18–0.83, P=0.015) (Figure 4C). In summary, we conclude from this retrospective analysis that *IDO2* host genotype appears to be a significant predictor of disease-free survival in PDAC patients who received adjuvant radiotherapy as part of their care.

## Discussion

Our results provide clear evidence linking IDO2 function to PDAC pathophysiology and therapeutic response. Locoregional degradation of tryptophan and accumulation of kynurenine catabolites have been broadly implicated in supporting cancer-promoting inflammation and immune escape (2,3,34). Unlike the IDO1 enzyme that has been the primary focus of attention, IDO2 is less widely expressed in human tumors but has been

reported in gastric, colon and renal cancers (35) as well as in PDAC where it appears to be widely overexpressed (14). Our genetic data from mice support the notion that IDO2 can play a contributory role in PDAC tumorigenesis. In contrast, in the case of familial PDAC, we have recently published evidence of increased risk being associated with an *IDO2* deficient genotype status although the trend for sporadic PDAC went in the opposite direction (30). A possible explanation might include a double edged model in which tumor initiation and tumor progression are located on either side of the IDO2 functionality spectrum. However, addressing the observed discrepancies between the mouse KC model, sporadic PDAC, and familial PDAC awaits detailed cellular and molecular interrogation of IDO2's various effects at the tumor cell, the microenvironment, and systematic immune system levels.

As a metabolic modifier of inflammation, IDO2 is likely to exert complex effects. However, it is notable that preclinical studies of the more extensively studied IDO1 enzyme have clearly established its role in mediating tumoral resistance to DNA-damaging chemotherapies and ionizing radiation which are immunogenic in nature (6,7). Correspondingly, a recent clinical study of non-small cell lung cancer (NSCLC) patients by Wang et al. reported that activity levels ascribed to IDO1 corresponded with responsiveness to radiotherapy (36). Specifically, their results showed significant correlations between lower kynurenine/tryptophan ratios assessed pre- and post-radiotherapy and prolonged overall survival. While no direct determination of the specific enzyme responsible for catabolizing tryptophan to kynurenine in these patients was provided in this study, prior evidence suggests that IDO2 is not likely to be directly responsible for this level of activity as it is enzymatically less active than either IDO1 or TDO and its loss has not been found to impact systemic kynurenine/tryptophan levels (37). However, IDO2 has been shown to enable the promotion of regulatory T cell activation by IDO1 (11). Thus, the possibility that IDO2 may be indirectly affecting the ability of IDO1 to regulate T cell function highlights the need to consider IDO2 genetic status in situations where IDO1 has been clinically implicated. Additionally, IDO2 has been shown to have a biological role in supporting pathogenic B cell antibody production in autoimmune settings (9,10). This finding is particularly noteworthy given that protumorigenic B cells, initially identified with squamous cell carcinomas of the vulva and the head and neck, have also been clinically associated with PDAC (18,38), and recent preclinical studies have implicated B cells as contributing to PDAC development (18,38). The striking absence of adenocarcinomas observed in female *Ido2*$^{-/-}$ KC mice precluded our ability to evaluate intratumoral B cells in these animals relative to their more susceptible counterparts, while the extremely low levels of B cells detected in the pancreas made comparative analysis of B cells the local microenvironment untenable. However, expression of oncogenic *Kras* in the pancreas was associated with differences in frequencies of other local immune cell populations, which in several instances appeared to be more pronounced in the animals lacking IDO2. Nevertheless, the trend in these data were not sufficiently robust to rule out the possibility of other functional contributions of IDO2 in this setting, perhaps including non-immune functions, that are yet to be elucidated. Additional studies will be needed to determine whether any of these associations are functionally relevant to the reduced incidence of PDAC associated with IDO2 loss in female mice. Elucidating the basis for PDAC resistance in this preclinical model may have direct

translational relevance given the significant absence of female R248W (*IDO2* deficient) SNP representation in our analysis of PDAC patients.

A major limitation of our study is the low number of PDAC samples evaluated. Still, our results may have important ramifications for PDAC treatment, most notably with regard to use of adjuvant radiotherapy where variable efficacy has been reported in patients. In the adjuvant setting, PDAC therapy has remained little changed for over the past two decades in relying mainly on 5FU or gemcitabine as monotherapies (39–41). Newer combinations such as gemcitabine/capecitabine have shown some promise but they produce higher rates of toxicity, limiting completion of treatment protocols for some patients (42). In the search for ways to improve standard of care, the benefits of adjuvant radiotherapy have been highly debated. Studies assessing the impact of radiotherapy on survival have produced results ranging from increased overall survival (43–45) to no response or even poorer response (46–48). In our pooled analysis of the TCGA-PAAD and TJUH cohorts, we identified a significant association between a functionally ablated host IDO2 genotype and a favorable response to radiotherapy. Given the significant natural variation in the IDO2 coding SNP genotypes among human populations (16), our findings offer a potentially incisive explanation for the large variability in efficacy reported for adjuvant radiotherapy. Thus, *IDO2* genotyping may provide a biomarker to personalize this modality for PDAC patients. Further evaluation of the host *IDO2* gene as a predictive biomarker is warranted to confirm and extend its utility in additional patient populations.

Finally, our results may also have implications with regard to the potential for developing IDO2 inhibitors for use in combination with radiotherapy to treat those PDAC patients harboring *IDO2* active alleles. Some support for this general concept is provided by a recent pilot study in which stratifying brain metastasis patients for the *IDO2* active genotype distinguished a positive trend in overall survival following whole brain radiotherapy together with co-administration of low-dose chloroquine, an indirect inhibitor of IDO2 but not IDO1 activity (31). While most experimental agents in clinical trials at present are selective for IDO1, and no IDO2-specific enzyme inhibitors are yet available, the IDO pathway inhibitor indoximod (D-1MT) has a different mechanism of action that may encompass IDO2 blockade (2,9,16,37,49). Indeed, D,L-1MT administration has been reported to improve the efficacy of radiotherapy in mouse tumor models (7,50). Thus, in theory, the *IDO2* genotype has the potential to serve as a decisional biomarker for distinguishing between PDAC patients who are more likely to respond to adjuvant radiotherapy alone versus those patients who may benefit from the coordinated blockade of IDO2. (5)

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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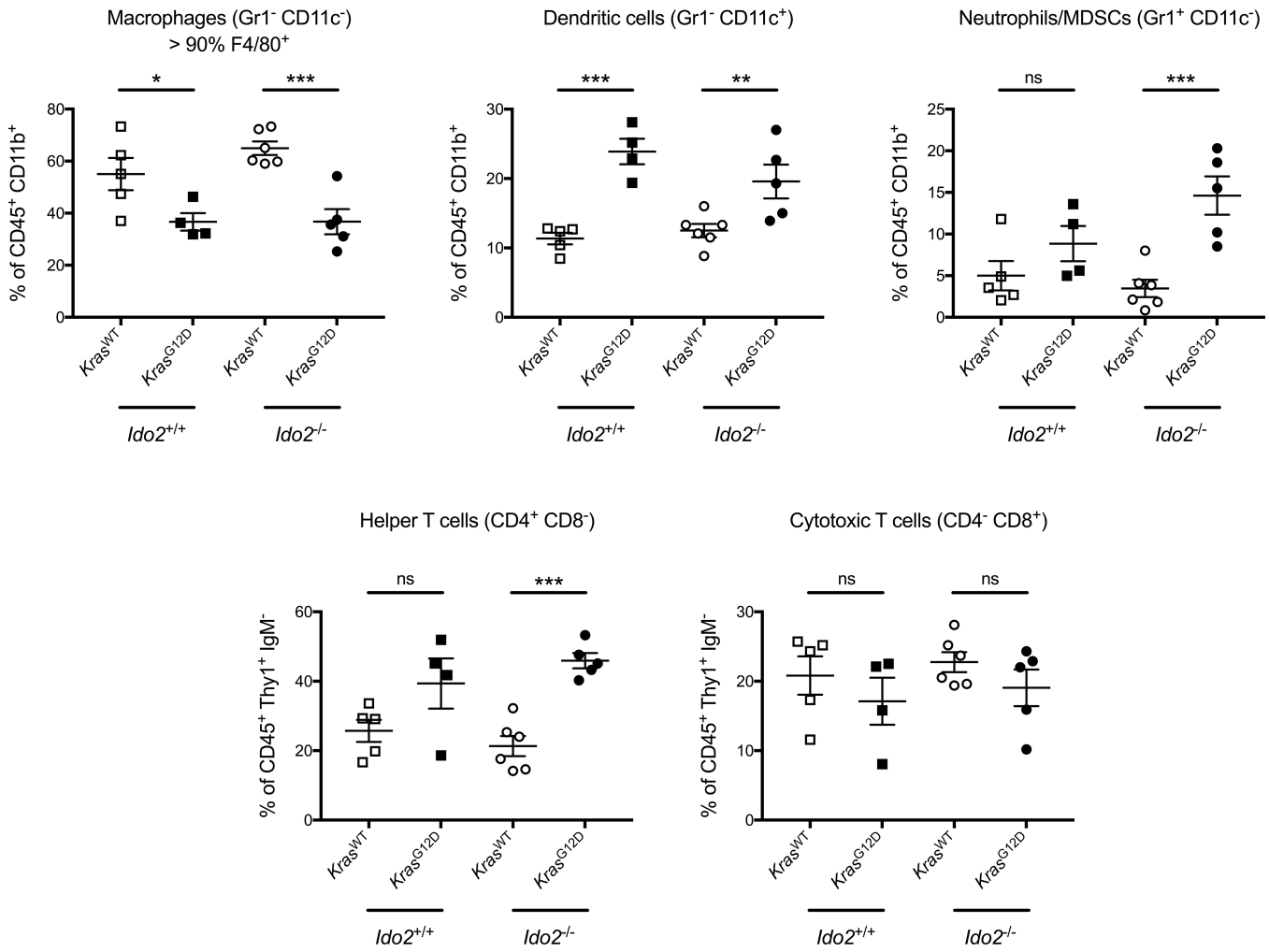
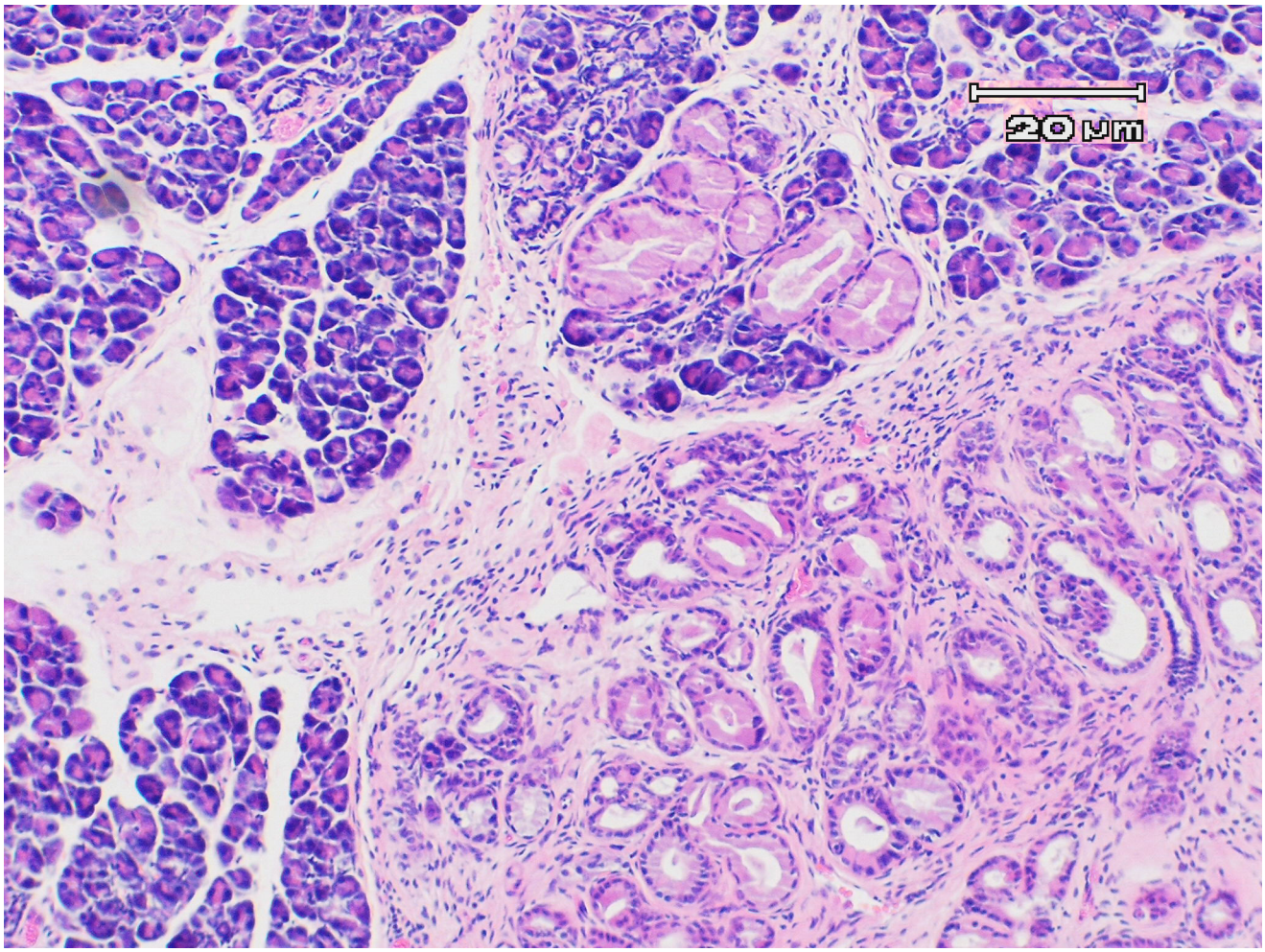
## Translational relevance

Sporadic pancreatic ductal adenocarcinoma (PDAC) develops into a lethal disease which has remained refractory to different treatment approaches including recent advances in cancer immunotherapy. Here we report evidence from both a mouse PDAC model and sequencing data from patient cohorts that the gene encoding IDO2 (an immunometabolic modifier) contributes to the development of *KRAS*-driven pancreatic tumorigenesis. Furthermore, for PDAC patients undergoing treatment, we have identified a significant association between IDO2 genetic deficiency and enhanced efficacy with adjuvant radiotherapy. These data have important translational ramifications due to the high prevalence of *IDO2* inactivating single nucleotide polymorphisms (SNPs) in the human population, meaning that IDO2 genotype analysis could provide a valuable biomarker for informing future treatment decisions.





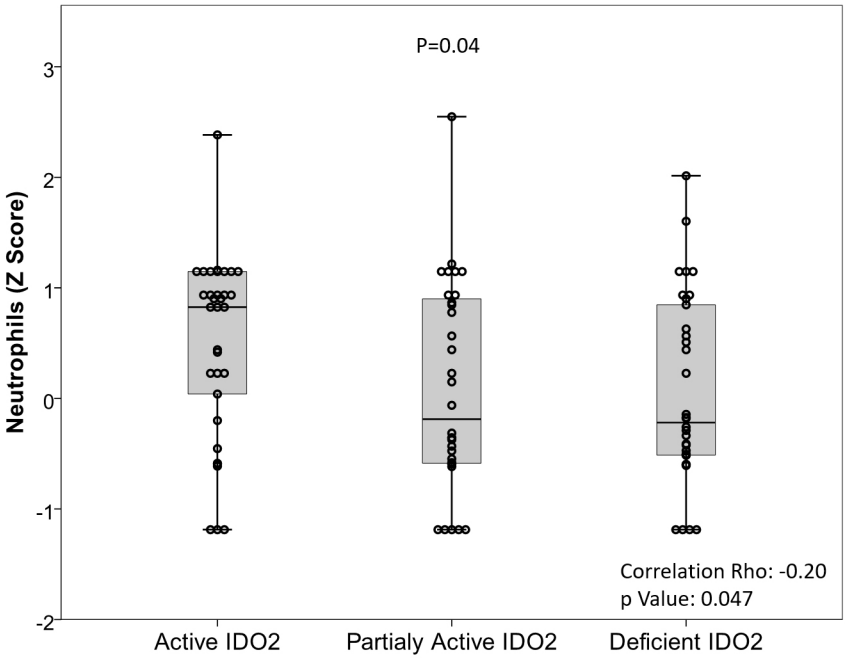

| All mice | *Ido2*$^{+/+}$ | *Ido2*$^{-/-}$ | Female mice | *Ido2*$^{+/+}$ | *Ido2*$^{-/-}$ | Male mice | *Ido2*$^{+/+}$ | *Ido2*$^{-/-}$ |
|---|---|---|---|---|---|---|---|---|
| Invasive PDAC ($P$ = 0.08) | 17/60 (28.3%) | 7/46 (15.2%) | Invasive PDAC ($P$ = 0.01) | 10/37 (27.0%) | 0/24 (0%) | Invasive PDAC ($P$ = NS) | 7/23 (30.4%) | 7/22 (31.8%) |
| Endpoint age (weeks ± SE) | 47.2 ± 2.4 | 47.4 ± 2.6 | Endpoint age (weeks ± SE) | 44.5 ± 2.1 | 47.0 ± 4.4 | Endpoint age (weeks ± SE) | 51.5 ± 5.1 | 47.9 ± 3.0 |

**Figure 1. *Ido2* deficient mice resist the development of KRAS-induced PDAC.**

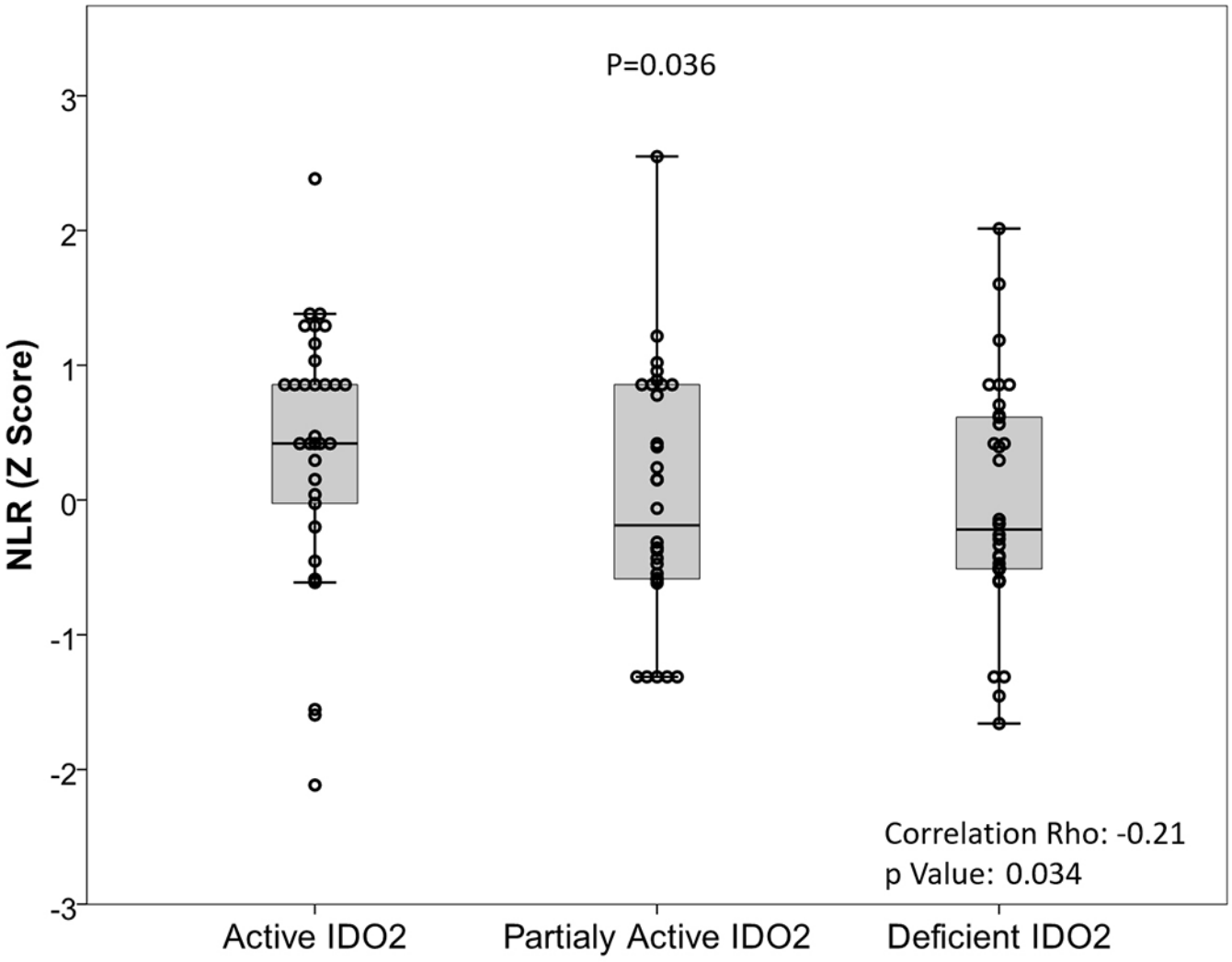
**(A)** Total leukocytes (CD45$^{+}$) obtained from dissociated pancreata from *Kras*$^{WT}$ or *Kras*$^{G12D}$ mice on either an *Ido2*$^{+/+}$ of *Ido2*$^{-/-}$ background were analyzed by flow cytometry for specific immune cell subsets as indicated. Representative examples of the gating strategy employed. are shown in Supplemental Fig 1. Results from evaluable samples are plotted together with the means ± SE (N ≥ 4). **(B)** Representative histologies of pancreatic tissue collected at necropsy. The panels show H&E stained tissues obtained from ♀ mice at necropsy diagnosed as moderately differentiated ductal adenocarcinoma (+/+) or PanIN1–3 exhibiting small duct proliferation only (–/–). **(C)** Quantitation of invasive carcinoma diagnosed in KC mice of *Ido2* wild-type (+/+) or nullizygous (–/–) genotype at 11–13 months of age (lifespan study). Complementing this work, when combining the TJUH

dataset with the TCGA dataset (n=200), we observed a significant absence of female patients harboring a homozygous R248W (*IDO2* deficient) genotype (OR 0.35, CI95% 0.17–0.74,P<0.01).

panel 1

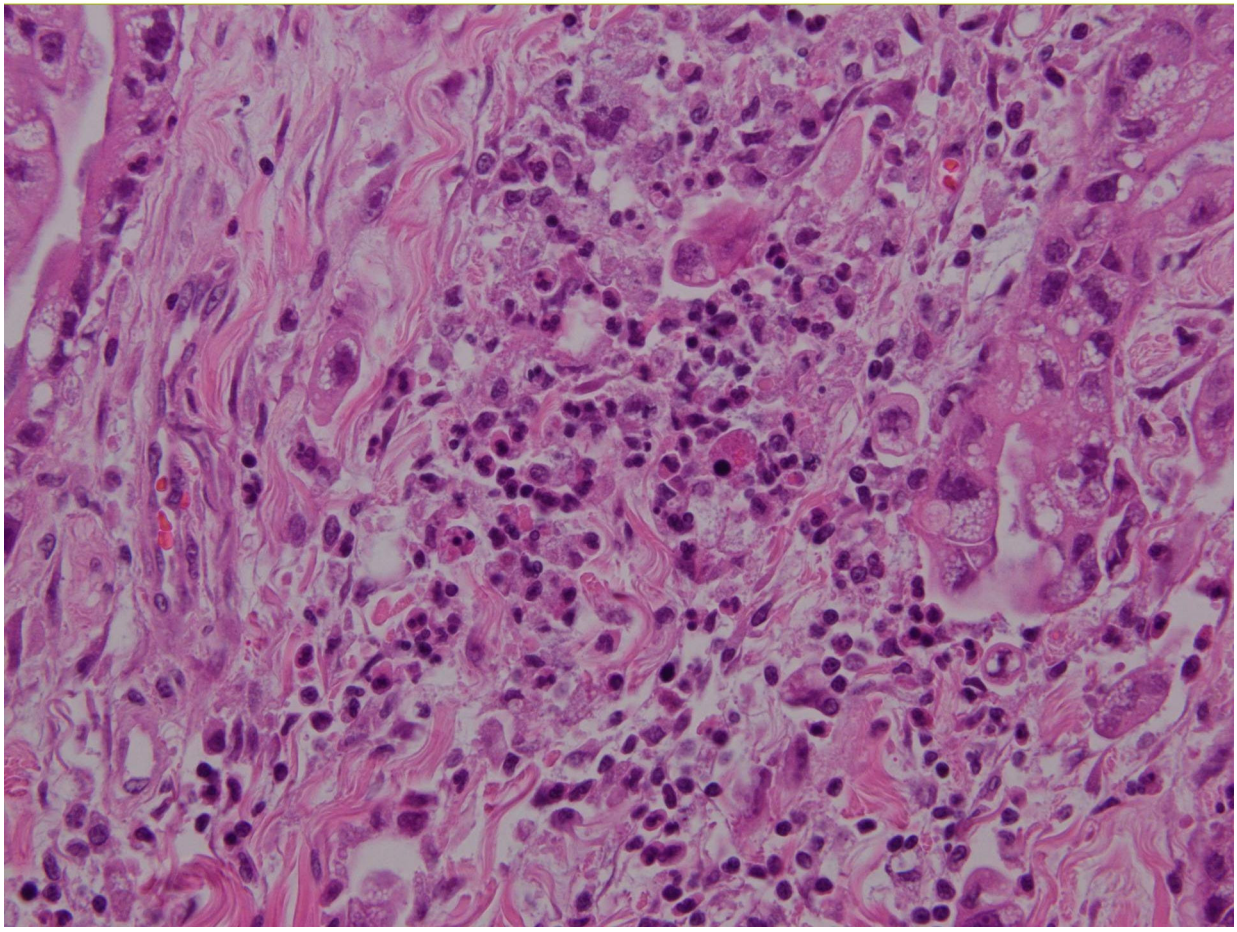


panel 2



panel 1

High NLR



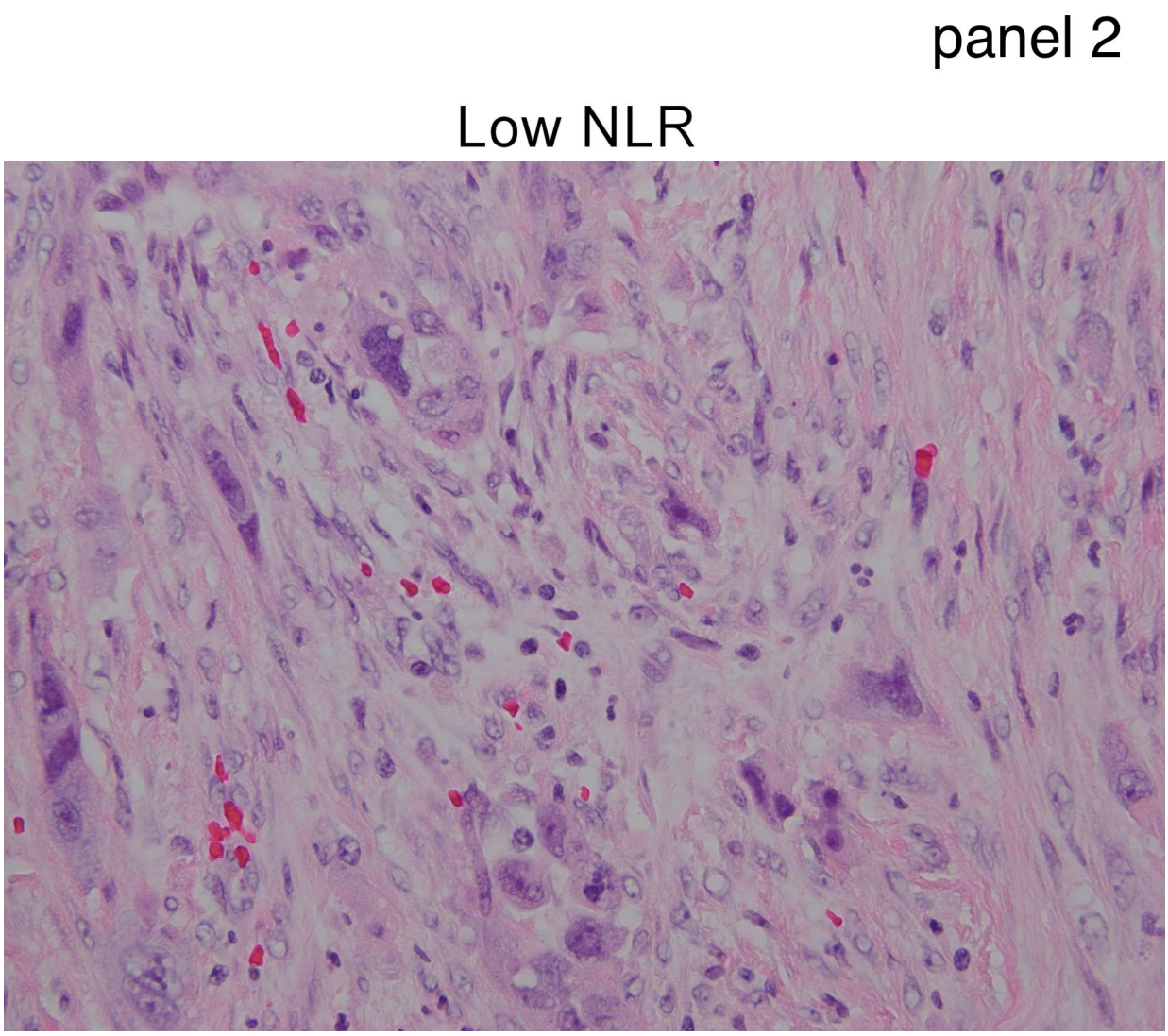



**Figure 2. *IDO2* genotype and histological neutrophil to lymphocyte ratio (NLR).**
**(A)** Evaluable cases from the TCGA and TJUH cohorts were subjected to histopathological analysis of H&E stained specimens to determine neutrophil-to-lymphocyte ratios (TCGA *N* = 95 *left*, TJUH *N* = 43 *right*). NLR scores were segregated according to *IDO2* functional genotype status as defined in Table S2. **(B)** Representative images scored for this analysis from the TJUH cohort.

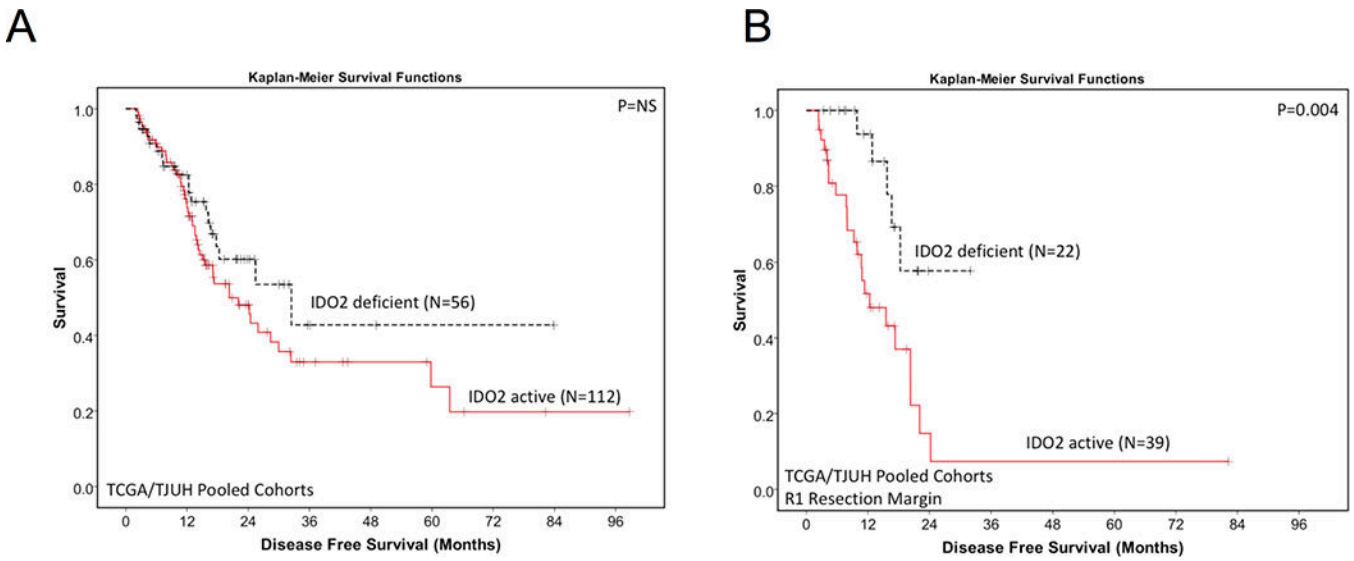



**Figure 3. *IDO2* deficient genotype in human PDAC is associated with improved disease-free survival of patients who receive radiotherapy.**

Disease-free survival analyses of the pooled TCGA-PAAD and TJUH cohorts stratified by IDO2 functional genotype. **(A)** Kaplan-Meier analysis of all cases (*N* = 168). **(B)** Kaplan-Meier analysis of cases with evidence of microscopic tumor involvement (R1 resection margins, *N* = 61).

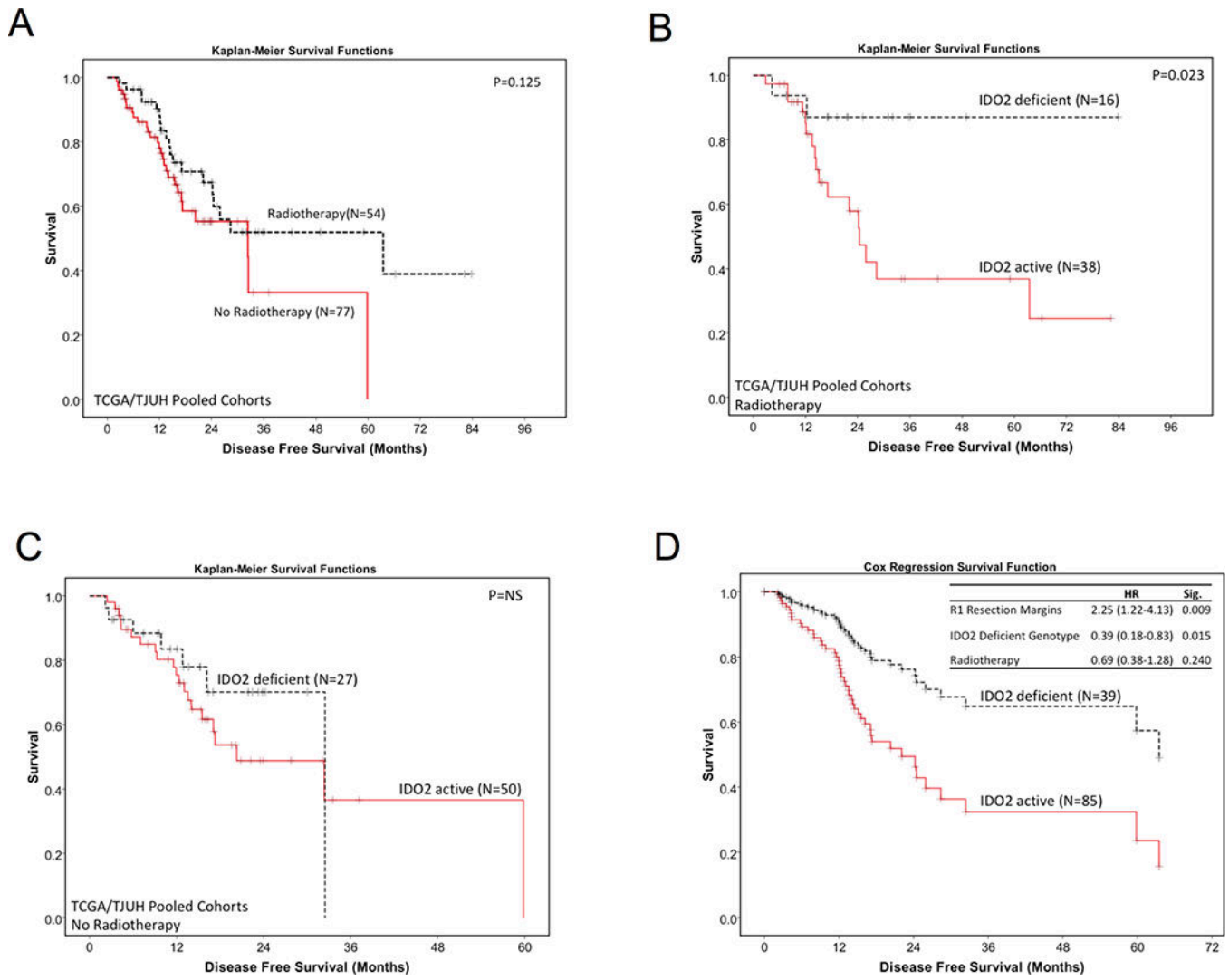



**Figure 4.** ***IDO2*** **deficient genotype in human PDAC is associated with improved disease-free survival of patients who receive radiotherapy.**

Disease-free survival analyses of the pooled TCGA-PAAD and TJUH cohorts stratified by IDO2 functional genotype. **(A)** Kaplan-Meier analysis of pooled cases (N=168). **(B)** Kaplan-Meier analysis of cases receiving radiotherapy (N=54). **(C)** Kaplan-Meier analysis of cases not receiving radiotherapy (N=77). **(D)** Cox multivariate hazard analysis (N=124).

## Table I.
## Patient and tissue specimen characteristics.

Demographics, genetic and clinicopathological characteristics are listed.

| | TJUH (N= 77) | TCGA (N=123) | P |
|---|---|---|---|
| | N (%) | N (%) | |
| **Gender** | | | |
| Male | 38 (49%) | 68 (55%) | *NS* |
| Female | 39 (51%) | 55 (45%) | |
| **Race** | | | |
| White/European | 67 (87%) | 109 (89%) | |
| African American | 5 (7%) | 5 (4%) | *NS* |
| Asian | 3 (4%) | 6 (5%) | |
| Other | 2 (2%) | 3 (2%) | |
| **Age (Years)** | 66.9 (±9.3) | 65.3(±11.2) | *NS* |
| **rs4503083 (Y359X)** | | | |
| Y359/Y359 | 52 (68%) | 84 (68%) | |
| Y359/359X | 18 (23%) | 37 (30%) | |
| 359X/359X | 7 (9%) | 2 (2%) | |
| **rs10109853 (R248W)** | | | |
| R248/R248 | 23 (30%) | 58 (47%) | |
| R248/248W | 39 (51%) | 46 (37%) | |
| 248W/248W | 15 (19%) | 19 (16%) | |
| **Familial PDA** | 14 (18%) | N/A | |
| **Histopathology** | | | |
| Tumor Size (cm) | 3.6 (±1.6) | 3.9 (±1.6) * | *NS* |
| Poor Tumor Grade | 20 (26%) | 36 (29.3%) | *NS* |
| Metastatic Lymph Nodes | 57 (74.0%) | 94 (76.4%) | *NS* |
| Lymph Node Ratio | 0.19 (±0.22) | 0.08 (±0.28) | *NS* |
| Positive Surgical Margins | 23 (29.9%) | 50 (43.5%) * | *NS* |
| Perineural Invasion | 64 (83.1%) | N/A | |

*TJUH:* Thomas Jefferson University Hospital. *TCGA:* The Cancer Genome Atlas. *N/A:* Non-Available.

* Missing TCGA data in 8–10 cases.