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Table 2. Associations between pneumococcal isolates with vaccine serotypes and BAPS clusters. The vaccine serotypes are those present in the seven valent pneumococcal vaccines (4, 6B, 9V, 14, 18C, 19F, and 23F), and all records present in the MLST database at the time of BAPS analysis were used to estimate ORs. Reestimation using records entered into the database since the initial analysis did not substantially alter the results (22).

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Vaccine serotypes</th>
<th>Totals</th>
<th>ORs</th>
<th>95% Confidence intervals</th>
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<td>1</td>
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<td>1357</td>
<td>0.54</td>
<td>0.475 to 0.622</td>
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<tr>
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<td>1027</td>
<td>1645</td>
<td>1.87</td>
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<td>730</td>
<td>0.90</td>
<td>0.768 to 1.061</td>
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Inhibition of Hedgehog Signaling Enhances Delivery of Chemotherapy in a Mouse Model of Pancreatic Cancer

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Pancreatic ductal adenocarcinoma (PDA) is among the most lethal human cancers in part because it is insensitive to many chemotherapeutic drugs. Studying a mouse model of PDA that is refractory to the clinically used drug gemcitabine, we found that the tumors in this model were poorly perfused and poorly vascularized, properties that are shared with human PDA. We tested whether the delivery and efficacy of gemcitabine in the mice could be improved by coadministration of IPI-926, a drug that depletes tumor-associated stromal tissue by inhibition of the Hedgehog cellular signaling pathway. The combination therapy produced a transient increase in intratumoral vascular density and intratumoral concentration of gemcitabine, leading to transient stabilization of disease. Thus, inefficient drug delivery may be an important contributor to chemoresistance in pancreatic cancer.

Pancreatic ductal adenocarcinoma (PDA) is among the most intractable of human malignancies. Decades of effort have witnessed the failure of many chemotherapeutic regimens, and the current standard-of-care therapy, gemcitabine, extends patient survival by only a few weeks (1–3). Oncology drug development relies heavily on mouse models bearing transplanted tumors for efficacy testing of agents. However, such models of PDA respond to numerous chemotherapeutic agents, including gemcitabine (4–9), which suggests that their predictive utility may be limited. Genetically engineered mouse models of PDA offer an alternative to transplantation models for preclinical therapeutic evaluation. We have previously described KPC mice, which conditionally express endogenous mutant Kras and p53 alleles in pancreatic cells (10) and develop pancreatic tumors whose pathophysiological and molecular features resemble those of human PDA (11). Here, we have used the KPC mice to investigate why PDA is insensitive to chemotherapy.

We first compared the effect of gemcitabine on the growth of pancreatic tumors in four mouse models: the KPC mice and three distinct tumor transplantation models (12, 13). Gemcitabine inhibited the growth of all transplanted tumors, irrespective of their human or mouse origin (Fig. 1A), but did not induce apoptosis (Fig. 1B). Rather, proliferation was substantially reduced in all transplanted tumors (Fig. 1A). In contrast, most tumors (15 of 17 tumors) in gemcitabine-treated KPC mice showed the same growth rate as in saline-treated controls (Fig. 1C). This is consistent with clinical results in which only 5 to 10% of patients treated with gemcitabine demonstrate an objective radiographic response at the primary tumor site (3). Two KPC tumors demonstrated a transient radiographic response at the primary tumor site (3).
response as detected by high-resolution ultrasound (13), which correlated with high levels of apoptosis (Fig. 1D and fig S1). Additionally, proliferation was diminished in gemcitabine-treated KPC tumors shortly after treatment but to a lesser extent than in transplantation models (fig S1).

Transplantation of low-passage cells derived from KPC tumors yielded subcutaneous tumors that were sensitive to gemcitabine treatment (Fig. 1A, syngeneics), suggesting that innate cellular differences are unlikely to explain the chemoresistance of KPC tumors. We therefore assessed the metabolism of gemcitabine [2′,2′-difluorodeoxycytidine (dFdC) to its active, intracellular metabolite, gemcitabine triphosphate [2′,2′-difluorodeoxycytidine triphosphate, (dFdCTP)], by means of high pressure liquid chromatography (HPLC). Consistent with the results of clinical studies (14), circulating gemcitabine in wild-type mice was rapidly deaminated to its inactive metabolite, 2′,2′-difluorodeoxyuridine (dFdU), resulting in a short half-life for gemcitabine (fig. S2, A and B). dFdCTP was present in transplanted tumor tissues and control tissues, but was undetectable in KPC tumors (table S1). Thus, dFdCTP accumulation in pancreatic tumor tissue distinguished transplantation and KPC models of PDA and correlated with the responsiveness to gemcitabine. Changes in the expression of genes involved in gemcitabine transport are unlikely to explain the difference in gemcitabine accumulation in transplanted and KPC pancreatic tumors (fig. S2, C and D).

Impaired drug delivery is another possible mechanism of chemoresistance (15, 16). We investigated drug delivery by characterizing tumor perfusion in each model. First, we delineated functional vasculature through the intravenous infusion of a plant lectin (Lycopersicon esculentum) in anesthetized mice; we followed this with the common fluorophore detection of blood vessels in harvested tissues using an antibody to CD31 (fig. S3). We found that transplanted tumors dem-onstrated a patent vasculature, whereas KPC tumors had a dysfunctional vasculature. Indeed, only 32% of CD31+ blood vessels in KPC tumors were labeled with lectin as compared with 78 and 100% of vessels in transplanted tumors and normal pancreas, respectively. Second, to evaluate whether intravascular delivery and penetration of small-molecule drugs is impeded in KPC tumors, we intravenously coadministered lectin with the autofluorescent drug doxorubicin (Fig. 2, A and B, and fig S4) (17). Confocal microscopy revealed a marked decrease in doxorubicin delivery to KPC pancreatic tumors as compared with adjacent control tissues and transplanted tumors, confirming an inefficient drug delivery over a short time course. Third, using high-resolution contrast ultrasound we found that the delivery of gas-filled liposomes (microbubbles with a mean diameter of 2.6 μm) was more efficient in transplanted tumors than in KPC tumors (Fig. 2, C and D, and fig. S5). Finally, we performed dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) on transplanted and KPC tumors (Fig. 2, E and F, and fig. S6). After administration of the contrast agent gadolinium-diethyltriaminepentaacetic acid (Gd-DTPA), we observed substantial signal enhancement in the periphery of transplanted tumors, whereas the tumor cores exhibited a variable heterogeneous pattern of enhancement, which is consistent with the central necrosis observed in histological sections (fig. S6C). In contrast, we observed efficient delivery of Gd-DTPA to the tissues surrounding KPC tumors with little or no signal enhancement within the tumor body, despite little necrosis in these tumors (fig S6D). Collectively, these results suggest that drug delivery is impaired in KPC pancreatic tumors.

We next investigated the vascular content and tissue architecture in transplanted and KPC tumors. The viable (peripheral) portions of transplanted tumors were densely vascularized (fig. S7A), and neoplastic cells made direct contact with blood vessels (Fig. 3A). In contrast, blood vessel density was markedly decreased within the parenchyma of KPC and human pancreatic tumors, and vessels were embedded within the prominent stromal matrix that is characteristic of these tumors and of primary human ductal pancreatic cancer (Fig. 3, B to D, and figs. S7 and S8). Neoplastic cells in both KPC and human pancreatic tumors were widely spaced from blood vessels as compared with those in transplanted tumors, reflecting these differences in stromal content (Fig. 3E). Using computer-aided image analysis, we confirmed the paucity of vasculature in an independent cohort of 18 human PDA specimens as compared with normal pancreatic tissues and chronic pancreatitis (CP) samples (Fig. 3F and fig S9). Our findings demonstrate that increased vascular content is not a prerequisite for ductal pancreatic cancer progression and suggest that the hypovascularity and vascular architecture of PDA tumors may impose an additional limitation to therapeutic delivery.

We hypothesized that disrupting the stroma of pancreatic tumors might alter the vascular network and thereby facilitate the delivery of chemotherapeutic agents. We studied an inhibitor of the hedgehog (Hh) pathway because pancreatic Hh signaling from neoplastic cells to stromal cells promotes stromal desmplasia (18, 19). Binding of Hh ligands to the Patched1 receptor relieves repression of the 12-transmembrane protein Smo (20), resulting in activation of the Gli family of transcription factors. Although Sonic Hedgehog (Shh) is overexpressed in the neoplastic cells of both human (20) and KPC (21) pancreatic tumors, Gli activity is restricted to the stromal compartment (21).

IPI-926 is a semisynthetic derivative of cyclopa-mine (the chemical structure is shown in fig. S10) that potently inhibits Smo (EC50 = 7 nM) with a long half-life (10.5 hours in CD1 mice) and a high volume of distribution (11 L/kg in CD1 mice). The detailed characterization of IPI-926 in cell-based and in vitro assays will be published separately. Daily oral administration of 40 mg/kg of IPI-926 to KPC mice resulted in a measurable accumulation of drug in PDA tissues (fig. S11A) and a significant decrease in the expression of Gli1, a transcriptional target of the Hh pathway (fig. S11B). The effects of Smo inhibition on tumor histopathology and perfusion were investigated in KPC mice after 8 to 12 days of treatment with IPI-926 or gemcitabine, alone or in combination (IPI-926/gem). In contrast to mice treated with vehicle or gemcitabine, which exhibited profuse desmoplastic tumor stroma, mice treated with IPI-926 or IPI-926/gem were depleted of desmoplastic stroma, resulting in densely packed ductal tumor cells (fig. S12, A to D). The effect of Smo inhibition on the stroma was also evidenced by a decrease in Collagen I content (fig. S12, E to H). These differences were not apparent in mice treated for only four days (fig. S13, I to L). However, co-immunofluorescence performed on tumors treated for four days with vehicle or IPI-926 found a reduced proliferation in α-smooth muscle actin (α-SMA)-positive stromal myofibroblasts (figs. S11C and S13, A and B). This decrease in proliferation was balanced by an increase in proliferation of α-SMA-negative cells (fig. S11D).

Smo inhibition also had a profound effect on the tumor vasculature, with a significantly higher mean vessel density (MVD) noted in the tumors from IPI-926-treated mice (Fig. 4A and fig. S14, A to D). This effect was most notable in IPI-926/gem treated mice, in which the MVD approximated that of normal pancreatic tissue. An increased CD31 content was also present after 4 days of treatment (fig. S14, E to H). At this early time point, numerous isolated CD31-positive cells were noted, which is consistent with the active formation of new endothelial precursors after Smo inhibition. Indeed, co-immunofluorescence for proliferation and endothelial markers confirmed a significant increase in proliferating endothelial cells after IPI-926 treatment (figs. S11E and S13, C and D). The increased MVD observed in IPI-926-treated mice also correlated with a more effective delivery of doxorubicin to tumor tissues (Fig. 4B and fig. S14, I to L).
Fig. 1. Pancreatic tumors in KPC mice are largely resistant to gemcitabine. Mice bearing pancreatic tumors were treated systemically with gemcitabine (*P < 0.05, Mann-Whitney U rank sum test). Solid lines indicate the mean and dashed lines indicate the mean without responders. (A) Percent change in tumor volume in transplantation models (13) treated with saline (blue) or 100 mg/kg gemcitabine, Q3Dx4 (red). (B) Gemcitabine treatment did not induce tumor cell apoptosis in the transplantation models as measured by immunohistochemistry (IHC) for CC3. (C) Percent change in volume of tumors in KPC mice treated with saline (blue), 50 mg/kg, or 100 mg/kg of gemcitabine, Q3Dx4 (red). Two of 17 KPC tumors responded transiently to gemcitabine, as assessed by ultrasonography (yellow). (D) Increased apoptosis was evident only in the KPC tumors that transiently responded to the drug (yellow).

Fig. 2. Pancreatic tumors in KPC mice are poorly perfused. Direct immunofluorescent detection of plant lectin (red) and doxorubicin (green) infused into transplanted (A) and KPC (B) tumors, along with hematoxylin and eosin–stained adjacent sections (inset). Scale bar, 200 μm. Doxorubicin was effectively delivered to transplanted tumors (n = 5 tumors) but poorly delivered to KPC tumors (n = 4 tumors) relative to surrounding tissue. Perfusion of microbubbles (green) into transplanted (C) and KPC (D) tumors visualized with contrast ultrasonography. Transplanted tumors were well perfused (n = 6 tumors) as compared with KPC tumors (n = 8 tumors). Tumors are outlined in yellow. Scale bars, 1 mm. DCE-MRI demonstrated increased perfusion and extravasation of Gd-DTPA (high delivery is indicated by white or yellow) in (E) transplanted tumors (n = 6 tumors) as compared with (F) KPC tumors (n = 6 tumors). Tumors are outlined in blue. Scale bars, 2 mm.
We found that the concentration of gemcitabine metabolites in KPC tumors was elevated by 60% after 10 days of pretreatment with IPI-926/gem (Fig. 4C) (P = 0.04, Mann-Whitney U rank sum test). These data suggest that depletion of pancreatic tumor stroma stimulates angiogenesis and consequently augments drug delivery. Whole-tissue mRNA microarray analysis revealed no significant differences in proangiogenic vascular endothelial growth factor (VEGF) expression between vehicle and IPI-926-treated tumors (table S2).

We then investigated the effects of Smo inhibition on cell proliferation and apoptosis. Although IPI-926 alone specifically decreased the proliferation of stromal myofibroblasts, it had little effect on overall cellular proliferation, which is consistent with the finding that conditional Smo deletion in pancreatic cells does not alter the progression of mutant Kras-induced pancreatic tumors (22). IPI-926/gem–treated tumors harbored many dead and dying cells as evidenced by a significant increase in staining for the apoptotic marker cleaved caspase 3 (CC3) (Fig. 4D).

Lastly, we performed an intervention survival study on KPC mice, monitoring tumor volume biweekly by means of three-dimensional ultrasonography. KPC mice treated with gemcitabine alone or IPI-926 alone showed no survival benefit in comparison with vehicle-treated controls. In contrast, combination treatment with IPI-926/gem extended the median survival of KPC mice from 11 days to 25 days (P = 0.001, log rank test), yielding a hazard ratio of 0.157 ± 0.458 95% confidence interval (CI) (Fig. 4E). Most IPI-926/gem–treated tumors (14 of 17 tumors) exhibited a transient decrease in size within 1 to 2 weeks of treatment (fig. S15). In contrast, only a minority of tumors treated with gemcitabine (2 of 10 tumors) or IPI-926 (2 of 10 tumors) demonstrated objective ultrasonographic responses to treatment. In addition, IPI-926/gem treatment resulted in a significant decrease in metastases to the liver (P = 0.015, Fisher’s exact test) (Fig. 4F).

Investigating the biology of tumors at endpoint, we found no differences in the expression of genes associated with gemcitabine resistance in IPI-926/gem–treated tumors (fig. S11G). However, the hypovascularity of IPI-926/gem–treated tumors was restored at endpoint because the MVD in IPI-926 was similar to controls (fig. S11H).

The general resistance of pancreatic cancer to systemic therapies is unusual among common carcinomas and was not predicted by preclinical models (23). Chemotherapeutic agents share two properties: a short half-life and a small therapeutic index (the range of concentration between efficacy and toxicity). Poor tissue perfusion will necessarily produce a substantial decrease in total exposure to drugs with a short half-life. We hypothesize that pancreatic tumors are poorly perfused relative to normal tissues and other tumors and that this is due to aspects of tumor architecture that are specific to the KPC model and to human PDA. Indeed, two groups using contrast-enhanced endoscopic ultrasound have recently reported that human pancreatic ductal adenocarcinomas are poorly perfused and that this feature distinguishes PDA from endocrine tumors and inflammatory diseases of the pancreas (24, 25).

A deficient nonangiogenic vasculature that limits drug delivery may also help explain why patients with pancreatic cancer show a poor response to anti-VEGF therapy (26).

To counteract this barrier to drug delivery, we propose that agents with a long half-life and a high therapeutic index should be the focus of preclinical investigations in pancreatic cancer. We have provided a proof-of-principle that a drug that disrupts a proposed determinant of poor perfusion in PDA, the desmoplastic stroma, can facilitate the delivery and enhance the efficacy of gemcitabine. Unexpectedly, this drug, which inhibits the Hh signaling pathway through effects on Smo, also increased tumor vascular density, contrasting with earlier work that demonstrated a pro-angiogenic role for Hh signaling during development and in adults (27, 28). Ultimately, the vascular content of KPC tumors returned to lower levels, suggesting that the tumors can adapt to chronic Smo inhibition. Although most of the tumors in KPC mice likewise resumed growth after a transient response, our results nonetheless may open new avenues for improving the delivery of...
Fig. 4. Smoothed inhibition facilitates gemcitabine delivery and extends survival. KPC mice were treated for 8 to 12 days with no treatment (NT), vehicle (V), gemcitabine (G), IPI-926 (I), or IPI-926/gem (IG). (A) MVD was elevated after IPI-926 or IPI-926/gem treatment \( (P < 0.05, \text{Mann-Whitney} \ U \ rank \ sum \ test) \). (B) Doxorubicin fluorescence was elevated after IPI-926 alone or IPI-926/gem treatment \( (P < 0.02, \text{Mann-Whitney} \ U \ rank \ sum \ test) \). (C) After treatment with the indicated 10-day regimens, all mice were administered a single dose of gemcitabine, and the concentration of fluorine-bearing metabolites was determined in extracted samples by means of \(^{19}\text{F} \) nuclear magnetic resonance. Gemcitabine metabolite concentration was elevated in IPI-926/gem–treated tumors \( (P = 0.04, \text{Mann-Whitney} \ U \ rank \ sum \ test) \). (D) IHC for CC3 revealed increased apoptosis in IPI-926/gem–treated tumors \( (P = 0.008, \text{Mann-Whitney} \ U \ rank \ sum \ test) \). (E) IPI-926/gem treatment significantly extended survival in KPC mice \( (P = 0.001, \text{log-rank test}; \text{hazard ratio} = 0.157 \pm 0.458, 95\% \text{CI} \ 0.363) \). (F) Fewer liver metastases were observed in IPI-926/gem KPC mice \( (* P = 0.015, \text{Fisher's exact test}) \).