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Specificity Delivers: Therapeutic Role of Tumor Antigen-Specific Antibodies in Pancreatic Cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDA) is among the most deadly cancers with less than 5% of the patients living beyond 5 years post-diagnosis. Lack of early diagnostic biomarkers and resistance to current therapies help explain these disappointing numbers. Thus, more effective and better-targeted therapies are needed quickly. Monoclonal antibodies offer an attractive alternative targeted therapy option for PDA because they are highly specific and potent. However, currently available monoclonal antibody therapies for PDA are still in its infancy with a low success rate of being approved. The challenges faced by these therapies include lack of predictive and response biomarkers, unfavorable safety profiles, expression of targets not restricted to the cancer cells, flawed preclinical model systems, drug resistance and PDA's complex nature. Additionally, discovery of novel PDA-specific antigen targets, present on the cell surface or in the extracellular matrix, is needed. Predictive and response markers also need to be determined for PDA patient subgroups so that the most appropriate effective therapy can be delivered. Serologic approaches, recombinant antibody producing technologies and advances in antibody engineering techniques will help identify these predictive biomarkers and aid in the development of new therapeutic antibodies. A combinatorial approach simultaneously targeting antigens on the PDA cell, stroma and immunosuppressive cells should be employed.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDA) is the fourth leading cause of death by cancer in the US. With its increasing incidence and lack of effective treatments, it is expected to overtake breast and colon cancers to become the second leading cause of cancer-related deaths by 2020¹. It is notably the most aggressive and debilitating malignant disease with a median survival of less than 6 months. Less than 5% of patients have an overall survival of more than 5 years². Inadequate early diagnosis, resistance to current chemoradiation therapies and ineffective treatment options account for these low survival statistics. Gemcitabine has been the established standard of care chemotherapy treatment for PDA since 1997³. The multidrug combination of FOLFIRINOX (oxaliplatin/irinotecan hydrochloride/5-fluorouracil/leucovorin) was recently shown to improve the overall survival over single drug gemcitabine⁴. However, it had an unfavorable safety profile and has not led to a significant impact on the overall treatment outcome for PDA. Nab-paclitaxel (Abraxane) given with gemcitabine is also an active combination and less toxic than FOLFIRINOX. This regimen was recently shown to improve survival but by only a few months more than single agent gemcitabine⁵. Thus, alternative treatment approaches are urgently needed for PDA; this compelling need has propelled the development of new, better-targeted therapies, especially as we understand more about the intra- and inter-cellular pathways involved in the pathogenesis as well as the role of the immune system in PDA.

Monoclonal antibodies are a promising class of targeted therapies for PDA. First, they are very specific in binding their targets on cell surfaces or in the extracellular matrix. This specific antibody-antigen binding results in a well-tolerated safety profile because the non-target tissues are spared. Second, antibody therapies are potent because they can directly and indirectly inhibit tumorigenesis and tumor progression. In addition to directly targeting the tumors, antibodies can mount an effective response against cancer cells through opsonizing, antigen presentation to T-cells, and mediating cell toxicity via natural killer cells or the complement system⁶.

Monoclonal antibody therapies are currently used either alone or in combination with other drugs for several other cancer types. Trastuzumab, a monoclonal antibody that targets human epidermal growth factor receptor 2 (HER2) has been used successfully in the treatment of HER2-positive early-stage and metastatic breast cancer⁷. Rituximab, the first US Food and Drug Administration (FDA) approved monoclonal antibody, has been used as a single agent for the treatment of B-cell non-Hodgkin's lymphoma or in combination for the treatment of chronic lymphocytic leukemia. Rituximab is an anti-CD20 antibody that specifically binds to CD20, a B-cell surface antigen^{8,9}. FDA has also approved cetuximab and panitumumab for the treatment of Kras wild-type colorectal cancers¹⁰⁻¹². Cetuximab and panitumumab target the human epidermal growth factor receptor (EGFR), which is frequently overexpressed in colorectal cancers¹³. The high specificity of the antibodies allows the cancer patients to be screened for the presence of these antigens and then, receive the targeted antibody therapy with minimal side effects. In addition to the targets present on the cancer cells, antibody therapies have also been developed for targeting antigens on immune cells to alter the immune milieu and hence, elicit an anti-tumor immune response.

Ipilimumab blocks the inhibitory cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) receptor on T cells and allows activated T cells to attack cancer cells. Ipilimumab has been approved for the treatment of advanced melanoma^{14, 15}.

In this review, we will discuss the current monoclonal antibody therapies that exist for the treatment of PDA, the challenges faced by these therapies and the approaches that can be utilized to successfully address the challenges, identify predictive biomarkers and new targets for antibody therapies.

CURRENT MONOCLONAL ANTIBODY THERAPIES FOR PDA TREATMENT

Monoclonal antibody developments facing challenges

Although tremendous efforts have been made in the clinical development of monoclonal antibody therapies to target neoplastic cells of PDA, the efficacy of these antibody therapies in treating PDA still needs to be demonstrated. Indeed, several monoclonal antibodies have already failed to prove their efficacy in phase III studies.

EGFR: Cetuximab—EGFR is part of the erbB receptor tyrosine kinase family. When a ligand, such as epidermal growth factor or transforming growth factor- α , binds to EGFR, EGFR dimerizes and a downstream signaling pathway is initiated via the phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways. Overexpression of EGFR has been observed in more than 70% of PDA patients and has been associated with a decreased overall survival. Cetuximab is a chimeric monoclonal antibody that prevents the binding between EGFR and its ligands and eventually, leads to the internalization of the receptor from the cell surface. Cetuximab has been shown to inhibit growth of cells in EGFR-expressing cell lines and tumor in xenograft models in preclinical studies^{16, 17}. A phase II clinical trial exhibited favorable outcomes in 76% of the patients receiving gemcitabine plus cetuximab versus only gemcitabine¹⁸.

However, a subsequent phase III study showed no gain in overall survival with gemcitabine plus cetuximab versus only gemcitabine¹⁹. Several phase II studies have shown that using a combination of cetuximab, gemcitabine and platinum-based drugs (cisplatin or oxaliplatin) has no effect on survival or response when compared to the drug combination without cetuximab²⁰⁻²². Currently, phase I and II studies combining cetuximab with other PDA therapies are being investigated in both the adjuvant as well as the neoadjuvant setting¹⁷.

It is not surprising to see the low success with cetuximab, considering that more than 90% of PDAs carry Kras mutations^{23, 24}. It has been shown that colorectal cancer patients whose tumors carry Kras mutations do not respond to the cetuximab treatment. A recent study has also correlated Kras mutation in codon 12 to decreased survival compared to patients with wild type Kras²⁵. In addition, several pathways utilized by PDA to gain resistance to cetuximab have been identified. Therefore, EGFR is not an ideal target for antibody therapies for PDA. Those resistance mechanisms for anti-EGFR therapies need to be targeted simultaneously to resensitize the patients to anti-EGFR therapies^{26, 27}.

Insulin-like Growth Factor 1 Receptor (IGF-1R): Ganitumab—IGF-1R belongs to the tyrosine kinase family of proteins. Binding of ligands, IGF-1 or IGF-2, leads to a conformational change and autophosphorylation of the receptor, which activates downstream signaling pathways such as the PI3K/Akt and the Ras/Raf/MEK/ERK pathways. These pathways are shown to inhibit apoptosis, and improve cell survival, cell proliferation and invasion^{28, 29}. IGF-1R, IGF-1 and IGF-2 are overexpressed in PDAs and preclinical studies have shown that the IGF-1R signaling cascade plays a pro-oncogenic role³⁰. Ganitumab is a fully human anti-IGF-1R monoclonal antibody that disrupts the binding between IGF-1R and its ligands. Ganitumab has been shown to inhibit growth and survival *in vitro* and *in vivo*²⁸. Following favorable preclinical data and a phase I study³¹, a phase II trial was carried out examining the effects of ganitumab and gemcitabine in metastatic PDA patients³⁰. Ganitumab in combination with gemcitabine showed improved 6-month survival, overall survival and tolerable toxicity. In this same study, the effect of conatumumab with gemcitabine was also examined. Conatumumab is a fully human monoclonal antibody agonist of human death receptor 5 that induces apoptosis and has been shown to improve the activity of gemcitabine in preclinical and phase I studies^{30, 32}. Similar to ganitumab, conatumumab also showed improved 6-month survival in the phase II trial³⁰. However, Amgen terminated the phase III study involving ganitumab and gemcitabine prematurely because they perceived no significant results at the end of the study³³.

It is imperative to have predictive genetic and proteomic markers as well as methods to evaluate the IGF-1R structure to help clinicians deduce the sensitivity of different tumors to IGF-1R³⁴⁻³⁶. Development of novel monoclonal antibodies that target receptors in this pathway other than IGF-1R are also needed³⁷. Finally, a better understanding of the alternate escape pathways upstream and downstream of IGF-1R will help us design better combination therapies³⁸.

Vascular Endothelial Growth Factor (VEGF): Bevacizumab—VEGF binds to and activates VEGF receptors, VEGFR-1 and VEGFR-2. Even though PDA is a hypovascular cancer, PDA overexpresses VEGF, VEGFR on not only the blood vessels but also on the cancer cells. An autocrine VEGF/VEGFR signaling pathway induces cancer cell growth and motility and plays a vital role in the invasion and migration of PDA^{39, 40}. Thus, VEGF is a potential therapeutic target. Bevacizumab is a recombinant humanized anti-human VEGF monoclonal antibody that disrupts the binding between VEGF and its ligand. Bevacizumab has been shown to have a synergistic effect in inhibiting tumor activity when combined with chemotherapies^{41, 42}. A phase II trial in advanced PDA patients showed that bevacizumab in combination with gemcitabine improved the response rate and the median survival time compared to gemcitabine alone⁴⁰. However, the phase III trial of 535 patients showed no difference in survival between the two groups⁴³. Phase II studies using bevacizumab plus capecitabine-based chemoradiotherapy, followed by gemcitabine showed no improvement in overall survival either⁴⁴. Erlotinib, a small molecule inhibitor of EGFR, has been shown to improve survival in PDA patients when used in combination with gemcitabine in a phase III trial⁴⁵. However, when bevacizumab was added to the mixture, there was no significant improvement in overall survival of the patients, even though the progression-free survival was longer with bevacizumab⁴⁶. Along similar lines, cetuximab and bevacizumab used in

combination with or without gemcitabine did not show favorable results⁴⁷. The failed phase III studies infused doubt in the favorable results of another phase II study that combined gemcitabine, capecitabine and bevacizumab leading the investigators to not move forward to a phase III trial⁴². Trials involving bevacizumab as a second-line therapy option have also failed to show much promise^{48, 49}. More recently, bevacizumab has been tested in combination with gemcitabine and other chemotherapies and radiation therapies in adjuvant settings and has continued to show no success⁵⁰⁻⁵².

Thus, the success witnessed in the preclinical PDA models are not being reflected in the clinical trials maybe due to a less complex tumor microenvironment system in the preclinical research settings⁴³. In addition, cancer cells also become resistant to anti-VEGF therapies by resorting to other escape pathways. Ongoing studies, using patient samples from the various discussed clinical trials, aim to elucidate predictive as well as resistance biomarkers to help screen for patients that will benefit from anti-VEGF based therapies as well as design an alternate treatment course once their cancer becomes resistant⁵³⁻⁵⁵.

New antigenic targets of antibody therapies under clinical development

There are several new monoclonal antibody therapies against promising PDA-specific antigens in preclinical and early clinical trials (**Table 1**). However, we may face the exact same challenges with these novel therapies as we did with cetuximab, ganitumab and bevacizumab. Thus, it is important for us to keep searching for new and better antibody therapies and targets.

Mesothelin: SS1P and MORAb-009—Mesothelin is a cell surface glycoprotein that is overexpressed in almost 100% of PDAs but not in normal pancreas. Mesothelin has recently been shown to play a role in cell proliferation and migration *in vitro* and *in vivo*⁵⁶⁻⁵⁸. The specific overexpression of mesothelin in PDA makes mesothelin an inviting therapeutic target. SS1P, a recombinant immunotoxin, comprises of an anti-mesothelin variable F region that is covalently linked to a truncated form of *Pseudomonas* exotoxin. SS1P is a type of an antibody-drug conjugate (discussed more in a following section). SS1P has been shown to kill mesothelin-expressing tumor in preclinical studies⁵⁹. Favorable anti-tumor activity and toxicity profile from recent phase I trials has warranted future trials in PDA patients expressing mesothelin^{60, 61}. However, SS1P suffered from drawbacks such as immunogenicity, toxicity with repeated dosing, and short half-life. Yet, SS1P demonstrated efficacy alone and in combination with other drugs. Thus, in order to avoid the immunogenicity, an improved version was developed and has entered a phase I study⁶². This study has shown significant tumor regression in mesothelioma patients⁶³.

MORAb-009 is a chimeric anti-mesothelin monoclonal antibody. Preclinical studies showed anti-tumor activity against mesothelin-expressing cancer when treated with MORAb-009 plus gemcitabine versus either treatment alone⁶⁴. These results were followed by a phase I trial that showed that MORAb-009 is relatively safe in PDA patients expressing mesothelin⁶⁵. These promising data has resulted in a recently completed phase II study, where MORAb-009 was tested in combination with gemcitabine in locally advanced and metastatic pancreatic patients⁶⁶. The results of this study are currently not available.

Additionally, a pilot study testing radiolabeled MORAb-009 in various mesothelin-expressing cancers, including PDA, has recently been completed⁶⁷.

Prostate Stem Cell Antigen (PSCA): AGS-1C4D4—PSCA, a glycosylphosphatidylinositol (GPI)-linked plasma membrane protein, was first identified as being overexpressed in prostate cancer. PSCA is overexpressed in 60% to 80% of PDA⁶⁸ but not expressed in normal pancreas⁶⁹; thus, PSCA is a potential therapeutic target. Targeting PSCA has yielded anti-tumor activity in preclinical studies. AGS-1C4D4, a fully human anti-PSCA monoclonal antibody, has favorably completed phase I trial in prostate cancer. A following phase II study has shown that AGS-1C4D4 plus gemcitabine improved 6-month survival rate compared to gemcitabine alone in previously untreated metastatic PDA patients⁶⁸.

Gastrin 17: G17DT—Gastrin is a peptide growth hormone that binds to the cholecystokinin-B (CCK-B)/gastrin receptor. Several studies have shown that CCK-B/gastrin receptors, progastrin, glycine-extended gastrin and amidated forms of gastrin are expressed in both PDA cells and resected PDA patient tissue. Gastrin has been shown to promote growth in PDA cell lines as well as tumor growth in mice via endocrine, autocrine and paracrine pathways^{70, 71}. Anti-gastrin antibodies have shown to inhibit PDA proliferation *in vitro* and *in vivo*⁷². Administration of an immunogen, G17DT, induces the formation of anti-gastrin antibodies in humans⁷³. These antibodies neutralize the glycine-extended and amidated forms of gastrin and disrupt their binding to their receptor, and are thereby thought to exert anti-tumor activity. An uncontrolled phase II study showed that an anti-gastrin antibody response was induced in patients receiving G17DT⁷³. G17DT was well-tolerated and patients responding to the immunogen had improved survival when compared with patients who did not respond. A follow up controlled phase III study also showed improved survival in patients developing an antibody response versus patients who did not or patients who received a placebo⁷⁴.

Connective Tissue Growth Factor (CTFG): FG-3019—CTFG plays an important role in the PDA stromal compartment through tumorigenic activities such as modulating cell adhesion, migration and cell growth. In addition, high levels of CTFG mRNAs have been observed in PDA tissue versus normal pancreas. Studies have shown that CTFG from cancer cells induce tumor growth and this pro-tumor effect and metastasis can be inhibited by FG-3019, a fully human anti-CTFG monoclonal antibody^{75, 76}. Very recently, a study also showed that CTFG in the tumor microenvironment confers resistance to gemcitabine in mouse PDA, which can be decreased by FG-3019 treatment⁷⁷. The results of a phase I study presented at 2013 ASCO Gastrointestinal Cancers Symposium involving a combination of FG-3019, gemcitabine and erlotinib showed that the drug combination is well-tolerated in previously untreated patients with advanced PDA⁷⁸.

LESSONS LEARNED FROM CURRENT ANTIBODY THERAPY DEVELOPMENTS

Multiple successful phase I and II trials have not resulted in any successful phase III trials. A thorough examination of the current monoclonal antibody PDA therapies sheds light on the challenges and drawbacks faced by these therapies. Understanding these issues will help design improved antibody drugs and trials going forward. At least five challenges stand out from this examination.

First, patients are not always screened for expression of the antibody targets; in some cases this is due to a lack of predictive and prognostic genetic and proteomic markers for the particular antibody therapy.

Second, preclinical data modeling antibody therapy does not appear to translate directly into the clinic setting as seen in the case of treatment of PDA using bevacizumab. The complexity of human PDA is not replicated in these preclinical models, which are often xenograft based immunocompromised mouse models, and provides a potential explanation for the failure seen in the case of VEGF inhibitors⁴³.

Third, some of the monoclonal antibody therapies are not cancer specific. The targeted proteins are not exclusively expressed by the PDA but can also be expressed on the normal pancreas or other tissues in the body. This non-specific expression often leads to an unfavorable safety profile and unacceptable toxicities in patients. Thus, it is vital to identify antibody targets that are highly specific to the tumor using the improved research tools that are now available for this purpose.

Fourth, there is also the issue of the tumor becoming resistant to a particular treatment as seen in the cases of cetuximab^{26, 27} and ganitumab³⁸. When one pathway in PDA is targeted, the tumor learns to find alternate pathways to become resistant to the treatment and survive. Ongoing research is elucidating the various escape mechanisms utilized by PDA in response to these various monoclonal antibody therapies. By finding different targets in these other pathways that can be inhibited, a strong case for multiple targeting therapies can be established.

Fifth, in contrast to several other solid tumors, PDA is hypovascular. Additionally, the small number of functional blood vessels, complemented with a very dense stroma surrounding the tumor, hinders the different therapeutic agents from being delivered effectively to the cancer cells^{79, 80}.

OVERCOMING THE CHALLENGES IN MONOCLONAL ANTIBODY THERAPY DEVELOPMENT FOR PDA

Where to look for potential antigen targets

A thorough genetic analysis of several PDAs revealed 12 cell signaling cascades that are frequently dysregulated in PDA²³. Proteins that are integral components of these commonly modified pathways provide a list of potential targets for PDA treatment²⁴. In addition, there

has been growing evidence in the literature pointing towards the important role of the stromal compartment in PDA progression and metastasis⁸¹⁻⁸³. The majority of PDA consists of the stroma instead of the PDA cells and greater amounts of stroma have been linked with poor disease prognosis. The stromal compartment consists of fibroblasts, pancreatic stellate cells, blood vessels, immune cells as well as the extracellular matrix that is composed of collagen, hyaluronan, fibrin and fibrinogen. Preclinical and clinical studies have shown an increase in effectiveness of PDA treatment achieved by altering the stromal components^{80, 84, 85}. Immunotherapies also relieve the immunosuppressive PDA tumor microenvironment resulting in anti-tumor activity in the clinic. Thus, not only antigens expressed on PDA cells, but also those present in the different stromal compartments, should be considered as potential therapeutic targets. By developing monoclonal antibodies against these antigens, it will be possible not only to disrupt intracellular pathways within the cancer cell but also the intercellular pathways that exist between the neoplastic cell and its stroma.

Several monoclonal antibody-based immunotherapy strategies aimed at creating a more anti-tumor immune environment are currently being tested in the clinic. In contrast to the view that PDA is a non-immunogenic cancer, several recent studies have shed light on the vital role of the tumor infiltrating immune cells in the PDA stromal compartment in immune suppression as well as anti-tumor activities. Immune suppression to various tumor-related antigens is associated with poor prognosis^{86, 87}. A large number of immune suppressive cells such as tumor-associated macrophages, regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) are present early in the PDA progression. Through different mechanisms, these suppressive cells result in a lower number of functional effector cells such as CD8+ and CD4+ T cells and natural killer cells in the stromal compartment⁸⁸. Tregs suppress effector T cells by direct contact through its inhibitory receptors, CTLA-4 and programmed death-1 (PD-1)⁸⁹. In addition, PDA cells also overexpress ligands such as programmed death receptor ligand 1 (PD-L1) that results in non-functional effector cells⁹⁰. Thus, immunotherapies aimed at creating an enhanced anti-tumor immune environment within PDA are currently being tested in the clinic (**Table 1**).

CTLA-4: Ipilimumab—Ipilimumab, a fully humanized anti-CTLA-4 monoclonal antibody, inhibits the inactivation of effector T cells. A functional CD8+ T cell can induce antigen-specific cancer cell cytotoxicity. Ipilimumab was shown to be effective as a single agent in metastatic melanoma and renal cell carcinoma. However, it failed to be effective as a monotherapy when used in advanced PDA patients⁹¹. Preclinical studies have shown that the effectiveness of anti-CTLA4 antibody is enhanced in the presence of granulocyte macrophage-colony stimulating factor (GM-CSF) cell-based vaccines. Several clinical trials involving an allogeneic, GM-CSF secreting PDA vaccine (GVAX) in resected PDA or metastatic PDA patients have shown the induction of a mesothelin-specific CD8+ T cell response that correlates with an improved disease free survival^{92, 93}. A phase Ib trial testing ipilimumab with or without GVAX was carried out in PDA patients⁹⁴. The mesothelin-specific T cell responses again correlated with survival. It is important to note that there were significant immune-related adverse events with ipilimumab with or without GVAX. The results of this trial showed objective responses together with a better survival in the ipilimumab plus GVAX group when compared to the ipilimumab alone group, thereby

reinforcing the enhanced effect of GVAX in the presence of ipilimumab as seen in earlier preclinical studies.

PD-1/PD-L1: Nivolumab, BMS-936559, CT-011, lambrolizumab and

MPDL3280A—Nivolumab (BMS-936558), a fully human anti-PD-1 monoclonal antibody, inhibited the inhibitory receptor PD-1 and allowed T cells to remain functional and induce tumor cell cytotoxicity in preclinical studies. A follow up phase I study showed that nivolumab was well-tolerated and suggested a potential anti-tumor activity⁹⁵. BMS-936559, a fully human anti-PD-L1 monoclonal antibody, binds to PD-L1 on pancreatic tumor cells and prevents its association with its receptor PD-1 resulting in an anti-tumor immune environment *in vitro*. A clinical trial in advanced cancer patients showed favorable clinical data⁹⁶. Another humanized anti-PD-1 antibody, CT-011, was shown to be safe in patients with advanced hematologic malignancies in a phase I trial⁹⁷. A following phase 2 trial testing CT-011 in diffuse large B-cell lymphoma patients has produced encouraging progression free survival data. CT-011 has undergone testing for various cancer patients, including PDA^{98, 99}. Very recently, a phase Ib trial testing lambrolizumab (formerly called MK-3475), Merck's humanized anti-PD-1 monoclonal antibody, showed promising clinical response in advanced melanoma patients¹⁰⁰. Completed and ongoing clinical trials involving Genentech's human anti-PD-L1 antibody, MPDL3280A, provides further ground for targeting this pathway¹⁰¹⁻¹⁰⁴. These results have paved the path for future trials using PD-1 and PD-L1 antibodies in treating various solid cancers, including PDA.

Nonetheless, it should be noted that PDA's tumor microenvironment is dominated with immune suppressive mechanisms and infiltrated with few effector T cells. It was thus not surprising to see that single agent anti-PD-1 and anti-PD-L1 antibodies did not show objective response in the small number of PDA patients that were tested in the early phase clinical trials. It may be critical to combine PD-1/PD-L1 blockade therapies with those treatments that simultaneously induce the effective T cell infiltration or inflammatory cytokine responses. Therefore, it is intriguing to test the combination of vaccine-based immunotherapy and anti-PD-1/PD-L1 antibodies.

CD40: CP-870,893—CD40 is a costimulatory plasma membrane receptor present on antigen presenting cells (APCs). CD4+ T helper cells initiate a signaling pathway via CD40 that leads to activation of APCs, which eventually results in functional effector CD8+ T cells. Activating CD40 antibodies have been shown to activate APCs without the need for CD4+ T helper cells¹⁰⁵. CP-870,893, is a fully human CD40 agonist monoclonal antibody. A phase I clinical trial using this CD40 agonist plus gemcitabine in previously untreated advanced PDA patients showed a favorable safety profile, an anti-tumor immune response as well as a preliminary therapeutic benefit^{106, 107}. It was surprising to see that CD40 agonist-activated macrophages, instead of the CD8+ T cells, were responsible for the anti-tumor activity via stromal depletion⁸⁵.

What to look for in potential antigen targets

Monoclonal antibodies offer a great therapeutic option because they are specific as well as potent⁶. Several characteristics should be considered when identifying potential antigens for

therapeutic antibody development: 1) the antigens should be overexpressed in PDA and have minimal expression in the normal pancreas; 2) the antigen should be present on the cell surface or in the extracellular matrix rather than inside the cell although it has been shown that antibodies can potentially target intracellular oncoproteins in mouse models^{108, 109}, 3) proteins that alter their cellular location from intracellular compartments to cell surface or extracellular compartments in PDA compared to normal pancreas would be excellent targets. For example, annexin A2 has been shown to translocate to the cell surface in PDA but is present inside the cell in the normal pancreas^{110, 111}. Thus, annexin A2 is a promising new target for PDA therapies.

Approaches to identify new antigenic targets for antibody therapies

Serologic approaches are gaining momentum in the identification of new cancer biomarkers¹¹²⁻¹¹⁷. These cancer biomarkers are beneficial for early detection, predicting response to immunotherapies including monoclonal antibody therapies and the determination of new targets for the development of biologically relevant therapies. These proteins have high potential as future targets for effective PDA treatment. This translational approach will advance the development of new drugs, vaccines and antibody-based therapies that will halt the progression and metastasis of the disease.

As described below, common serologic approaches to identify immunogenic proteins are: serological screening of cDNA expression library (SEREX), protein arrays and seroproteomics/serological proteome analysis (SERPA)^{112, 113, 115, 117}. Although patient sera is a common component in these methods, the source of protein used can vary¹¹⁶. It is imperative to validate antigens found using serologic approaches to prevent the issue of misidentification by mass spectrometry analysis. To confirm that the identified cancer-specific antigen is correct, one can employ western blot, immunohistochemistry or enzyme-linked immunosorbent assay (ELISA) analyses to validate their targets¹¹⁶.

SEREX—SEREX involves expression of tumor cDNA phage library in bacteria¹¹⁸. These expressed proteins are transferred to a membrane and are screened against patient sera. The most well known application of SEREX utilizes sera, from untreated cancer patients or individuals with known genetic susceptibilities for cancer, to screen for cancer-associated proteins that elicit an antibody response. NY-ESO-1 is a famous protein that was found from an esophageal cancer cDNA library using the SEREX technology¹¹⁷. SEREX, however, utilizes proteins expressed in bacteria and thus, does not account for human posttranslational modifications.

Protein Arrays—Protein arrays come in many forms with more choices in the protein source that can be used and are comparatively more high-throughput¹¹². Some protein arrays use tumor cell lysate fractions, which identify proteins in their native conformation with the correct posttranslational modifications. However, these arrays do not identify which specific protein in the fraction instigates the immune response and thus, it would be hard to pinpoint the specific antibody-antigen. In contrast, other protein arrays are printed with recombinant human proteins expressed in bacteria or yeast, allowing the user to know the identity of each protein contained in a spot.

SERPA/Seroproteomics—Seroproteomic approaches utilize and thrive on the technological advances in the field of proteomics^{116, 119}. SERPA involves 2-dimensional electrophoresis (2-DE) followed by mass-spectrometry analysis¹¹². Either patient tumor or cell line lysates are subjected to 2-DE to separate the proteins in the whole cell lysates. Using western blot analysis, the differential antibody response is observed and finally, the proteins of interest are identified by mass spectrometry. Our group has used SERPA to discover annexin A2 as a potential therapeutic target in PDA¹¹⁰ (**Figure 1**). Briefly, pre- and post-vaccination sera from patients that showed favorable clinical and immune responses to GVAX in a phase II clinical trial were used⁹². Cell lysates were obtained from the PDA cell lines that were used for the generation of the vaccine.

The cell lysates were subjected to 2-DE, followed by western blot analysis, where the pre- and post-vaccination sera was compared. Proteins that showed an increased antibody response post-vaccination were identified via mass spectrometry. The discovery of annexin A2 has not only helped identify a new monoclonal antibody therapy for PDA but also led to a better understanding of PDA progression and metastasis^{110, 111}. Ongoing research is aimed at identifying predictive biomarkers for a favorable response to GVAX in PDA patients as well as novel therapeutic targets for PDA.

One major drawback of SERPA is its inability to identify membrane proteins. Both SEREX and SERPA identify linear epitopes, are relatively low throughput and semi-quantitative. Thus, the development of a new high throughput quantitative comprehensive approach identifying native epitopes is needed.

Novel approaches to identify native anti-tumor antibodies

Based on recent advances, it is now possible to produce recombinant monoclonal antibodies from single B cells of human origin¹²⁰⁻¹²². These recombinant antibodies can potentially serve as new monoclonal antibody therapy options for PDA. Briefly, this method involves separating single B cells by flow cytometry, followed by amplification of the different immunoglobulin chains via reverse transcription polymerase chain reaction (RT-PCR). These amplified genes are cloned into an expression vector to produce human monoclonal antibodies *in vitro*. One can imagine an application of this B cell technology to identify and produce monoclonal antibodies for therapeutic use in PDA. For example, single B cells, obtained from laser-capture microdissected PDA tissue¹²³ or from the tumor draining node followed by flow cytometry, could provide a rich source of diverse antibodies that are PDA-specific. However, similar to the validation steps for the seroproteomic approaches, this method necessitates thorough *in vitro* and *in vivo* studies to ensure that the recombinant antibodies produced indeed bind to cancer cells with high specificity and are effective anti-tumor agents. It is also important to note that the isotype of the antibody used should be carefully examined because a change in an antibody's isotype can dramatically change its efficacy¹²⁴.

Lung cancer studies have also used a modified serologic approach where tumor-specific antibodies from tumor infiltrating B cells were used instead of the antibodies from the patient sera¹²⁵. It was shown that the change in antibody source resulted in fewer autoantigens being identified that were also expressed on normal cells and hence, an

increased specificity towards identifying cancer-specific antigens was observed. A similar study in colorectal cancer has also shown that tumor infiltrating B cells are active and secrete tumor antigen-specific immunoglobulins¹²⁶. Thus, using antibodies from the tumor microenvironment, rather than the sera antibodies, may provide a more direct way of identifying biologically relevant PDA-specific antigens that could be targeted using monoclonal antibody therapies.

Advanced antibody engineering technologies

In addition to discovering new PDA antigens that could be targeted, recent advancements in antibody engineering, including the technology developments for antibody-drug conjugates, antibody-cytokine fusions and bispecific/trispecific antibodies, could be utilized for PDA treatment.

Antibody-drug conjugates (ADCs)—The effectiveness of chemotherapies is thwarted by the ineffective drug delivery to the cancer site and the systemic toxicities observed due to the non-discriminatory nature of these therapies. ADCs help overcome both this hurdle. ADCs combine monoclonal antibody to a cytotoxic drug via a stable labile linker. Thus, when the antibody binds the cancer-specific antigen, it is internalized, where the attached drug is released only to specific antigen-expressing cells. Once released, the drug can hinder cell signaling pathways, leading to cytotoxicity. ADCs are thought to reduce the systemic side effects caused by the cytotoxic drugs by the increased specificity and thereby provide a greater therapeutic index. Preclinical and clinical trials using ADC alone have produced better anti-tumor outcomes compared to the trials involving the corresponding unconjugated monoclonal antibody¹²⁷⁻¹³⁰.

The monoclonal antibody can be attached to radioactive particles, chemotherapy drugs or cell toxins. Ibritumomab tiuxetan and iodine-tositumomab are radiolabeled monoclonal antibodies used for the treatment of B-cell non-Hodgkin's lymphoma¹³⁰. FDA has approved brentuximab vedotin and trastuzumab emtansine for the treatments of lymphomas and HER2-positive breast cancers, respectively¹³¹. Brentuximab vedotin links anti-CD30 monoclonal antibody to an anti-mitotic agent, monomethyl auristatin E. Trastuzumab emtansine connects anti-HER2 antibody to maytansine (DM-1), an anti-microtubule agent¹³². Trastuzumab emtansine was shown to have greater efficacy in the clinic with reduced toxicities. The success of trastuzumab emtansine has paved the path for the development of several ADCs in the treatment of blood and solid tumors, including PDA¹³³.

Preclinical studies have produced favorable results for mesothelin-targeting ADCs: MDX-1204 and BAY 94-9343⁵⁸. MDX-1204 is a human anti-mesothelin antibody, MDX-1382, that is linked to duocarmycin, a DNA damaging agent. BAY 94-9343 is a fully human anti-mesothelin antibody that is connected to DM4, an anti-tubulin agent. Both these ADCs are effective anti-tumor agents specific for mesothelin-expressing cells, including PDA, *in vitro* and *in vivo*. Thus, a phase I study testing the acceptable dosage and safety of BAY 94-9343 in advanced solid tumor patients is ongoing.

Cantuzumab mertansine is an ADC that links the humanized huC242 monoclonal antibody to DM-1¹³⁴. HuC242 binds specifically to CanAg, an antigen that is predominantly

expressed on pancreatic, colon and biliary tumors but is expressed at very low levels on normal tissues. *In vivo* studies have demonstrated the improved anti-tumor effects of cantuzumab mertansine over unconjugated huC242 alone or in combination with other chemotherapy options. Subsequent phase I trials have shown cantuzumab mertansine to be a well-tolerated treatment option, providing the rationale for future studies in PDA patients expressing CanAg¹³⁴⁻¹³⁶.

Epithelial cell adhesion molecule (EpCAM) is highly expressed in several solid tumors, including PDA. It is also expressed by PDA stem cells. Although adecatumumab, an anti-EpCAM antibody, was shown to be safe in solid tumor patients, a phase II trial failed to show tumor regression in metastatic breast cancer patients. Thus, an ADC, chiHEA125-Ama, was developed¹²⁷. This ADC links the chimeric anti-EpCAM antibody, chiHEA125, to amanitin, an inhibitor of DNA transcription. It was shown to be an effective anti-tumor agent *in vitro* and *in vivo* and thereby, warranting further evaluation through clinical trials.

Antibody-cytokine fusions—In addition to linking antibodies to drugs, strategies where antibodies are fused to anti-tumor cytokines, such as interleukin-2 (IL-2) or tumor necrosis factor-alpha (TNF- α), have also been tested¹³⁷. Systemic administration of IL-2 has serious toxicities in patients and reduced effectiveness due to its fast clearance from the body¹³⁸. However, tumor-specific administration, via antibodies, has shown to be clinically favorable. L19-IL2 is a fusion protein, where IL-2 is fused to a single-chain variable antibody fragment (L19) that binds specifically to extr domain B (ED-B) of fibronectin¹³⁹. ED-B is expressed on tumor vasculature and stroma whereas it is absent from the fibronectin in the plasma and normal tissues (except certain female reproductive organs)¹³⁸. L19-IL2 is biodistributed in a tumor-specific manner *in vivo* and in patients¹⁴⁰. Enhanced anti-tumor effects and slow clearance rate was observed in preclinical studies. A phase I clinical trial showed that L19-IL2 was well-tolerated in patients with solid tumors¹³⁹. Subsequent animal and patient studies have further shown the efficacy of L19-IL2 in treating hematologic and solid cancers when used in combination with chemotherapies, immunotherapies and other monoclonal antibodies^{137, 140-142}. The recruitment of anti-tumor immune cells following treatment of L19-IL2 in the PDA stroma coupled with the strong PDA-specific expression of ED-B provides a strong rationale for testing L19-IL2 in PDA patients¹³⁸ (**Table 1**).

Bispecific/trispecific antibodies—Bispecific antibodies are recombinant antibodies that can bind two different antigens at the same time. These antibodies can activate/inhibit multiple pathways and increase avidity-based binding by involving two different receptors on cell surfaces. The most commonly used bispecific antibodies recruit effector T cells to the tumor cells by dually binding CD3 on T cells and cancer-specific overexpressed antigens such as EGFR and EpCAM^{143, 144}. Catumaxomab, a bispecific anti-EpCAM and anti-CD3 antibody, has shown to inhibit tumor growth and proliferation in hepatocellular carcinoma xenografts¹⁴⁵. Catumaxomab inhibited tumor growth of PDA xenografts and stem cells. It was also able to enhance effector cytokine production and extend the contact time between the T cells and the cancer cells *in vivo* and *in vitro*^{146, 147}. Catumaxomab has received approval in Europe for use in the treatment of malignant ascites in EpCAM-expressing cancer patients^{148, 149}. A multicenter, phase I/II trial testing catumaxomab for treatment of

peritoneal carcinomatosis in cancer patients, including PDA, has produced favorable clinical outcomes and safety profile¹⁵⁰. Ongoing efforts aim to develop novel bispecific antibodies not only targeting other PDA-specific antigens, such as CD133 (a PDA stem cell marker)¹⁵¹ but also recruiting other T cell populations (such as $\gamma\delta$ T-cells)¹⁵² to the site of action. Bispecific antibodies also provide an alternate solution to overcome the challenges seen with monoclonal antibody therapies such as cetuximab. Bispecific antibodies that are both anti-EGFR and anti-CD3 have shown promising anti-tumor activity at a reduced dosage when compared to cetuximab alone *in vivo*¹⁵³. With advances in antibody engineering technologies, trispecific antibodies are also being developed and evaluated for use in different cancer treatments¹⁵⁴⁻¹⁵⁷.

CONCLUSION AND FUTURE DIRECTIONS

Despite the numerous preclinical studies showing strong efficacy data for new monoclonal antibody therapies, similar successes have not been mirrored in clinical trials. Furthermore, favorable phase I and II clinical trials have not translated into successful phase III studies. PDA is a very complex cancer where not only the tumor cells, but also the tumor microenvironment, contribute substantively to PDA initiation, progression and metastasis. This complexity helps explain why single agent antibody therapies are not successful. In addition, the lack of predictive and response biomarkers prevent the selective treatment of PDA patients that are most likely to benefit from these therapies. Thus, further work needs to be done in two critical areas to help make monoclonal antibody therapies a success for PDA patients. First, combination therapies need to be developed. Using serologic approaches and new recombinant antibody producing methods, novel PDA-specific antigens need to be identified and monoclonal antibodies need to be developed. Advances in the production of antibody-drug conjugates, antibody-cytokine fusions and bispecific/trispecific antibodies will help evolve antibody therapy further. Combination therapy should involve multiple antibodies targeting both intracellular signaling pathways within the cancer cell as well as intercellular pathways between the stroma and the cancer cell. This approach can also be coupled to existing and new chemotherapies, radiation therapies and vaccines. Using this multipronged approach, antibodies against the stromal targets will degrade the stroma and the chemotherapy or the antibody targeting the cancer cell will be effectively delivered to the tumor cell, thereby killing it. At the same time, antibody therapies targeting the immune cells will make the immune environment less suppressive, thereby allowing the effector cells to mount an effective immune response against the cancer. Second, predictive markers need to be developed for the various antibody therapies. Doing so will enable PDA patients to be screened for the marker or a panel of markers, and get directed to the most effective treatment or at least know what treatments they will not respond to without wasting time and hope.

We are experiencing a technological revolution that has allowed the therapeutic antibody field to make great progress in many cancers. Taking PDA's unique biology into account when developing antibody approaches is key to the success of antibody therapy for this difficult cancer.

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Figure 1. The application of seroproteomics for the discovery of PDA-specific antigens and novel monoclonal antibody therapies using the PDA-GVAX study as a representative example

2-dimensional electrophoresis (2-DE) is performed using the cell lysate obtained from the PDA GVAX cell lines. Discovery patient sera is obtained from patients that have favorable clinical and immune responses to the vaccine. Pre- and post-vaccination discovery patient sera is used for the western blot analysis. Mass-spectrometry analysis is used to identify PDA-specific antigens that elicit a favorable antibody response in patients. These antigens are subsequently validated using recombinant protein technology and sera from other patients in the clinical trial (both responders and non-responders to the vaccine). Next, the PDA antigen is subjected to immunohistochemical and functional studies to establish it as a biomarker whereas a monoclonal antibody, against the antigen, is tested as a potential antibody therapy. Sideway arrows indicate the flow of reagents into the process whereas vertical arrows represent the process flow.

Table 1

Summary of recent and ongoing clinical trials with antibody therapies for PDA.

Antigenic PDA target	Monoclonal antibody therapy	Clinical trial phase	Targeted PDA patient group	Summary of clinical data	Up to date clinical trial results
Mesothelin	SS1P (Antibody-drug conjugate)	Phase I, single agent (bolus)	Mesothelin-positive PDA patients	MTD: 45µg/kg/dose i.v. QOD × 3 doses DLT: pleuritis 4 MR and 19 SD out of 33 patients No CR	Hassan, et al. (2007) ⁶⁰
Mesothelin	SS1P (Antibody-drug conjugate)	Phase I, single agent (c.i.)	Mesothelin-positive PDA patients	MTD: 25 µg/kg/dose given as c.i. over 10 days 1 PR out of 24 patients No CR	Kreitman, et al. (2009) ⁶¹
Mesothelin	MORAb-009	Phase I, single agent	Mesothelin-positive PDA patients	MTD: 200 mg/m ² 11 SD out of 24 patients	Hassan, et al. (2010) ⁶⁵
Mesothelin	MORAb-009	Phase II, randomized, gemcitabine vs. gemcitabine and MORAb-009	Locally advanced and metastatic PDA patients	Data not yet available	Morphotek (2013) ⁶⁶
PSCA	AGS-1C4D4	Phase II, randomized, gemcitabine vs. gemcitabine and AGS-1C4D4	Previously untreated, metastatic PDA patients	6-month SR: 44.4% vs. 60.9% (p=0.03) OS: 5.5 vs. 7.6 months	Wolpin, et al. (2013) ⁶⁸
Gastrin 17	G17DT	Phase II, randomized, G17DT vs. placebo	Advanced PDA patients unsuitable for or unwilling to take chemotherapy	Median Survival (unadj): 151 vs. 82 days (p=0.03)	Gilliam, et al. (2012) ⁷⁴
CTFG	FG-3019	Phase I, combination of FG-3019, gemcitabine and erlotinib	Previously untreated, locally advanced or metastatic PDA patients	No serious adverse events PFS: 4.3 months OS: 9.4 months	Dimou, et al. (2013) ⁷⁸
CTLA-4	Ipilimumab	Phase Ib, ipilimumab vs. ipilimumab and GVAX	Locally advanced or metastatic PDA patients after failed chemotherapy	Median OS: 3.6 vs. 5.7 months (p = 0.072) 1 year OS: 7% vs. 27%	Le, et al. (2013) ⁹⁴
PD-1	CT-011	Phase 2, gemcitabine and CT-011	Patients who have had surgically resected PDAs	Trial currently recruiting participants	Khleif, et al. (2013) ⁹⁹

Antigenic PDA target	Monoclonal antibody therapy	Clinical trial phase	Targeted PDA patient group	Summary of clinical data	Up to date clinical trial results
			without any follow-up treatments		
PD-L1	BMS-936559	Phase I, single agent	Selected solid tumors including advanced PDA patients with or without previous therapy	No objective response seen in PDA patients	Brahmer, et al. (2012) ⁹⁶
PD-L1	MPDL3280A	Phase I, single agent	Selected solid tumors including locally advanced or metastatic PDA patients	No objective response seen in PDA patients	Herbst, et al. (2013) ¹⁰¹
CD40	CP-870,893	Phase I, gemcitabine and CP-870893	Chemotherapy-naïve advanced PDA patients	MTD: 0.2 mg/kg dose level 4 PR out of 22 patients No CR	Beatty, et al. (2013) ¹⁰⁶
Fibronectin ED-B	L19-IL2 (Antibody-cytokine fusion)	Phase I, gemcitabine and L19-IL2	Locally advanced or metastatic PDA patients not eligible for surgical resection	Trial currently recruiting participants	Philogen (2012) ¹⁵⁸

Abbreviations: PSCA- Prostate Stem Cell Antigen, CTFG- Connective Tissue Growth Factor, CTLA-4- Cytotoxic T Lymphocyte-associated Antigen 4, PD-1- Programmed Death 1, PD-L1- Programmed Death receptor Ligand 1, ED-B- Extradomain B, MTD- Maximum Tolerated Dose, DLT- Dose-Limiting Toxicities, QOD- Every Other Day, I.V.- Intra-Venous, C.I.- Continuous Infusion, SR- Survival Rate, OS- Overall Survival, PR- Partial Response, CR- Complete Response, MR- Minor Response, SD- Stable Disease, Unadj- Unadjusted, PFS- Progression Free Survival.