



# Altering the response to radiation: radiosensitizers and targeted therapies in pancreatic ductal adenocarcinoma: preclinical and emerging clinical evidence

Adam R. Wolfe, Terence M. Williams

Department of Radiation Oncology, The Ohio State University Medical Center, Arthur G. James Comprehensive Cancer Center and Richard J. Solove Research Institute, Columbus, OH 43210, USA

*Contributions:* (I) Conception and design: All authors; (II) Administrative support: TM Williams; (III) Provision of study materials or patients: All authors; (IV) Collection and assembly of data: All authors; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

*Correspondence to:* Terence M. Williams, MD, PhD. Department of Radiation Oncology, The Ohio State University, 460 W. 10<sup>th</sup> Avenue, Rm D252L, Columbus, OH 43210, USA. Email: [terence.williams@osumc.edu](mailto:terence.williams@osumc.edu).

**Abstract:** Radiation therapy continues to have an evolving role in pancreatic ductal adenocarcinoma. While metastatic failure likely contributes to the majority of patient mortality, achieving local control through surgery and/or radiation appears to be important as certain studies suggest that mortality is contributed by local failure. Many studies support that pancreatic cancer is a relatively radiation resistant tumor type. In addition, the ability to further improve radiation through dose escalation strategies in the non-metastatic setting is hampered by closeness of normal organs, including small bowel and stomach, to the tumor. Thus subverting molecular pathways that promote radiation resistance will be critical to further success of radiation in this disease. There is a wealth of preclinical data supporting the targeting of various molecular pathways in combination with radiation therapy, including DNA repair, cell cycle checkpoint proteins, receptor tyrosine kinases, oncoproteins, stem cells, and immunomodulation. A number of clinical trials have been completed or are on-going with novel molecular inhibitors. In this review, we summarize existing preclinical and clinical molecular strategies for improving the efficacy of radiation in pancreatic cancer, and highlight recent and ongoing clinical trials combining radiation and various targeted therapies.

**Keywords:** Pancreatic cancer; radiation; radiosensitizer; small molecule inhibitor; KRAS; epidermal growth factor receptor (EGFR); poly (ADP-ribose) polymerase (PARP); Wee1; Chk1/2; PI3K; AKT; mTOR

Received: 05 January 2018; Accepted: 08 August 2018; Published: 31 August 2018.

doi: [10.21037/apc.2018.08.02](https://doi.org/10.21037/apc.2018.08.02)

View this article at: <http://dx.doi.org/10.21037/apc.2018.08.02>

## Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a lethal cancer exemplified by a 5-year survival rate of 7%. Survivorship has increased only slightly since 1975 when the 5 year overall survival (OS) was only 4% (1). Furthermore, PDAC incidence is rising, and by 2030 it is projected PDAC will be the number two leading cause of cancer-related death, second only to lung cancer (2).

One of the major obstacles contributing to the poor survival in PDAC is the high percentage of patients who

present with metastatic or unresectable disease. Nearly 60% of patients present with metastatic disease, and these patients survive an average of only six months. Surgical resection remains the only potentially curative approach for non-metastatic PDAC, however only 15% to 20% of patients present with resectable disease, with the remaining 40% of patients presenting with borderline resectable or locally-advanced (unresectable) disease (3). Another major challenge is that PDAC is highly resistant to our current chemotherapies and radiotherapy. DNA damaging agents,

such as radiation and gemcitabine, are commonly used in the treatment of pancreatic cancer. However, failure rates with existing therapies is high. For example, local failure rates following tri-modality therapy ranged from 47–63% in three large randomized controlled phase III clinical trials for resectable PDAC treated with surgery and adjuvant chemotherapy with concurrent 5-FU and external beam radiation (4-6). Thus, novel therapeutic approaches are needed to improve survival outcomes for PDAC.

### The controversial role for radiation in PDAC

The utilization of radiation in PDAC as a component of adjuvant therapy or as definitive therapy in unresectable cases is still controversial. In the adjuvant setting, clinical trial results have been inconclusive for the benefit of radiation in treating PDAC. In the ESPAC-1 trial, adjuvant chemoradiation compared to chemotherapy alone was detrimental to OS (7). In North America, RTOG 9704 implemented chemoradiation for all patients following one cycle of either gemcitabine or 5-FU chemotherapy, and the results of the trial suggested chemoradiation improved local control in the setting of higher positive margin rates compared to other adjuvant studies (ESPAC-1, CONKO-001) (8). For unresectable PDAC, the ECOG 4201 trial showed gemcitabine combined with radiation resulted in improved survival over gemcitabine alone, although the trial closed early due to poor accrual and potentially was not adequately powered to address the role of radiation in unresectable PDAC (9). In contrast, the larger LAP07 clinical trial showed no significant survival benefit with the addition of radiation to chemotherapy, despite a slight improvement in local control (10). Despite this, one interesting autopsy study showed that about one-third of PDAC deaths can be attributed to local disease progression rather than metastatic disease progression (11). Therefore, chemoradiation is still being tested as a means to improve local control, decrease margin negative resection rate, and ultimately improve survival in ongoing clinical trials, including RTOG 0848 (adjuvant setting, NCT01013649), Alliance A021501 (neoadjuvant setting, NCT02839343), and PREOPANC-1 (neoadjuvant setting, Dutch trial).

### Dose escalation

One way to improve local control is to increase the radiation delivered through dose escalation. The advent of

intensity modulated radiation therapy (IMRT) has allowed for increasing doses of radiation to the tumor site while sparing critical structures. Krishnan *et al.* reported an association between higher biologically effective dose (BED) with respect to OS and local failure in locally advanced PDAC. This study evaluated 200 patients treated at MD Anderson with induction chemotherapy (FOLFIRINOX or gemcitabine) followed by chemoradiation, with a median dose of 50.4 Gy in 28 fractions (BED =59 Gy). 47 patients were dose escalated to a BED >70 Gy using IMRT. They reported a 3-fold increase in OS in patients receiving a radiation BED >70 Gy. Three-year OS was 31% in the patients receiving BED >70 Gy compared to 9% in the group receiving a BED <70 Gy. Median local-regional recurrence-free survival was 10.2 *vs.* 6.2 months in the high *vs.* low BED groups respectively. Despite this, recurrence-free survival and distant recurrence-free survival were not statistically different in the two groups. Grade 2 and 3 GI toxicities were uncommon in the dose escalated group at 28% and 2% respectively (12). In a Korean study, 497 locally advanced PDAC patients were reviewed retrospectively. Median OS for patients receiving concurrent chemotherapy with radiation to a dose >61 Gy was 21.9 months compared to 14.8 months for those patients receiving <61 Gy. There was no significant differences between the two groups for acute or late toxicities (13).

Other studies have not shown a significant benefit to dose escalation. The Gastrointestinal Tumor Study Group's (GITSG) clinical trial for locally advanced PDAC showed a survival benefit with the addition of concurrent chemoradiation. However, no significant benefit was found comparing doses of 40 *vs.* 60 Gy. A caveat to this study was that radiation was delivered in an uncommonly used 2-week split course (14). Hall *et al.* obtained records for 977 unresectable PDAC patients from the National Cancer Database and compared OS based on radiation dose. On multivariate analysis, radiation doses >50 Gy delivered had superior OS outcomes compared to doses of <40 Gy. However, there was no OS benefit for doses of >40 Gy (15).

### Stereotactic body radiation therapy

Conventionally-delivered dose escalated radiation for PDAC is burdensome, lasting 5–6 weeks and the benefits are unclear. Stereotactic body radiation therapy (SBRT) is a promising new advance for PDAC due to the ability to offer comparable or improved local control with shorter treatment times. One of the early SBRT dose escalation

studies was done by Koong *et al.* at Stanford University. Seven patients were treated to 25 Gy in one fraction and all of these patients had control of their primary pancreatic tumor, and developed distant metastases as the site of first progression (16). Subsequent single and multi-institutional studies showed excellent responses using single and multi-fractionated SBRT regimens to treat unresectable PDAC patients. Radiation doses ranged from 22 to 45 Gy in one to five fractions. Local control rates ranged from 57% to as high as 94%, and median OS ranged from 5.7 to 18.4 months in these studies. However, late grade 2 or greater GI toxicities were high, ranging from 25–94% (16–23). A prospective phase II multi-institutional trial recently reported by Herman *et al.* treated locally advanced PDAC patients with up to three cycles of gemcitabine followed by SBRT, followed by additional gemcitabine in 49 locally advanced PDAC patients. The total dose was 33 Gy delivered in 5 fractions, which was felt to be safer by delivering the dose in more fractions, and with rigid constraints on luminal organs (stomach/small bowel). Median OS was favorable at 13.9 months, and 1-year local progression free survival was 83%. In addition, the reported toxicity was relatively minimal, with only 11% of patients demonstrating grade 2 or greater toxicities (24). This has led to a randomized trial prospectively testing of the role of this radiation regimen in the neoadjuvant setting for borderline resectable pancreatic cancer (NCT02839343).

Due to location of the pancreas relative to small bowel and other critical structures, further dose escalation strategies even with newer SBRT techniques remains challenging. We hypothesize that radiosensitizers and targeted therapies have the ability to further enhance tumor control and outcomes through “biological dose escalation” to the tumor while sparing the surrounding critical structures.

### PDAC, a radioresistant disease

One of the major challenges to designing effective therapies is PDAC’s hallmark of desmoplasia which comprises a dense network of stromal cells, inflammatory cells, and endothelial cells. This dense extracellular matrix forms a microenvironment that may contribute resistance to chemotherapies and radiation. One study showed a negative correlation between survival in metastatic PDAC patients and high expression of collagen I, one of the key components of the desmoplasia (25). The vasculature of this desmoplastic environment has also shown to be abnormal leading to decreased delivery of chemotherapy agents to the

PDAC cells (26).

Hypoxia is also a known factor in contributing to a radioresistant environment. Indeed, re-oxygenation is considered one of the classical “R’s” of radiobiology. Tumors can be made more radioresistant by making them more hypoxic, while radiosensitization can be improved by decreasing hypoxia (27). PDAC has been reported to be a cancer that is unusually hypoxic. In one study, Eppendorf electrode probe pO<sub>2</sub> measurements made intraoperatively of seven human pancreatic tumors measured significantly less pO<sub>2</sub> than the corresponding normal pancreatic tissues. The percentage of tumor measuring less than 2.5 mmHg ranged from 24% to 94% in PDAC compared with a range of 0% to 16% in the normal pancreas (28). In addition, multiple reports confirm the unusually hypoxic environment of PDAC based on poor perfusion by radiological contrast agents. For example, pancreatic cancers typically hypoenhance on contrast-enhanced CT scans relative to the rest of the pancreas (29,30). Preclinical studies have supported that hypoxia drives the aggressive nature of PDAC through increasing metastasis and enhanced stromal formation in murine models (31,32).

Recently, PDAC has been shown to be a relatively radioresistant tumor type. Yard *et al.* profiled the radiosensitivity of 533 human cancer cell lines from 26 cancer types using high-throughput clonogenic survival techniques. Survival was integrated as a function of dose and each cell line generated a radiation survival value. In their analysis, PDAC sorted near the top of all tumor types based on radioresistance (33). Another recent study by Torres-Roca *et al.* developed a predictive assay for inherent radiosensitivity using a multigene expression model, which they termed the radiosensitivity index (RSI). They validated the RSI in esophageal and rectal cancer cohorts by showing RSI could predict for pathological response after neoadjuvant radiation, whereby RSI could be used to separate patients into groups based on radiation responders *vs.* non responders (34). They further characterized tumor types based on radiation sensitivity by combining the RSI with the linear quadratic model to derive what they termed the genomic-adjusted radiation dose (GARD). GARD was derived using the linear quadratic model, RSI, and the standard of care radiation dose and fractionation schedule (i.e., typically 45 Gy for GI cancers). A higher GARD predicted for a higher therapeutic effect after radiation. They grouped gastrointestinal cancers into the 45 Gy group which included esophageal, gastric, colon, kidney, rectal, and pancreas. In this group of tumors, PDAC had

the lowest median GARD score, supporting other studies showing PDAC is a relatively radioresistant tumor type. Importantly they validated GARD in a PDAC cohort and showed GARD was significant on multivariate analysis for OS for PDAC patients treated with adjuvant radiation (35). This review article will therefore focus on the current and potential opportunities for improving response to radiation in PDAC.

### PDAC genomics and targetable mutations

PDAC tumorigenesis is driven by a large number of genetic mutations activating oncogenic pathways and inactivating tumor suppressor pathways. Over the past 2 decades with the discovery of high-throughput genome sequencing, there has been an explosion of data elucidating genetic alterations in PDAC. One of the first landmark PDAC genetic studies from the Johns Hopkins University performed genetic analysis of 24 PDACs and identified 12 cellular signaling pathways altered in the majority of the PDAC samples. The major pathways mutated included KRAS signaling, regulation of G1/S phase transition, hedgehog signaling, TGF-beta signaling, and the Wnt/Notch pathway (36). Additional attempts have been made to classify PDAC into subtypes based on molecular profiling similar to breast and lung cancer. Utilizing microarray data sets Collison *et al.* developed a 62-gene signature to classify PDAC samples into three subtypes: classical, quasi-mesenchymal, and exocrine-like. The classical subtype has a high expression of adhesion and epithelial genes, and the quasi-mesenchymal has a high expression of mesenchymal-associated genes, while the exocrine-like expresses tumor-cell derived digestive enzyme genes. The classical subgroup had the longest OS in their patient cohort compared to the quasi-mesenchymal, which had the lowest survival of the three subgroups (37).

Over the past 5 years the Australian Pancreatic Cancer Genome Initiative (APDACGI) has provided the largest characterization of genomic events in PDAC through whole-genome sequencing of 100 PDACs. The four most commonly mutated genes included KRAS, TP53, SMAD4, and CDKN2A, similar to prior genomic studies. Further characterization based on structural chromosomal rearrangements distributed PDACs into four subtypes: stable, locally rearranged, scattered, and unstable. They elegantly showed that for PDACs with high level of BRCA pathway mutations (or low BRCA signature rank), that response to platinum agents was higher compared to PDACs with low BRCA mutational status (high BRCA

signature rank). They hypothesized that targeting cancers with the BRCA mutation with agents that target alternate DNA repair pathways (such as PARP inhibitors) would enhance tumor response (38). The APDACGI followed up the original study with genomic analysis of 456 PDACs using both whole-genome and deep-exome sequencing followed by RNA-seq for 96 PDACs (39). Genetic mutations clustered into 10 molecular pathways. KRAS again was the highest mutated gene at 92% followed by G1/S checkpoint genes (TP53, CDKN2A, and TP53BP2) that were mutated in 78% of PDACs. TGF-Beta signaling was also mutated in 47% of PDACs and the BRCA pathway mutated in 12%. Other less observed potential targetable mutations were in the chromatin remodeling SWI/SNF pathway (ARID1A), the WNT pathway, and RNA processing genes (SF3B1, U2AF1, and RBM10). RNA expression through RNA-seq clustered the 96 PDACs into 4 subtypes, squamous, pancreatic progenitor, immunogenic, and aberrantly differentiated endocrine exocrine (ADEX). These subtypes nicely overlapped with the previous classified subgroups identified (39).

Finally, the highly anticipated results from the Cancer Genome Atlas (TCGA) were recently published in 2016. DNA alterations, DNA methylation, mRNA, miRNA, lncRNA, and protein expression profiles of 150 PDACs were reported. Importantly when excluding KRAS mutations, they reported 42% of patients had at least one known targetable alteration. Once again they observed mutations in BRCA2 and ATM which they highlighted as potential targets for platinum-based agents or PARP inhibitors. For the few patients with wild type KRAS they observed increased levels of activated mTOR pathway proteins which could be a potential target for therapeutic intervention (40). The results of these genomic studies have provided the framework for designing therapeutic targets which we hypothesize will improve PDAC radiosensitization. We summarize the most recent and ongoing clinical trials for combining radiation and targeted therapies in Table 1.

### Molecular targets for radiosensitization in PDAC

#### *Poly (ADP-ribose) polymerase 1 (PARP1)*

The targeting of DNA repair pathways has been shown to enhance radiosensitization in multiple tumor types. DNA repair is complex and involves hundreds of proteins in order to coordinate the recognition of DNA repair. Cancer

**Table 1** Clinical trials combining molecularly-targeted agents or immunotherapy with radiation in PDAC

Class	Trial	Phase	Stage	Drug therapy	Target	Radiation	Outcomes	Reference
Parp	NCT01585805	I	Unresectable	Veliparib	PARP	36 Gy in 15 fractions	Completed	
	NCT00047307	I	Unresectable	Alvociclib	CDK9	50.4 Gy in 28 fractions	Completed	
Cell cycle checkpoints	NCT02037230	II	Unresectable	MK-1775	Wee-1	52.5 Gy in 25 fractions	Recruiting	
	MD Anderson	II	Locally advanced	Cetuximab	EGFR	50.4 Gy in 28 fractions	Median OS 19.2 months (95% CI, 14.2 to 24.2 months) 1-, 2-, and 4-year actuarial overall survival rates were 66.0%, 25.02%, and 11.3%	(41)
Tyrosine kinase	Penn state	I	Locally advanced	Erlotinib	EGFR	50.4 Gy in 28 fractions	Median OS 1.1 years (95% CI, 0.62–1.59)	(42)
	NCT02868632	II	Unresectable locally advanced	MEDI4736 and tremelimumab	PD-L1 and CTLA4	SBRT 30 Gy in 5 fractions	Recruiting	
Checkpoint inhibitors	NCT02866383	II	Metastatic refractory to prior chemotherapy	Nivolumab and ipilimumab	PD-1 and CTLA4	SBRT 15 Gy in 1 fraction	Recruiting	
	NCT02311361	II	Unresectable locally advanced	Durvalumab +/- tremelimumab	PD-1 and CTLA4	SBRT 8 Gy in 1 fraction or 25 Gy in 5 fractions	Recruiting	
Cancer vaccine	NCT02305186	II	Resectable or borderline resectable pancreatic cancer	Pembrolizumab	PD-1	Neoadjuvant chemoradiation with capecitabine and radiation 50.4 Gy in 28 fractions	Prelim data: Similar toxicities. No reported grade 4 toxicities	(43), ASCO Meeting 2017
	NCT01072981 (IMPRESS)	III	Surgically resected	Algenpantucel-L	alpha-1,3-galactosyltransferase	50.4 Gy in 28 fractions	No difference in OS between groups. Median OS 27.3 vs. 30.4 months.	
Cytokines	NCT02648282	II	Locally advanced	GVAX + pembrolizumab	granulocyte-macrophage colony-stimulating factor	SBRT 33 Gy in 5 fractions	Recruiting	
	NCT02446093	II	Borderline resectable	GMCI	AdV-ik (aglatimagene besadenovec) + valacyclovir	50.4 Gy in 28 fractions	Recruiting	
Multi-Institutional	ACOSOG Z05031	II	Resected	IFN-alpha	-	50.4 Gy in 28 fractions	All-cause grade ≥3 toxicity rate was 95%. Median disease-free survival and overall survival were 14.1 months (95% CI, 11.0–20.1 months) and 5.4 months (95% CI, 23.4–34.1 months)	(44)
	Multi-Institutional	III	Unresectable	TNFERade	TNF-alpha	50.4 Gy in 28 fractions	No difference in median PFS and OS. PFS 6.8 vs. 7.0 months. OS 10 months in both arms	(45)

PDAC, pancreatic ductal adenocarcinoma; EGFR, epidermal growth factor receptor.



cells replicate at a rate higher than that of normal cells; therefore, due to this replicative stress, cancer cells acquire DNA single-strand and double-stranded breaks (DSB) more frequently. In cancer cells unable to repair these double-stranded breaks through homologous recombination (HR) and/or non-homologous end-joining (NHEJ)-mediated repair, the progression of unrepaired single-stranded breaks to double-stranded breaks due to stalled replication forks is especially harmful, leading to fork collapse, potentially promoting genome instability and triggering post-mitotic death or apoptosis (46). One such protein shown to be important in DNA repair following radiation damage is the poly (ADP-ribose) polymerase (PARP) enzyme. PARP is critical for recognition and activation of pathways responsible for single strand break repair (47). Indeed, both PARP1 and PARP2 are key players in the base excision repair (BER) pathway. Loss of *BRCA1/2* in cancer cells is a pertinent example of a DSB deficient cancer, since *BRCA1/2* are important components of the HR repair pathway. *BRCA1/2* deficient cancer cells are vulnerable to persistent single stranded DNA lesions caused by PARP1/2 inhibition leading to synthetic lethality (48). Moreover, cancer cells that are deficient in other HR deficiency (e.g., *ATM*, *PALB2*) may be also susceptible to synthetic lethality in the presence of PARP1/2 inhibition. The mechanism for synthetic lethality is supported by numerous preclinical and clinical studies in breast cancer and other tumor types (48). Chemotherapy, including platinum agents, inflicts DNA damage via lesions including adducts and cross-linking, which causes PARP1 up-regulation to promote base-excision repair of the DNA damage. Inhibition of PARP1 disables DNA base-excision repair, allowing replication fork collapse leading to DNA DSBs. In the absence of proper HR repair activity (e.g., *BRCA1/BRCA2*) cell death ensues. In PDAC, genomic studies have revealed numerous mutations in HR repair pathway genes, including *BRCA1*, *BRCA2*, *ATM*, and *PALB2*. Mutations in these genes account for ~10–15% of patients with pancreatic cancer (49). In addition, others have developed molecular signatures predicting loss of HR repair (or a BRCA-like phenotype) in tumor cells. In one particular study in pancreatic cancer, a BRCA-signature was shown to predict for sensitivity to platinum based therapies (38). Preclinical studies indicate the utility of combining PARP inhibitors with other DNA damaging therapies or tumors deficient in certain DNA repair pathways. Farmer *et al.* reported inhibition of PARP in *BRCA1* or *BRCA2* deficient PDAC cells resulted in cell cycle arrest, apoptosis, increased DNA double strand

breaks, and reduced growth *in vivo* (50). PARP inhibition was likewise shown to enhance radiosensitization in pre-clinical PDAC models when combined with checkpoint inhibitors targeting Wee1 or Chk1. In these studies, PARP inhibition alone did not improve radiosensitization suggesting checkpoint inhibition is critical for the potential radiosensitizing effects of PARP inhibition (51,52). Another potential mechanism to utilize PARP inhibition with radiation is targeting patients whose tumors have BRCA mutations to take advantage of synthetic lethality. Phase I trials of PARP inhibitors have now established safety and efficacy for several solid tumors including metastatic PDAC (53,54). One case report published of a patient with a *BRCA2* germline mutation who progressed through adjuvant chemoradiation was treated with combination PARP inhibitor, iniparib, and gemcitabine and remained disease free for 32 months (55).

A prospective phase II study of stage III–IV PDAC patients with a BRCA mutation is underway looking at the combination of the PARP inhibitor, veliparib twice daily in combination of cisplatin and gemcitabine (NCT01585805) (56). Another phase II study is testing olaparib in patients with pancreatic cancer who have a “BRCAness phenotype” (strong family history or somatic mutations in homologous recombination repair genes such as *BRCA*, *ATM*, *RAD51*, etc.) (NCT02677038). In addition, a phase III double blinded study using the PARP inhibitor olaparib in germline *BRCA1/2* mutated metastatic PDAC patients who have not progressed on first line platinum-based chemotherapy is underway (NCT02184195) (57).

Clinical investigations combining PARP inhibitors and radiation are underway in multiple cancers, including breast, colon, and brain (58). In PDAC, there is currently a phase I trial testing the combination with veliparib, gemcitabine, and radiation for borderline-resectable and locally-advanced PDAC (NCT01908478). The clinical data from other cancer sites as well as pre-clinical data in PDAC show the potential for radiosensitization using PARP inhibitors, specifically in BRCA- or HR-mutated PDAC patients.

#### *Cell-cycle checkpoint proteins (Wee1, Chk-1/2)*

Checkpoint kinase 1 (Chk1) and Wee1 are both cell cycle checkpoint mediators which also respond to DNA damage by inhibiting the G2-transition through the cell cycle (59). DNA damage activates DNA damage response signaling events that subsequently activate Chk1 and Wee1, which induce cell cycle arrest through inhibition

of cdk1. Chk1 and Wee1 kinase inhibitors sensitize tumor cells to chemoradiation through cell-cycle redistribution, by preventing G2 arrest that normally allows cells to repair DNA damage before progressing to mitosis (60). Unrepaired DNA damage that is propagated to daughter cells ultimately leads to mitotic catastrophe or apoptosis. Chk1 inhibitors were shown to sensitize PDAC cells and xenografts to radiation through a mechanism of homologous repair inhibition (61). This effect was enhanced with gemcitabine-based chemoradiation (62). Wee1 inhibition by the small molecule inhibitor AZD1775 radiosensitizes *TP53* mutant solid tumors (63). Multiple studies have suggested that *TP53* plays an important role in the sensitivity of tumor cells to Chk1 or Wee1 inhibition (64,65). This preferential sensitization occurs by virtue of the function that p53 normally protects cells from DNA damage by initiating cell cycle arrest at the G1 checkpoint to promote DNA repair. However, p53 mutant cells lack the G1 checkpoint and rely on the other main checkpoint, the G2 checkpoint, to repair DNA damage. As a result, TP53 mutant cells are more sensitive to inhibitors that block initiation of the G2 checkpoint (66). Interestingly, as mentioned above, simultaneous inhibition of Wee1 (via AZD1775) and PARP (with olaparib) produced highly significant radiosensitization in PDAC cell lines and xenografts (51). An ongoing phase II trial at the University of Michigan is currently studying the Wee1 inhibitor, AZD1775, in combination with gemcitabine and radiation for unresectable PDAC (NCT02037230).

#### *Ataxia telangiectasia and Rad3-related (ATR) kinase*

Ataxia telangiectasia and rad3-related (ATR) protein is a serine-threonine protein kinase that belongs to the phosphatidylinositol 3-kinase-related kinase (PIKK) protein family (67). ATR is activated in response to DNA damage particularly single strand breaks, and serves to induce cell cycle arrest. ATR is activated at single strand breaks induced from radiation, but also stalled replication forks or during the processes of nucleotide excision repair and homologous recombination repair. Once ATR is activated, it typically phosphorylates Chk1, beginning a signal transduction cascade that culminates in G2 cell cycle arrest as described above. Similar to Chk1 and Wee1, inhibition of ATR may be an attractive approach to potentially radiosensitize tumor cells. Preclinical studies support this in pancreatic cancer. Prevo *et al.* showed that ATR inhibition with a novel small molecule inhibitor, VE-

821, could prevent Chk1 phosphorylation and subsequent G2 arrest after radiation or gemcitabine, and effectively radiosensitized multiple pancreatic cell lines to radiation and gemcitabine in both normoxic and hypoxic conditions (68). Increased DNA damage and inhibition of homologous recombination repair was observed. Similarly, a study by Fokas *et al.* showed that treatment of pancreatic cancer cells with an analog of VE-821, VE-822 (also called VX-970), inhibited ATR activity both *in vitro* and *in vivo*, with resultant increased DNA damage and decreased homologous recombination repair (69). Radiosensitization mediated by ATR inhibition was shown to be enhanced in tumor compared to normal tissue cells using intestinal irradiation models, and VE-822 in combination with radiation resulted in marked tumor growth delay in xenografts without causing significant normal tissue toxicity. Taken together, this data supports the use of ATR inhibitors in combination with radiation or chemoradiation for the treatment of pancreatic cancer. To our knowledge, there are currently no trials evaluating ATR inhibitors in combination with radiation.

#### *DNA-dependent protein kinase*

DNA double strand breaks (DSBs) induced after radiation are commonly repaired by the non-homologous end-joining (NHEJ) repair pathway. A central mediator of NHEJ repair is DNA-dependent protein kinase catalytic subunit (DNA-PKcs), which is commonly activated after radiation (70). DNA-PKcs is another member of the PIKK family of serine/threonine kinases. DNA-PKcs forms a holoenzyme with Ku70/Ku80 heterodimers, called DNA-PK, which subsequently catalyzes the joining of non-homologous ends to effect DNA repair of double strand breaks. In a preclinical study by Li *et al.*, they found that pancreatic cancer cells rely on the NHEJ pathway to rapidly repair DNA DSBs (71). Pharmacological inhibition or genetic silencing of DNA-PK resulted in accumulation of DNA damage, inhibition of tumor cell growth, and apoptosis in pancreatic cancer cells. This data likewise supports DNA-PK inhibition alone or in combination with radiation for the treatment of pancreatic cancer. Similar to ATR inhibitors, there are no currently ongoing trials with a DNA-PK inhibitor with radiation to our knowledge.

#### *Receptor tyrosine kinases*

The epidermal growth factor receptor (EGFR) is a well-

studied receptor tyrosine kinase (RTK) over-expressed in a wide variety of solid tumors. EGFR-targeted therapies in combination with radiation, including either antibodies designed to inhibit the binding of EGF ligand to the receptor, or small molecules which block receptor activation, have been tested in head and neck, lung, breast, rectal, and anal cancers with varying success. Cetuximab (anti-EGFR antibody) was the first biologic therapy approved in combination with radiation for the treatment of head and neck squamous cell cancer on the basis of a large phase III clinical trial showing improved OS compared to radiation alone (41). In PDAC, the overexpression of EGFR has been shown to occur in ~50% of tumors (42). Pre-clinical studies have shown promise with targeting EGFR as a radiosensitizer in PDAC preclinical studies. Lapatinib, an EGFR inhibitor FDA approved for HER-2/neu positive advanced breast cancer, inhibited cell growth *in vitro* of 4 PDAC cell lines, but only radiosensitized wild-type *KRAS*-expressing T3M4 cells (72). Upwards of 90% to 95% of PDAC patients have mutated *KRAS*, resulting in constitutive activation of *KRAS* independent of upstream EGFR signaling. These results could explain the lack of benefit and minimal response shown with EGFR inhibition in clinical trials of PDAC. Indeed, the NCI of Canada phase-III clinical trial found only a small 2 weeks (but statistically significant) improvement in OS in metastatic PDAC patients who received both erlotinib and gemcitabine compared to gemcitabine alone (73). Likewise, erlotinib did not show any clinically benefit in the adjuvant setting in either of the phase III clinical trials, RTOG 0848 or CONKO-005 (74). In addition, the combination of erlotinib and gemcitabine in the locally-advanced setting showed no benefit in the LAP07 trial (9).

Despite this, preclinical studies of the combination of either cetuximab or erlotinib with concurrent gemcitabine and radiation significantly reduced tumor growth *in vivo* compared to gemcitabine and radiation alone. This effect was mediated through decreased phosphorylated EGFR and AKT, suggesting that inhibition of these kinases can attenuate survival signals after radiation (75). Studies such as these support development of clinical trials combining EGFR inhibition with radiation. A phase II trial conducted at MD Anderson tested the combination of cetuximab with gemcitabine, oxaliplatin, and capecitabine-radiation (50.4 Gy) for patients with locally-advanced pancreatic cancer, and showed a very favorable median survival time of 19.2 months with acceptable toxicity (76). Recently, a phase I trial showed combination of capecitabine and erlotinib with radiation to a

total dose of 50.4 Gy was well-tolerated in locally advanced PDAC with a median OS of ~13 months (77).

Targeting of alternate RTKs may be of benefit. Preclinical and clinical data has shown efficacy for combining radiation and targeting agents directed against HGFR, PDGFR, VEGFR, and FGFR (78-81). Another example sunitinib, a tyrosine kinase inhibitor of VEGFR and PDGFR, inhibited phosphorylation of Akt and Erk in PDAC preclinical studies. Sunitinib further radiosensitized PDAC cell and delayed tumor growth *in vivo* after radiation treatment (82). More studies combining EGFR or other RTK inhibition with radiation may be warranted in pancreatic cancer to further test the radiosensitizing ability of EGFR/RTK inhibitors concurrent with radiation.

### *KRAS pathway*

*KRAS* mutations are found in greater than 90% of PDAC patients (38,40). The *RAS* gene family consists of three members, *NRAS*, *HRAS*, and *KRAS*. Mutations affecting *RAS* proteins promotes tumorigenesis through activation of downstream oncogenic pathways which include a numerous number of proliferation associated proteins. *RAS* members show a tumor-type specificity. *NRAS* mutations are predominant in hepatocellular carcinoma, *HRAS* in papillary thyroid carcinoma, and *KRAS* in pancreatic, non-small cell lung cancer, and colorectal (83). In PDAC, mutations in *NRAS* and *HRAS* are very rare, occurring in less than 1–2% (39,40). Activated *RAS* results in activation of downstream RAF-MEK-ERK (p42/44 MAPK) pathway signaling and parallel PI3kinase-AKT-mTOR signaling. Activation of the *RAS* pathway is thought to be the driver mutation for PDAC proliferation and metastatic spread (84,85). Early studies from the 1980s showed the mutated *RAS* oncogene increases resistance to tumor cells treated with radiation (86). In order for *KRAS* to function, it requires prenylation at one of the cysteine residues leading to plasma membrane attachment. Pre-clinical data suggests inhibiting prenylation of *KRAS* with farnesyltransferase inhibitors (FTIs) results in enhanced radiosensitivity (87). However, cells have alternate mechanisms of prenylation, through geranyl-geranyl transferases, allowing *KRAS* to be become activated even in the presence of FTIs, which may have contributed to the failure of FTIs in clinical testing (88). Indeed, a phase-III trial of combination gemcitabine plus the farnesyltransferase inhibitor, tipifarnib, showed no improvement in survival in locally advanced, unresectable, or metastatic PDAC patients (89). Developing therapeutic



agents which directly inhibit KRAS still remains a challenge, but there are new agents in development. One promising class of drug targets the specific *KRAS*<sup>G12C</sup> mutation and inhibits the conformational change required for activation (90). Unfortunately, the *KRAS*<sup>G12C</sup> mutation is rare in PDAC (91). New strategies for targeting RAS include small molecule inhibitors that bind to a unique pocket on the RAS:Son of Sevenless (SOS):RAS complex and disrupt downstream signaling (92), and small molecule inhibitors targeting the prenyl-binding RAS chaperone PDE $\delta$  (93).

Due to the difficulties targeting KRAS, inhibitors of the downstream effectors have been developed. MEK inhibitors possess radiosensitizing properties in several tumor types (94,95). Low dose radiation was shown to increase transient activation of both AKT and ERK (82,96). MEK inhibition resulted in radiosensitization of multiple preclinical PDAC tumor models, and when combined with an AKT inhibitor resulted in enhanced radiosensitization (96). In addition, radiosensitization by MEK inhibition was shown to occur via inhibition of multiple DNA repair pathways, such as NHEJ and HR repair. Unfortunately, trametinib, a MEK inhibitor, showed no clinical benefit in a phase II trial in combination with gemcitabine compared to gemcitabine alone in advanced PDAC patients (97). A phase II trial studying the combination of EGFR and MEK inhibitors in refractory PDAC patients showed only modest benefit with a median progression free survival reported as 1.9 months (98).

Phosphoinositide 3-kinases (PI3Ks) once activated modulate the activity of a wide range of signaling pathways, including the activation of oncogenic drivers Akt and mammalian target of rapamycin (mTOR) (99). In cancer cells, once stimulated, PI3K leads to activation of AKT through phosphorylation and activation of the downstream kinase mTOR. mTOR is increased in around 50% of PDAC samples, and particularly enriched in patients with *KRAS* wild-type pancreatic cancer (40,100). Hayman *et al.* showed the mTOR inhibitor, INK128, could enhance radiosensitivity *in vitro* and *in vivo* through a mechanism of inhibiting DNA repair (101). In addition, Park *et al.* have shown effective radiosensitization with PI3-kinase inhibitor in pancreatic cancer models (102). Other studies have shown that dual inhibition of PI3K and mTOR improves tumor oxygenation and tumor vascular normalization leading to enhanced radiosensitization (103). Targeting AKT also led to radiosensitization of multiple cancer cell lines (104). Gupta *et al.* showed that inhibiting the PI3K with a targeted agent led to radiosensitization of mutant *RAS* cells but showed no effect in wild type *RAS* cells (105).

Taken together, inhibitors of the downstream effectors of KRAS such as MEK, ERK, PI3K, AKT, and mTOR are attractive targets to induce radiosensitization in PDAC despite the lack of clinical efficacy noted in the advanced/metastatic stage when not used in combination with radiation.

### *Immunotherapies*

As mentioned earlier, desmoplasia (i.e., fibrosis) is one of PDAC's hallmarks. In addition to dense fibroblastic stroma, PDAC is thought to arise in a relatively immunosuppressed microenvironment. PDAC appears to induce this immunosuppression through interfering with the process of antigen presentation (106), secreting soluble immunosuppressive cytokines such as interleukin (IL)-10, increasing concentrations of the immunosuppressive transforming growth factor-beta (TGF-beta) (107,108), inducing tolerance to tumor associated antigens (109), overexpressing immune checkpoint ligands such as programmed cell death ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (110), and increasing cell populations of immunosuppressive tumor-associated macrophages, myeloid-derived suppressor cells (MDSCs) and T-regulatory cells (43). Immunotherapy combined with radiation as a strategy to improve local control rates or using radiation to improve immunotherapy distant control rates (through potentiation of an *in situ* personalized tumor vaccine) are both ideas currently being explored in PDAC.

Klug and colleagues found low dose irradiation (2 Gy) led to the recruitment of tumor-reactive T cells in murine models of PDAC. In addition macrophages were polarized from the M2 (pro-tumorigenic) phenotype into the M1 (anti-tumorigenic) phenotype following irradiation. Tumor vascularization was also normalized following irradiation (111). This data suggests radiation can prime the tumor microenvironment to enhance immunogenicity against PDAC cells. Sharabi *et al.* published a comprehensive review on radiation and checkpoint blockade immunotherapy (112). To briefly summarize, radiation enhances antigen presentation, activates dendritic cells, increases tumor infiltrating T cells, and increases the infiltration of T-regulatory cells in the tumor microenvironment.

Immune checkpoint inhibitors targeting CTLA-4, PD-1, and PD-L1 have demonstrated substantial efficacy in solid tumors, including non-small cell lung cancer, melanoma, and urothelial cancer leading to FDA approval for these

checkpoint inhibitors in various disease settings. Antibodies which target these proteins have been shown to induce anti-tumor immunity (113,114). In addition, the abscopal effect is a phenomenon defined as regression of one tumor site distant from the primary site of radiation (115). Case reports in melanoma (and other disease sites) have reported regression of disease at an un-irradiated distant site following irradiation to a tumor, provoking excitement that radiation may serve as an *in situ* tumor vaccine (44,116,117). Indeed, the APCGI identified that the immunogenic molecular subtype of PDAC demonstrated up-regulated CTLA-4 and PD-1 pathways, suggesting opportunities for therapeutic intervention (39). There are numerous phase I or II PDAC clinical trials accruing patients testing checkpoint inhibitors with anti-CTLA-4 or anti-PD-1 antibodies in both the non-metastatic and metastatic settings. Several of these clinical trials are examining the combination of checkpoint inhibition and radiation. NCT02311361 is a phase-I trial combining SBRT, either 8 Gy  $\times$  1 or 5 Gy  $\times$  5, and durvalumab, PD-L1 inhibitor, with or without tremelimumab, a CTLA-4 inhibitor, in unresectable PDAC (45). NCT02305186 is a phase 1b/2 multi-institutional trial for resectable or borderline resectable PDAC which randomizes patients 2:1 to either pembrolizumab (PD-1 inhibitor) every 3 weeks with concurrent capecitabine and radiation (50.4 Gy in 28 fractions) or chemoradiation alone prior to surgery (118). NCT02648282 is a phase II trial of locally advanced PDAC following completion of mFOLFIRINOX or gemcitabine/abraxane based chemotherapy combining cyclophosphamide, pembrolizumab, granulocyte-macrophage colony-stimulating factor vaccine (GVAX) and SBRT (6.6 Gy in 5 fractions). These future studies will help determine whether combining radiation or chemoradiation with checkpoint inhibitors represent an effective strategy for treating PDAC.

Other strategies for combining radiation and immunotherapy pathways in PDAC are underway. Gene-mediated cytotoxic immunotherapy (GMCI) uses the delivery system of aglatimagene besadenovec (AdV-tk), an adenoviral vector containing the herpes simplex virus thymidine kinase gene followed by an antiherpetic prodrug valacyclovir. This therapy stimulates an immune response and was shown to be efficacious in glioblastoma patients (119). NCT02446093 is an ongoing phase I/II trial of patients with borderline or unresectable locally advanced PDAC where patients receive neoadjuvant mFOLFIRINOX with GMCI followed by gemcitabine with radiation.

Interferon-alpha (IFN-alpha) is another class of immune

stimulators being used in a variety of cancer types, including PDAC (120). A preclinical study has shown that IFN-alpha enhanced radiosensitization and chemosensitization in eight human PDAC cell lines (121). Additionally, IFN-alpha enhanced radiosensitization in MiaPaca-2 and Panc-1 cells by roughly 2-fold (122). The Virginia Mason Clinic published the results of a phase II study of adjuvant cisplatin, 5-FU, IFN-alpha, and radiation following pancreaticoduodenectomy which resulted in a remarkable 5 year OS of 55% (123). Ten year updated follow up revealed an impressive 10 year OS of 28% (124). These promising results led to a phase II multi-institutional trial, ACOSOG Z05031 of 89 patients with resected PDAC (125). The trial demonstrated a median OS of 25.4 months (much lower than the first study), with an all-cause grade  $\geq$ 3 toxicity rate that was substantially high at 95%. Because the investigators could not replicate the median survival observed in the original single institutional setting, and due to the substantial toxicity, this concept has not advanced to the phase III setting.

Tumor necrosis factor (TNF) is another anti-neoplastic cytokine tested in a wide variety of cancer types, but have not yet shown benefit in PDAC (126). The pancreatic Cancer Res group from Johns Hopkins set out to determine if TNF blockade could improve survival in locally advanced pancreatic cancer. Their phase III trial used an adenovirus vector containing TNF-alpha cDNA incorporated into the early growth response protein 1 (Egr-1) promoter, termed TNFerade. Patients were enrolled to receive either TNFerade in combination with standard 5-FU chemoradiation or chemoradiation alone. Median OS and progression free survival were identical, 10 months and 7 months respectively (127).

### *Pancreatic stem cells*

Cancer stem cells (CSCs) are a small subset of tumor cells responsible for tumor initiation, self-renewal, and have been shown to be resistant to chemotherapy and radiation. Al-Hajj *et al.* was the first study to identify CSCs in solid tumors (128). In this study they identified a rare subset of breast cancer cells which expressed CD44<sup>+</sup>CD24<sup>-</sup>/low ESA<sup>+</sup> that could form tumors in immunodeficient mice with few numbers of cells implanted. Li *et al.* identified a similar subpopulation of PDAC that expressed a CD44<sup>+</sup>CD24<sup>-</sup>/low ESA<sup>+</sup> phenotype. They showed that these cells (0.2–0.8% of total PDAC cells) had a 100-fold higher tumorigenic potential compared to the differentiated cell population.

They also found these cells upregulated self-renewal pathways such as NOTCH, PTEN, and Wnt (129). Hermann and colleagues discovered a separate population which expressed CD133 that also displayed CSC properties and had 14% overlap with the CD44<sup>+</sup>CD24<sup>-</sup>/low ESA<sup>+</sup> phenotype (130). CD133<sup>+</sup> cells have been shown to be highly resistant to radiation in leukemia, brain, breast, and colon cancers (131-133). Li and colleagues found CD44 expression was elevated in PDAC tissue compared to adjacent normal pancreas and patients with high levels of CD44 had worse OS outcomes than those patients with low levels of CD44. They also found that in PDAC xenografts, anti-CD44 antibodies reduced recurrence rates following radiation (134).

In terms of CSC pathway inhibitors, there are now two hedgehog pathway inhibitors (LDE225/sonidegib and GDC-0449/vismodegib) FDA approved for treating basal cell carcinoma (135). Clinical trials have also tested CSC-targeted agents inhibiting FAK and PI3K/mTOR pathways which are thought to be important for CSC maintenance (136). There are now 3 clinical trials underway testing FAK inhibitors with immune checkpoint blocking antibodies (NCT02546531, NCT02758587, and NCT02943317). NCT02546531 is a phase I study testing the combination of a FAK inhibitor (defactinib) in combination with pembrolizumab and gemcitabine for advanced PDAC. There are still many unanswered questions for the optimal methods to target CSCs in PDAC, and certainly more research in this area is needed.

## Conclusions

PDAC is a devastating cancer, with high rates of local, regional, and distant disease failure despite current therapies. Dose escalation with conventional techniques has shown minimal improvement. Due to PDAC being a disease typified by high metastatic rates of disease failure, certainly there is an urgent need to develop systemic therapies to better control metastatic disease. While conventional chemotherapies have made some improvements in clinical outcomes (i.e., FOLFIRINOX, gemcitabine-Abraxane combinations), these improvements have been modest. However, despite metastatic failures, strategies aimed at improving local control may also be important in improving outcomes in PDAC, since local destruction and disease morbidity resulting from local progression can affect quality of life and potentially survival. Thus, further development of novel biologic radiosensitization strategies

are important given the relative radioresistance inherent to PDAC. Many agents reviewed here and elsewhere have been tested without radiation in PDAC and have shown little to no efficacy, leading investigators to conclude that these agents should not be tested further in PDAC. However, such agents could have radiosensitizing functions completely independent of their other mechanisms of action, and thus have the potential to significantly improve radiation efficacy. We believe immunotherapy and novel targeted agents inhibiting DNA repair and/or oncogenic dependent signaling pathways hold great promise for improving radiation efficacy in PDAC and warrant further consideration and testing.

## Acknowledgments

*Funding:* This work was supported in part by NIH/NCI grant R01CA198128-01.

## Footnote

*Provenance and Peer Review:* This article was commissioned by the Guest Editor (Richard Tuli) for the series “Radiotherapy and Pancreatic Adenocarcinoma” published in *Annals of Pancreatic Cancer*. The article has undergone external peer review.

*Conflicts of Interest:* Both authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/apc.2018.08.02>). The series “Radiotherapy and Pancreatic Adenocarcinoma” was commissioned by the editorial office without any funding or sponsorship. The authors have no other conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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doi: 10.21037/apc.2018.08.02

**Cite this article as:** Wolfe AR, Williams TM. Altering the response to radiation: radiosensitizers and targeted therapies in pancreatic ductal adenocarcinoma: preclinical and emerging clinical evidence. *Ann Pancreat Cancer* 2018;1:26.