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Oncolytic adenovirus as pancreatic cancer-targeted therapy: Where do we go from here?

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ABSTRACT

Pancreatic cancer remains one of the deadliest cancers with extremely high mortality rate, and the number of cases is expected to steadily increase with time. Pancreatic cancer is refractory to conventional cancer treatment options, like chemotherapy and radiotherapy, and commercialized immunotherapeutics, owing to its immunosuppressive and desmoplastic phenotype. Due to these reasons, development of an innovative treatment option that can overcome these challenges posed by the pancreatic tumor microenvironment (TME) is in an urgent need. The present review aims to summarize the evolution of oncolytic adenovirus (oAd) engineering and usage as therapeutics (either monotherapy or combination therapy) over the last decade to overcome these hurdles to instigate a potent antitumor effect against desmoplastic and immunosuppressive pancreatic cancer.

1. Introduction

Pancreatic cancer is highly desmoplastic, immunosuppressive, hypoxic, chemoresistant, and metastatic, thus standard treatment options, such as chemotherapy and radiotherapy, only offer patients with a minor survival advantage [1,2]. Due to these reasons, pancreatic cancer is notoriously difficult to cure and 5-year survival rate of patients remain at less than 10%, despite the recent advancements in treatment paradigm [3,4]. Even immune checkpoint inhibitors (ICIs), which have significantly improved the prognosis of cancer patients with diverse tumor types, are ineffective for the treatment of pancreatic cancer [5]. Currently, programmed cell death protein 1 (PD-1)/programmed cell death ligand 1 (PD-L1)-targeted ICIs only benefit extremely small fraction of pancreatic cancer patients with mismatch repair deficiency (dMMR) and high microsatellite instability (MSI-H) [6]. With pancreatic cancer incidence rate expected to steadily rise with time and become the second highest cause of cancer-related deaths in the United States [7], there is an urgent and a growing need for the development of effective treatment options that can overcome the multifaceted challenges posed by the complex tumor microenvironment (TME) of pancreatic cancer.

Oncolytic virus (OV), which exerts cancer-specific cytopathic effect, therapeutic gene expression, and immune response, is a promising candidate to overcome the challenges posed by the TME of pancreatic cancer [8,9]. In particular, OV's ability to convert immunologically 'cold' tumors into 'hot' tumors is widely believed to be a key factor that can unlock the therapeutic potential of other commercialized immunotherapeutics, like ICIs and chimeric antigen receptor (CAR)-T cells, against tumors with immune-dessert phenotype [10,11]. Additionally, OV as a monotherapy can be armed with countless possible combinations of therapeutic genes to exert multifaceted biological functions, which would be difficult to achieve with conventional cancer treatment options, like antibodies and chemotherapeutics [12–14]. These attributes of OV make them a promising candidate to simultaneously address multiple treatment constraints that are posed by the complex TME of pancreatic cancer.

To date, four different types of OVs (herpes simplex virus, adenovirus (Ad), reovirus, and parvovirus) have been investigated in clinical trials that specifically enrolled pancreatic cancer patients. Among these viruses, Ad has been most extensively evaluated in clinical trials as both replication-incompetent Ad (dAd) and oncolytic Ad (oAd) are under

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active investigation in ongoing clinical trials for the treatment of pancreatic cancer. Based on these backgrounds, the present review aims to provide in-depth summary of the advancements made with oAd's application as either monotherapy or combination therapy for the treatment of pancreatic cancer over the last decade.

2. Strategies to enhance the therapeutic efficacy of oAd against pancreatic cancer

Arming oAds with anticancer therapeutic genes is a promising strategy to enhance the therapeutic efficacy of the virus, as preferential replication of the virus leads to exponential amplification of these gene products in a cancer-specific manner [15,16]. As the amplification of the therapeutic genes is dependent on viral replication, oAds with sufficient cancer specificity induce limited off-target therapeutic gene expression in normal cells where virus cannot replicate (Fig. 1) [17]. Furthermore, arming the oAds with different type of therapeutic genes can be a promising approach to reprogram the hostile tumor milieu of pancreatic cancer, as these gene products can exert novel or adjuvant biological functions, whereas the therapeutic efficacy of unarmed oAds is limited to replication-mediated cytolytic effect and induction of pro-inflammatory response. Alternatively, combination of oAd with other cancer treatment options, like radiation, chemotherapeutic, and immunotherapeutics, are also actively being investigated to potentiate the efficacy of oAds against pancreatic cancer [8,18,19]. This section of the review will explore how different types of therapeutic genes can be utilized to enhance cancer cell killing efficacy and overcome the barriers posed by the TME of pancreatic cancer, as well as how these viruses are utilized in combination therapy regimen to maximize the therapeutic efficacy of oAd against pancreatic cancer (Fig. 2).

2.1. Strategies to induce apoptosis of pancreatic cancer

Pancreatic cancer is highly proliferative and metastatic, allowing the tumor to expand rapidly and infiltrate nearby and distant tissues. To address these challenges, oAds armed with therapeutic transgenes that induce apoptosis of pancreatic cancer have been extensively investigated [20–28].

Apoptosis-inducing or tumor suppressor genes have been widely adopted as therapeutic genes for oAd to enhance anticancer efficacy of the virus against pancreatic cancer [26–29]. For instance, oAd-induced expression of function p53, which is a tumor suppressor gene that induces cell cycle arrest, senescence, and apoptosis [29], was shown to be beneficial for the treatment of pancreatic cancer where dysfunctional p53 signaling pathway is a common phenomenon [30]. To this end, p53-expressing OBP-702, which was constructed using OBP-301, a human telomerase reverse transcriptase (TERT) promoter-regulated oAd in a phase 2 clinical trial, was shown to effectively induce apoptosis of pancreatic cancer [31]. OBP-702 induced superior pro-apoptotic function over the unarmed OBP-301 due to more efficient adenovirus early region 1A (E1A)-dependent suppression of anti-apoptotic MDM2 expression and p53 therapeutic gene expression. Currently, it has not been clearly elucidated why E1A of OBP-702 induced more potent E1A-dependent inhibition of anti-apoptotic proteins compared to E1A of OBP-301, but p53-mediated activation of p300/CBP [cyclic adenosine monophosphate response element-binding protein (CREB)-binding protein] histone acetyltransferases may explain this difference between two oAds. Further, OBP-702 induced efficient apoptosis of chemo-resistant or metastatic pancreatic tumors, resulting in potent tumor growth inhibition [32,33]. OBP-702 was shown to induce superior antitumor immune response compared with OBP-301 due to p53 transgene expression causing more robust immunogenic cell death (ICD) of pancreatic cancer cells that ultimately augmented cluster of differentiation (CD)8^+ T cell infiltration into the immunologically 'cold' pancreatic tumors [34]. Importantly, the combination of OBP-702 with PD-L1 blockade was demonstrated to exert superior inhibition of gemcitabine-resistant pancreatic tumor growth over either monotherapy options by inhibiting intratumoral accumulation of immunosuppressive myeloid-derived suppressor cells (MDSC). These findings demonstrate that oAd-mediated expression of p53 can be a promising strategy to enhance induction of ICD against pancreatic tumors that frequently lack functional p53 signaling pathway.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor superfamily, has also been utilized as a pro-apoptotic therapeutic gene for OV therapy [35,36]. TRAIL-expressing oAds armed with an additional therapeutic gene or their combination with chemotherapeutics have been widely investigated to induce apoptosis of pancreatic cancer [37–39]. For instance, oAd co-expressing TRAIL and ST13 (CD55-ST13-TRAIL) was investigated for the treatment of pancreatic cancer based on the clinical evidence where low intratumoral expression level of ST13 was associated with poorer prognosis of pancreatic cancer patients [37].

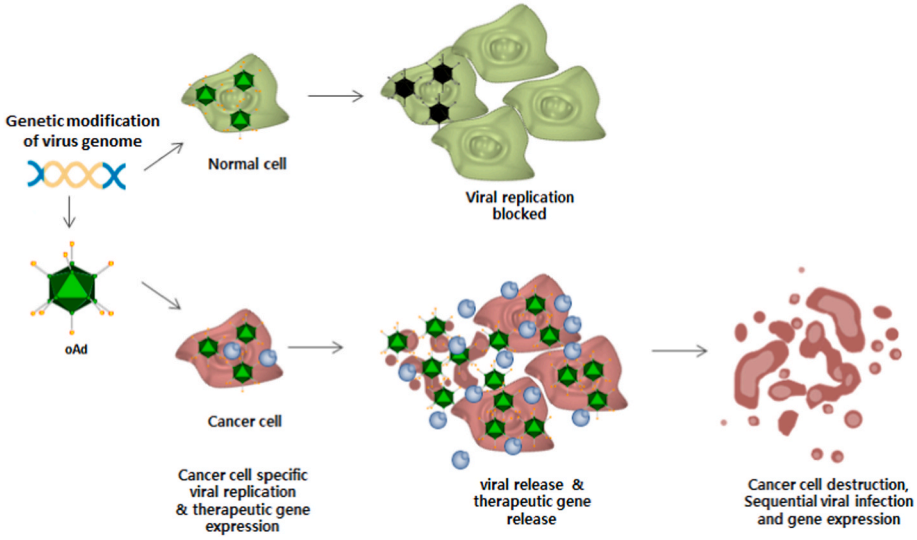

Fig. 1. An oncolytic adenovirus (oAd) is a virus that has been genetically engineered to preferentially replicate in and lyse cancer cells. As the infected cancer cells are lysed by oAd, they release new infectious virus particles to infect and destroy the surrounding cancer cells. Additionally, oAd can be used as a vector system to express therapeutic genes, ultimately resulting in high expression level of therapeutic genes in cancer cells upon successful viral replication.

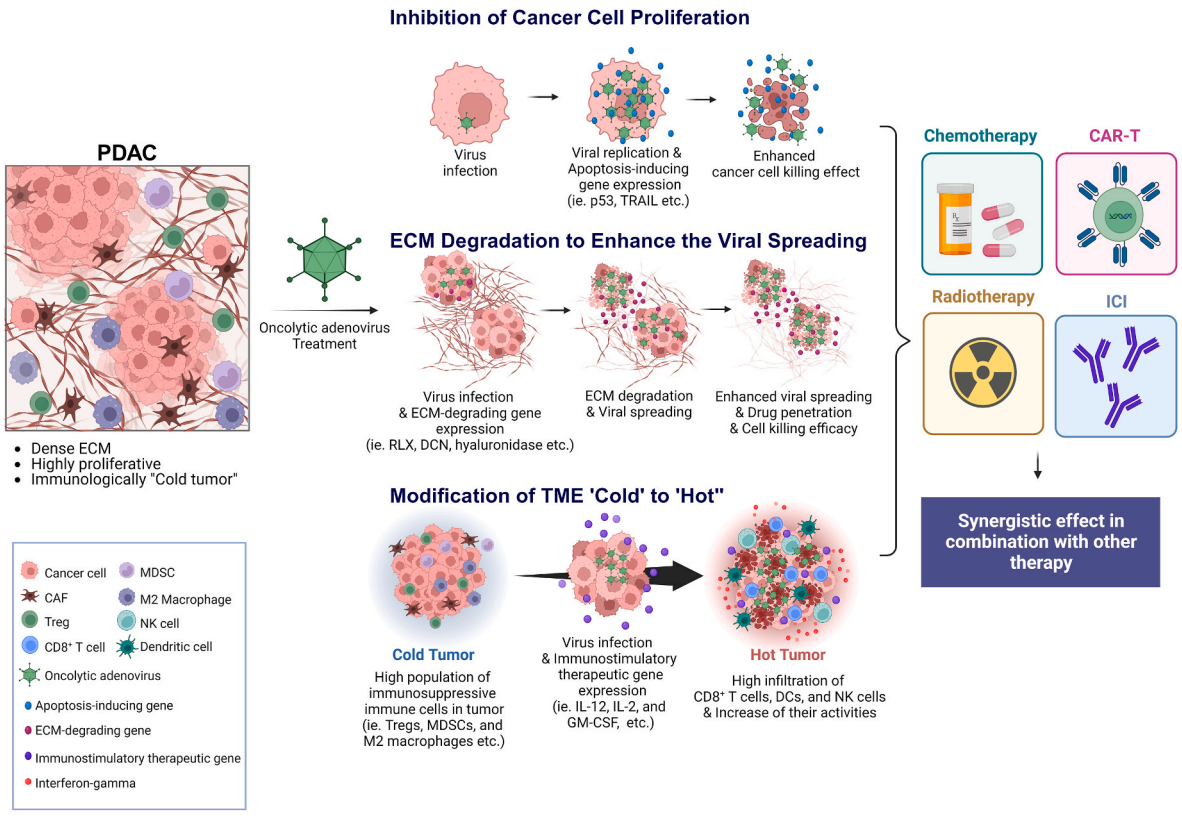

Fig. 2. Different types of therapeutic genes can be expressed by oAds to enhance their cancer cell killing efficacy or overcome the barriers posed by the tumor microenvironments of pancreatic cancer. Further, oAds can exert synergistic therapeutic effect in combination with other cancer treatment options, like chemotherapy, radiotherapy, and immunotherapy. The illustration was created with BioRender.com.

CD55-ST13-TRAIL more effectively induced the apoptosis of pancreatic cancer cells compared with oAd expressing only one of the therapeutic gene (CD55-ST13 or CD55-TRAIL), while no apoptotic cells were observed in the normal cell population. Importantly, CD55-ST13-TRAIL significantly inhibited tumor growth and improved the survival of pancreatic tumor-bearing mice. In a similar approach, the oAd co-expressing TRAIL and another proapoptotic gene Smac (ZD55-TRAIL-IETD-Smac) has been shown to effectively induce apoptosis of pancreatic cancer, exerting more potent antitumor efficacy than control oAds expressing one of the therapeutic genes, showing that co-expression of multiple therapeutic genes using armed oAd can be a promising strategy to improve the overall efficacy of the virus against pancreatic cancer [38,39]. More importantly, ZD55-TRAIL-IETD-Smac in combination with cyclin-dependent kinase (CDK) inhibitor SNS-032, which selectively inhibits activity of CDK-2, -7, and -9 and shown to exert moderate therapeutic activity in phase II clinical trials as monotherapy [40,41], was shown to exert synergistic anticancer property that superseded those observed in respective monotherapy due to more robust suppression of anti-apoptotic molecules, like CDK-2, CDK-9, Mcl-1 and XIAP. These findings demonstrated that pro-apoptotic oAds expressing right combination of therapeutic genes can be utilized in combination with clinically relevant chemotherapy to exert synergistic anticancer effect against pancreatic cancer.

Suicide gene, which is a gene that can express a biological molecule that can convert inactive and non-toxic prodrugs into active and cytotoxic metabolite, is another promising strategy to induce cell death of pancreatic cancer [42]. To this end, an oAd-mediated expression of these suicide genes can induce preferential overexpression of suicide genes in the tumor cells over normal cells due to exponential amplification of therapeutic gene product necessitating cancer-specific replication of the virus. Indeed, intratumoral administration of E1B 55 kDa-deleted oAd co-expressing yeast cytosine deaminase (yCD)/mutant sr39 herpes simplex virus thymidine kinase fusion gene (yCD/mutTKSR39rep) and adenovirus death protein (ADP; Ad5-yCD/mutTKSR39rep-ADP) in combination with gemcitabine and two prodrugs (5-fluorocytosine (FC) and gancliclovir) to patients with locally advanced pancreatic cancer was shown to be extremely safe up to 1×10^{12} viral particles; (VP) in this dose-escalation phase I clinical trial [43]. In detail, no grade 3 or 4 adverse event was reported even at the highest viral dose and maximum tolerated dose was not reached, showing that dual suicide gene therapy using oAd is extremely safe. Among the nine enrolled patients (three patients each at following dose level; 1×10^{11}, 3×10^{11}, and 1×10^{12} VP), 8 patients had stable disease and 1 patient had partial response at 12 weeks after the treatment initiation. Remarkably, six cases of stable disease and one case of partial response was retained up to 6.5 months after the virus administration, achieving overall response rate of 11% and disease control rate of 100%. Based on this promising phase I clinical trial results, phase II trial is ongoing (NCT04739046) and highlights that oAd-mediated suicide gene therapy can be a promising approach for the treatment of pancreatic cancer in clinical setting.

In conclusion, proapoptotic genes expressed by oAds effectively killed pancreatic cancer cells via apoptotic mechanism that is different from innate cytolytic activity of the virus. Thus, cytotoxic genes-armed oncolytic Ads are attractive therapeutics for cancer treatment.

2.2. *Strategies to overcome desmoplasia of pancreatic cancer*

The TME of desmoplastic pancreatic cancer is characterized by dense stromal compartment and aberrantly high extracellular matrix (ECM), which function as physical barrier against penetration of immune cells and cancer therapeutics as well as spread of OVs in pancreatic tumors [44,45]. To this end, a number of studies have provided additional evidence that degradation or rearrangement of ECM is advantageous for the dissemination of drugs [46].

Relaxin (RLX) is a peptide hormone released during pregnancy and its expression by oAd was the first report to demonstrate that degradation of tumor ECM was viable using oncolytic virotherapy [47]. The degradation of tumor ECM has been shown to dramatically improve the distribution of oAd throughout the entirety of the tumor mass [47] and sensitize gemcitabine-resistant pancreatic tumor to gemcitabine therapy [48]. Of note, oAd-mediated degradation of tumor ECM via expression of RLX was shown to improve the intratumoral penetration, accumulation, and distribution of other cancer therapeutics, like trastuzumab, ICI, and doxorubicin, throughout the pancreatic tumors [48,49], which likely contributed to chemosensitization of gemcitabine-refractory pancreatic tumors [48]. In multiple gemcitabine-refractory pancreatic tumor models, oAd expressing RLX was shown to cause synergistic induction of apoptosis within the tumor mass and induce more effective degradation of tumor ECM than respective monotherapy, showing that desmoplasia and chemoresistance of pancreatic cancer can be overcome by utilization of RLX-expressing oAd [48].

During the last two decades, decorin (DCN), which antagonizes transforming growth factor (TGF)-β activity, has also been extensively investigated as therapeutic transgene of oAds to enhance virus dispersion in desmoplastic pancreatic tumors [22,50]. In detail, an oAd expressing DCN (oH(E)mT-DCN) was shown to effectively lower the expression level of ECM components, such as collagen I/III, elastin and fibronectin, and effectively induce apoptosis of pancreatic xenograft tumors and patient-derived tumor explants [22]. In the subsequent study, the potency of DCN-expressing oAd was further augmented against pancreatic tumors by arming the virus with Wnt decoy receptor as second therapeutic gene to inhibit Wnt/β-catenin signaling pathway (oAd/DCN/LRP) [23]. oAd/DCN/LRP was shown to effectively inhibit Wnt/β-catenin signaling pathway to prevent epithelial-to-mesenchymal transition (EMT) of pancreatic cancer. Importantly, intraperitoneal administration of oAd/DCN/LRP was shown to effectively inhibit metastatic dissemination of orthotopic pancreatic tumor xenografts with minimal hepatotoxicity, showing that effective co-inhibition of Wnt/β-catenin signaling pathway and TGF-β-mediated desmoplasia by single oAd can be an efficient strategy to prevent metastatic progression of pancreatic cancer. Further, oAd/DCN/LRP in combination with gemcitabine was shown to inhibit orthotopic pancreatic tumor growth more effectively than respective monotherapy, showing that the virus can function in adjuvant manner with standard-of-care chemotherapy. The superior anticancer effect of the combination of oAd/DCN/LRP and gemcitabine was achieved in a similar manner as those observed with RLX-expressing oAd [48] as oAd/DCN/LRP-mediated degradation of tumor ECM was shown to enhance the intratumoral penetration and accumulation of systemically administered chemotherapeutic [23].

Hyaluronic acid, which is one of the ECM components, is known to accelerate malignant progression of pancreatic cancer by stiffening the tumor tissue and promoting invasion and drug resistance. Therefore, hyaluronidase, which degrades hyaluronic acid, has also been explored as a therapeutic gene to induce ECM degradation and attenuate stiffness of pancreatic tumors [51–54]. Clinically evaluated VCN-01, which has genetically engineered Ad E1A promoter with additional E2F binding sites and Rb biding site-mutated E1A gene for enhanced cancer specificity, expresses hyaluronidase as the therapeutic gene to degrade the tumor ECM [55]. In a preclinical study, intravenously or intratumorally administrated VCN-01 demonstrated potent antitumor activity and high level of tumor ECM degradation in human pancreatic tumor xenograft model and syngeneic pancreatic tumor model in immunodeficient mice and immunocompetent Syrian hamsters, respectively [55]. Furthermore, VCN-01-induced degradation of pancreatic tumor stroma has been shown to increase extravasation of therapeutic agents, such as chemotherapy and therapeutic antibodies, into the tumor mass [56]. In a phase I clinical trial, intratumoral administration of VCN-01 combined with standard-of-care chemotherapy (gemcitabine and nab-paclitaxel) was well-tolerated and disease stabilization was observed with several pancreatic cancer patients. These patients showed time-dependent elevation of their serum hyaluronidase level and lower level of stiffness in post-treatment tumor biopsies versus pre-treatment levels, indicating that VCN-01-mediataed expression of hyaluronidase efficiently disrupt tumor stroma of pancreatic tumor. Unfortunately, the study did not assess whether VCN-01-induced softening of pancreatic tumor tissues promoted more effective accumulation of chemotherapeutics in the tumor mass, which has been only demonstrated in preclinical environment to date.

In another phase I clinical trial, intravenous administration of VCN-01 with or without nab-paclitaxel plus gemcitabine was shown to exhibit acceptable safety profile when VCN-01 was administered as monotherapy or combined with chemotherapy after 7-day recovery period following systemic administration of VCN-01 [57]. In contrast, concomitant systemic administration of VCN-01 (1×10^{13} VP) and chemotherapy (gemcitabine and nab-paclitaxel) on the same day was shown to be unsafe with 2 out of 6 patients experiencing grade 4 neutropenia or grade 5 enterocolitis and thrombocytopenia. These findings suggest that systemic administration of VCN-01 did induce transient side effect in pancreatic cancer patients, which can make these patients vulnerable to other traumatic events during recovery phase. The occurrence of grade 4 or 5 neutropenia or thrombocytopenia, which are side effects associated with intravenous administration of Ads [58–60], after concomitant systemic administration of VCN-01 and chemotherapy suggests transient, but potentially compounding, virus-associated safety concerns. Still, intravenous VCN-01 combined with chemotherapy achieved 50% overall response rate for the treatment of pancreatic cancer patients, which is promising despite the trial not being powered to accurately assess the therapeutic efficacy, and its benefit should outweigh the potential safety concerns [57]. In line with these findings, the combination of VCN-01 and chemotherapy has received orphan drug designation by United States Food and Drug Administration (FDA) for the treatment of pancreatic cancer in 2023, and is under investigation in a randomized phase IIb trial for the treatment of patients with metastatic pancreatic cancer (NCT05673811).

Collectively, these studies demonstrate that several different therapeutic gene candidates, like RLX, DCN, and hyaluronidase, for oAd that can induce effective degradation of tumor ECM to enhance both intratumoral accumulation of the virus itself and other conventional cancer therapeutics, like cancer-targeted antibodies, ICIs, and chemotherapeutics. The early clinical data from VCN-01 trial also demonstrated that this approach is viable against highly desmoplastic pancreatic tumors in clinical environment, suggesting that these ECM degrading oAds could be promising candidates to potentiate the clinical efficacy of conventional treatment options against pancreatic cancer in future trials.

2.3. *Strategies to overcome immunosuppression of pancreatic cancer*

Immunosuppressive features of pancreatic cancer, such as low intratumoral tumor infiltrating lymphocyte (TIL) counts and high level of immunosuppressive cell infiltration in the tumor tissues, pose significant challenges to the development of effective immunotherapy strategies [61,62]. This is highlighted by the fact that to date, only one immunotherapy product (PD-1-targeting ICI, pembrolizumab) has been approved for the treatment of pancreatic cancer by the US FDA [6]. Unfortunately, pembrolizumab is only beneficial in an extremely small subset of pancreatic cancer patients with dMMR and hMSI, and other immunotherapeutics approved for other cancer indications have failed to demonstrate clinical benefits [6,63,64]. Dense ECM layer of the TME also functions as a physical barrier against immune cell infiltration [65–68]. Due to these limitations, the development of novel immunotherapy that can inflame immunologically ‘cold’ tumors is a topic of immense interest within the field of cancer immunotherapy.

To this end, oAd, like other OVs, has been proposed as a promising therapeutic solution due to the following strengths: the virus is innately immunogenic and known to induce pro-inflammatory response through

generation of danger- or pathogen-associated molecular pattern (DAMP and PAMP, respectively) and that virus-induced cytolysis of tumor cells lead to generation of tumor-associated antigens that can be recognized by antigen-presenting cells, such as dendritic cell (DC), to induce adaptive immunity against cancer [65,67]. Importantly, the induction of antitumor immune response by oAd can be greatly augmented by arming the virus with immunostimulatory therapeutic genes, like cytokines and chemokines, to induce synergistic antitumor immune response.

Interleukin (IL)-12, which is a well-known and potent antitumor cytokine, induces T helper type 1 (Th1) immunity and promotes cytotoxic T-lymphocyte (CTL) response against cancer [69,70]. Accordingly, several clinical trials have investigated recombinant IL-12 cytokine as a cancer immunotherapy option, but short half-life in the bloodstream and high level of systemic toxicity led to early termination of these trials [71–73]. To this end, arming OVs with potent, but pleiotropic, pro-inflammatory cytokine like IL-12 is a promising method to circumvent the systemic toxicity associated with recombinant cytokine therapy, as OV-mediated transgene expression is restricted to tumor tissues due to high level of gene expression necessitating active replication of the virus [74–76].

In support, phase I clinical trial investigating intratumorally administered E1B 55 kDa-deleted oAd co-expressing mutTKSR39rep and IL-12 (Ad5-yCD/mutTKSR39rep-hIL-12) in combination with systemic prodrug 5-FC and standard-of-care chemotherapy (FOLFIRINOX or gemcitabine plus nab-paclitaxel) demonstrated good safety profile in patients with metastatic pancreatic cancer [92]. Specifically, majority of the adverse events (~94%) observed in this study were grade 1 or 2 requiring no medical intervention with serious adverse events of grade 3 or 4 occurring only in 5% or 1% of patients, respectively. In terms of immune activation, higher level of proliferating $CD8^+$ T cells in peripheral blood mononuclear cells (PBMCs) was detected between 7 and 21 days after virus administration at all viral dose levels (1×10^{11} to 1×10^{12} VP) compared to the baseline, which is suggestive of immune system activation. Additionally, the patients also exhibited virus dose-dependent elevation in serum levels of cytokines IL-12 and interferon (IFN)-γ, which also demonstrated virus-induced immune response. Of note, a time-dependent increase in the expression level of checkpoint molecule, T-cell immunoglobulin and mucin domain (TIM)-3, was observed with $CD8^+$ T cell population of patients that received the lowest dose of Ad5-yCD/mutTKSR39rep-hIL-12, suggesting T cell exhaustion. At the higher dose levels, TIM-3 expression level of $CD8^+$ T cells stayed at the baseline level or decreased with time. These findings suggest that inadequate or transient oAd-induced immune response can lead to T cell exhaustion and that a high level of immune activation that passes certain threshold is required to sustain T cell activation in a prolonged manner. Additionally, it should be noted that local administration of Ad5-yCD/mutTKSR39rep-hIL-12 did induce serious adverse events (grade 3 or higher) in patients at the higher dose levels, albeit at low frequency. These findings suggest that oAd-induced immune response is a double-edged sword where inadequate activation of the immune system cannot overcome immunosuppressive tumor microenvironment of pancreatic cancer to elicit therapeutic response, while excessive immune activation is associated with increased safety hazard.

The protein engineering of IL-12 can be a promising approach to attenuate off-target toxicity while retaining its potent antitumor efficacy, as several innovative approaches enabled tumor-specific immune activation by IL-12 and these variants are showing promising therapeutic activity with improved safety profile over unmodified IL-12 in early phase clinical trials [77,78]. In lieu of these trends, a recent study has investigated N-terminal signal peptide truncation of IL-12 (nsIL-12) can enhance its safety profile as therapeutic transgene for oAd [79]. The signal peptide was deleted to ensure that nsIL-12 was not secreted into extracellular space, as an additional layer of safety for the therapeutic transgene. The local or systemic delivery of oAd expressing the nsIL-12 (TD-nsIL-12) to subcutaneous or peritoneally disseminated orthotopic pancreatic tumors, respectively, were shown to exert similarly potent antitumor effect as oAd expressing secretable IL-12 (TD-IL-12), showing similar T cell activation and antitumor immunity. These findings demonstrate that nsIL-12 exhibits a similar biological activity as the parental IL-12 *in vivo*. Notably, intraperitoneally administered TD-nsIL-12 induced markedly lower level of hepatotoxicity compared with TD-IL-12. Even when TD-IL-12 was systemically administered at a 5-fold lower viral dose than TD-nsIL-12, TD-IL-12 still induced higher level of blood vessel congestion, eosinophilic degeneration, and apoptosis of hepatocytes. Another interesting feature of systemically administered TD-nsIL-12 was its ability to induce steadily elevated serum IL-12 level over 5-day period after the virus treatment, whereas serum IL-12 levels peaked on day 1 for TD-IL-12 and exhibited time-dependent decrease that eventually led to it showing similar level as those of TD-nsIL-12 on day after treatment. The sharp increase in serum IL-12 by TD-IL-12 was associated with induction of hepatotoxicity at early time-point, whereas steadily elevated serum IL-12 at a moderate level by TD-IL-nsIL12 prevented damaging of the liver tissues. Together, these findings demonstrate that simple protein engineering strategy, like removal of signal peptide, can drastically improve the safety of IL-12 as therapeutic transgene for the systemic application of oAd.

As an alternative approach to enhance the safety of IL-12-expressing Ad, co-expression of suicide therapeutic gene, herpes simplex virus tk (HSV)tk, was utilized as a safety switch that could terminate the virus replication via prodrug-induced killing of virus infected cells [80]. In detail, binary usage of oAd lacking therapeutic transgene and a helper-dependent Ad co-expressing IL-12, HSVtk, and single chain variable fragment (scFV) antagonizing PD-L1 (CAdTrio) was utilized and its combination with human epidermal growth factor receptor (HER) 2-targeted chimeric antigen receptor (CAR)-T cell for the treatment of pancreatic cancer [80]. Binary Ad system is utilized to exploit the large transgene insertion capacity of helper-dependent Ad, which is devoid of most Ad genome, to circumvent limited transgene insertion capacity of oAds due to viral packaging limitations. In this binary system, co-infection of host cell with oAd and helper-dependent Ad enable replication of otherwise replication-incompetent helper-dependent Ad as viral proteins, like E1A, that are essential for viral replication are provided *in trans*. The administration of CAdTrio was shown to enhance chemotaxis and recruitment of HER2-specific CAR-T cells to the pancreatic tumors, resulting in combination of CAdTrio and HER2-specific CAR-T cell therapy exerting superior antitumor efficacy over respective monotherapy. Unfortunately, no lethal or serious adverse events that required safety intervention with prodrug were necessary in this study, thus it could not be clearly elucidated as to whether the co-expression of HSVtk gene by binary Ad system is sufficient safety measure to control high level of immunotoxicity in this study. Importantly, intratumoral administration of CAdTrio to the primary tumor-induced systemic antitumor immune responses, which led to sensitization of distant tumors to HER2-specific CAR-T cell-mediated tumor growth inhibition, in a bilateral pancreatic tumor model. Considering that CAR-T cell therapy has been largely inefficacious in controlling the growth of solid tumors in clinical environment [184], CAdTrio's ability to sensitize immunosuppressive pancreatic cancer in a systemic manner to CAR-T cells is highly promising.

The preliminary phase I clinical trial data of CAdTrio for the treatment of advanced solid tumors at an extremely low viral dose totaling 5×10^9 VP (more than 100-fold lower than other oAd doses from clinical trial) was shown to exert promising therapeutic effect in first four patients treated (3 patients with metastatic breast cancer and 1 with recurrent head and neck squamous carcinoma) in a relatively safe manner (no grade 4 toxicity was observed). In terms of efficacy, one complete remission (CR) and two partial responses (PR) were observed at the injected tumor site [81]. Importantly, strong abscopal effect against distant or visceral metastases was observed in two patients, demonstrating induction of potent and systemic adaptive immune response against the tumors. The patient suffering from head and neck squamous cell carcinoma achieved confirmed overall CR with no

residual or distant disease without further treatment at 15 months after the virus administration. The potent and durable systemic antitumor immune response was likely facilitated by CAdTrio treatment attenuating the number of circulating immunosuppressive T cells with regulatory T cell (Treg)-associated phenotype ($CD4^+$, $CD25^{High}$, and CTLA-4^{High}) to the levels well-below the pretreatment values from one week after virus administration in all patients. Currently, no clinical data has been reported for CAdTrio treatment in pancreatic cancer patients, but preclinical data of the virus monotherapy and its combination with HER2-targeted CAR-T cells and the encouraging clinical data in other advanced solid tumors highlight that CAdTrio could be a promising candidate for the treatment of advanced pancreatic cancer in the future.

Similar to CAdTrio, co-expression of IL-12 along with combination of other therapeutic genes can be a promising strategy to improve the overall therapeutic efficacy of oAd against highly desmoplastic and immunosuppressive pancreatic tumors. To this end, an oAd co-expressing RLX and two antitumor cytokines (IL-12 and granulocyte-macrophage colony-stimulating factor (GM-CSF; oAd/IL12/GM-RLX)) has been shown to induce synergistic and durable antitumor immune response with αPD-1 in a pancreatic tumor model that is refractory to ICI treatment, inducing complete tumor regression in large fraction of treated animals with excellent safety profile [158]. This was achieved by oAd-mediated enhancement of αPD-1 accumulation in pancreatic tumor and spleen tissues, which led to more robust activation of $CD4^+$ and $CD8^+$ T cells in tumor draining lymph nodes and TIL population in the combination therapy arm. These findings demonstrated that RLX-expressing oAd could drastically enhance αPD-1 accumulation in tumor and immune organs to activate and induce T cell-mediated antitumor immune response against desmoplastic and immunosuppressive pancreatic tumors more readily. Additionally, the study demonstrated that co-expression of all three therapeutic genes played an integral role in promoting antitumor immune response as oAd/IL12/GM-RLX induced superior infiltration of $CD8^+$ T cell into tumor tissues compared with oAd co-expressing IL-12 and GM-CSF (oAd/IL12/GM) or oAd expressing RLX alone, showing that ECM degradation also facilitated immune cell infiltration into the tumor tissues. Alternatively, a recent study has highlighted that RLX gene therapy can change the intratumoral macrophage composition to reprogram the TME into more immunogenic status, like higher level of CTL infiltrate, and responsive to PD-L1-targeted ICI [82]. These findings suggest that oAd/IL12/GM-RLX may also reprogram the macrophage population within the TME of pancreatic cancer and that this aspect could be one of the contributing factors to its superior CTL response over oAd/IL12/GM, which warrants further investigation in the future to better understand oAd/IL12/GM-RLX-mediated inflaming of immunosuppressive pancreatic cancer.

Other than IL-12, recombinant IL-2 has been extensively investigated in clinical trials, as early as in the 1990s, for treatment of cancer [77,78,83]. Similar to systemic therapy with recombinant IL-12, high-dose IL-2 therapy was associated with severe toxicity in cancer patients [84]. Despite high level of toxicity associated with IL-2, therapeutic benefit was only observed in less than 20% of the treated patients at the highest dose [85]. Although IL-2 has been approved by the US FDA for the treatment of renal cancer and melanoma, its severe toxicity has led to subsequent development of IL-2 variants with better safety profiles and these new products are now under evaluation in clinical trials [86,87]. In general, most widely adopted strategies attenuate IL-2's ability to bind with immunosuppressive Tregs in order to induce preferential proliferation and activation of T cell populations (e.g., $CD4^+$ or $CD8^+$ T cells) that can instigate antitumor immune response [77,78].

These IL-2 variants (vIL2) can avoid nonspecific consumption by Treg and does not induce proliferation of Tregs, thus lower level of vIL-2 can be administered to preferentially induce effector-like T cell response in a safer manner over the wild-type IL-2 [77,78]. In lieu of these trends, an oAd armed with IL-2 variant (vIL2) with attenuated Treg binding (Ad5/3-E2F-d24-vIL2) has been developed and evaluated for the treatment of pancreatic cancer [88]. Notably, Ad5/3-E2F-d24-vIL2 exerted superior inhibition of pancreatic tumor growth over control oAd expressing wild-type IL-2 (Ad5/3-E2F-d24-IL2), despite Ad5/3-E2F-d24-IL2 inducing higher level of $CD8^+$ T cell accumulation in the tumor due to inferior binding affinity of vIL-2 to IL-2Rβγ receptors in comparison to wild-type. Still, Ad5/3-E2F-d24-vIL2 treatment induced suppression of total $CD4^+$ T cells (the lack of reagent availability for Syrian hamster prevented discrimination of the Th1 $CD4^+$ T cells from Tregs in this study) accumulation in the tumor and lower level immunosuppressive genes (CD11B, ARG1, CD206, and HCK) being expressed in the pancreatic tumor tissues over Ad5/3-E2F-d24-IL2, while both induce similar elevation of antigen presentation-related genes (CD80, CD86, and CD40). These findings suggested that Ad5/3-E2F-d24-vIL2 induced more efficient antigen presentation and subsequent antitumor immune response over Ad5/3-E2F-d24-IL2 by preventing immunosuppressive cell accumulation in the pancreatic tumor tissues.

Ad5/3-E2F-d24-vIL2 in combination with standard-of-care chemotherapy (nab-paclitaxel and gemcitabine) was also shown to exert superior survival benefit than respective monotherapy for the treatment of pancreatic cancer [89]. In detail, the combination therapy was shown to induce higher level of mitotic slippage and immunogenic cell death compared with Ad5/3-E2F-d24-vIL2 monotherapy in pancreatic cancer cells. The combination of two therapeutics did not induce additional side effects compared with respective monotherapy, demonstrating that these therapies can be combined in a safe manner. Importantly, combination with Ad5/3-E2F-d24-vIL2 prevented induction of chemotherapy-induced T and natural killer (NK) cell exhaustion. Specifically, the combined chemotherapy arm (gemcitabine and nab-paclitaxel) showed higher frequency of PD-1^+ or lymphocyte-activation gene 3 $CD4^+$ and $CD8^+$ T cells and NK cells being detected among TIL population in comparison with negative control arm, but this effect was suppressed to basal level observed in negative control arm when chemotherapy was combined with Ad5/3-E2F-d24-vIL2. Similarly, Ad5/3-E2F-d24-vIL2 was shown to effectively inhibit chemotherapy-induced recruitment of immunosuppressive CAF subsets to the basal level observed in negative control arm. One major caveat of this study was that majority of the statistical analysis was only conducted between chemotherapy versus combination therapy groups, whereas combination therapy versus oAd monotherapy has been omitted. In general, chemotherapy addition did not seem beneficial for the induction of T cell-mediated response, as the combination therapy arm and virus monotherapy arm exhibited similar T cell regulation profiles. Unexpectedly, chemotherapy and combination therapy groups led to superior level of NK cell infiltration into the tumor over virus monotherapy that failed to elevate NK cell infiltration compared to the levels observed in the negative control arm [89]; this was surprising since both oAd and its transgene vIL2 are known to induce NK cell recruitment in the literatures [77,78]. Based on the available preclinical evidence, the combination therapy seems to exert superior antitumor efficacy over respective monotherapy due to it exerting both T and NK cell-mediated immune response, while respective monotherapy only induces activation of single pathway [89].

Both CD40 and 4-1BB are targets of cancer immunotherapy that are under active clinical investigation with several agonistic antibodies targeting CD40 under clinical trial for the treatment of pancreatic cancer patients, showing promising clinical activity in early phase clinical trials [90–93]. A clinically evaluated LOAd703 has been armed with membrane-anchored and trimeric forms of CD40 ligand (CD40L) and 4-1BB ligand (4-1BBL) as therapeutic genes [94]. LoAd703 was shown to disrupt desmoplasia of pancreatic cancer by infecting and lysing pancreatic stellate cells (PSCs) and enhancing migration and cytotoxicity of T cells and NK cells by inducing the secretion of lymphocyte-recruiting chemokines and activation of DCs. According to the reports from the 2022 ASCO Annual meeting, combining

intratumoral injections of LOAd703 with standard intravenous nab-paclitaxel/gemcitabine chemotherapy was shown to be safe (NCT02705196) [95]. The adverse events were short-lived and grade 1/2, except for a single grade 3 transaminase elevation in one subject receiving the highest dose of 5×10^{11} VP. Objective response rate (ORR) among those treated at the highest dose level was 55% (5/11 subjects), thus meeting the predefined criterion for efficacy. Among all response evaluable patients, overall response rate (ORR) was 44%, and disease control rate (DCR) was 94%. With this clinical protocol, proportion of T effector memory cells was increased, while proportion of T regulatory cells and myeloid-derived suppressor cells decreased. Currently, a phase I/II clinical trial combining LoAd703, chemotherapy (nab-paclitaxel and gemcitabine), along with PD-L1-targeted ICI atezolizumab is ongoing (NCT04123470).

Recently, oAd has been utilized to generate reprogrammed somatic-cell-derived tumor cell and utilize it as a prophylactic cancer vaccine for the treatment of pancreatic cancer [96]. In detail, induced pluripotent stem cells (iPSC) were generated from autologous normal cells then these cells were gene edited to harbor oncogenic mutations ($KRas^{G12D}$ and $p53^{R127H}$) that ultimately instigated carcinogenic transformation into pancreatic cancer-like cells (PCLCs). These cells were infected with oAd as prime or oncolytic vaccinia virus (oVV) as boost to enhance the immunogenicity of these cells as cancer vaccines. Their findings demonstrate PCLCs exhibited highly similar antigen repertoire as pancreatic tumors developed from KPC transgenic mice, which is one of the most extensively employed transgenic pancreatic cancer model. Infection of PCLCs with either oAd or oVV was shown to exhibit ICD-like features as evidenced by elevated release of ATP, high mobility group box 1 (HMGB1), and calreticulin (CRT) into the extracellular space. Their findings demonstrated that sequential administration of oAd-infected PCLCs as prime and oVV-infected PCLCs boost was shown to induce highest level of adaptive immunity against tumor cells of same genetic background compared to other regimens with different treatment order and/or combination of viruses utilized to infect PCLCs. Prophylactic vaccination of KPC transgenic mice with oAd-infected PCLCs as prime and oVV-infected PCLCs as boost prior to tumor lesion formation was shown to delay tumor development compared to unvaccinated KPC mice, thus resulting in prolonged survival of vaccinated mice. Importantly, their findings demonstrate that infection of PCLCs with OVs was critical to successful induction of T cell-mediated adaptive immunity against pancreatic cancer, as vaccination regimen with uninfected PCLCs failed to induce antitumor immune response and provide survival benefit. In terms of safety, no weight loss was observed in vaccinated mice and no evidence of autoimmune disorders was noted. In particular, no tumor lesions were found at the vaccination administration site, suggesting that PCLCs are not carcinogenic. These findings highlighted the potential of oAd's utility in a cancer vaccination-based approach. Alternatively, the expression of multiple tumor-derived or -associated antigens as therapeutic genes could also be useful for oAd-based cancer vaccination of pancreatic cancer in the future, as Ad-based cancer vaccine has been shown to elicit promising anticancer activity in both preclinical and clinical environment for several different cancer types and Ad-based antigen expression system for COVID-19 vaccination has demonstrated excellent antigen-specific immune response with good safety profile [97–102].

Collectively, these studies demonstrate that oAd can be armed with different set of therapeutic genes to exert novel and synergistic anticancer functions that supersede the efficacy of unarmed virus and enable these armed oAds to address the critical barriers that severely hamper the overall efficacy of therapeutics against highly proliferative, desmoplastic, immunosuppressive, and drug-resistant pancreatic cancer. Based on the early clinical data, armed oAds have shown promising therapeutic effects against pancreatic cancer in combination with standard-of-care treatment options in a safe manner. Many of the innovations seen in preclinical setting, such as strategies to enhance the potency and specificity of the virus against pancreatic cancer, also highlight the bright future and utility of oAd as a promising next-generation therapy option to address many of the limitations of conventional therapies in the treatment of pancreatic cancer.

3. Conclusion

Pancreatic cancer with its aggressive, desmoplastic, and immunosuppressive phenotype poses a significant challenge toward development of efficacious therapeutic options. Despite many hurdles that arise from the complex TME of pancreatic cancer, oAd has been extensively engineered to enhance its specificity and efficacy against pancreatic cancer over the last decade. Emerging preclinical and clinical evidence suggests that arming oAd with an optimal set of therapeutic genes can be a promising approach to overcome uncontrolled proliferation, desmoplasticity, and immunosuppression in pancreatic cancer. Removal of desmoplasia and immunosuppression can improve the efficacy of other pancreatic cancer therapy, and studies utilizing oAd in combination therapy regimens have been yielding promising results. Currently, there are several innovative immunotherapeutics, like bispecific antibodies, ribonucleic acid neoantigen vaccine, antibody-drug conjugates, and toll-like receptor agonists among others, with promising clinical trial results for the treatment of pancreatic cancer and oAd in combination with these novel therapies seem promising and warrant in-depth investigation. Lastly, systemic therapy of pancreatic cancer using oAds now seem within our reach, as tumor-targeted delivery of the virus in a safe manner has been demonstrated using various genetic engineering approach and carrier-based systems.

CRediT authorship contribution statement

A-Rum Yoon: Writing – original draft. **JinWoo Hong:** Writing – original draft. **Bo-Kyeong Jung:** Visualization, Conceptualization. **Hyo Min Ahn:** Conceptualization. **Songnam Zhang:** Validation. **Chae-Ok Yun:** Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: JinWoo Hong, Bo-Kyeong Jung, and Hyo Min Ahn are employees of GeneMedicine Co., Ltd. Chae-Ok Yun is the CEO of GeneMedicine Co., Ltd. The funders had no role in the writing of the manuscript.

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