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Original Article

Multimodal immune activation abilities and characteristics of reovirus

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Abstract: Reovirus is a ubiquitous, non-pathogenic, double stranded RNA virus with anti-tumor properties. The virus's replicative potential is regulated by phosphorylation of protein kinase receptor (PKR). In cancers with *RAS* pathway activation which leads to dysregulation of PKR, the virus maintains its protein translational potential and induces oncolysis. Systemic chemotherapy remains the standard of care for metastatic colorectal cancer with the addition of biologic agents in *KRAS* wildtype subtypes. In *KRAS* mutant colorectal cancers, there has been no added benefit to biologic agents. The therapeutic potential of reovirus (Reolysin®, pelareorep, Oncolytic Inc., Calgary, Canada), which induces its oncolysis with *RAS* activation through multimodal immune mechanisms, has been demonstrated in preclinical and clinical studies. In this review, we outline the specific immune mechanisms of reovirus induced oncolysis and provide both preclinical and clinical data on its applications in metastatic colorectal cancer patients.

Keywords: Reovirus, KRAS, colorectal cancer, immune profile, lymphocytes

Introduction

Colorectal cancer (CRC) is the fourth most common cancer in the U.S. with 149,500 estimated new cases and 52,980 estimated deaths in 2021 [1]. Approximately 50%-60% of patients develop metastatic disease [2, 3]. While surgical resection is widely accepted as potential curative therapy for isolated liver and lung metastases, systemic chemotherapy remains the standard of care for patients with unresectable disease [3-7]. Treatment options for systemic therapy include FOLFOX, FOLFIRI, FOLFOXIRI, and CAPEOX, with addition of biologics depending on the mutational spectrum, which has become an integral part of directed or target therapy [8-15]. In patients with *RAS* wildtype metastatic CRC, *EGFR* inhibitors, panitumumab and cetuximab have demonstrated clinical benefit based on TAILOR, CALGB/SWOG 80405, and PRIME trials [16, 17, 22]. Approximately 45% of colorectal cancers are characterized, by mutations in *KRAS* and these patients are resistant to EGFR inhibitors [18-22]. In mutant KRAS, the addition of panitumumab to chemotherapy was associated with a reduced progression free survival (PFS) compared to chemotherapy alone (HR 1.29, P=0.02), with also a trend for decreased overall survival (OS) (HR 1.24, P=0.068) [20]. Treatment for such patients remains a challenge.

Respiratory Enteric Orphan virus (REOvirus) is a non-enveloped, widely prevalent, double-stranded RNA virus with benign pathologic implications in humans that has demonstrated anti-tumor activity through oncolysis [23-25]. The mechanism of its anti-tumor activity is multifactorial involving both the innate and adaptive immune systems [23]. The virus selectively replicates in *RAS* mutated cells through the regulation of protein kinase receptor (PKR). In normal cells with intact *RAS* pathway, PKR is phosphorylated leading to inhibition of viral protein translation, but in *RAS* mutant cells, the phosphorylation is inhibited, allowing viral translation and replication [26]. Due to its benign pathologic implications and its selective replication in *RAS* mutant cells, reovirus has been studied as a potential thera-

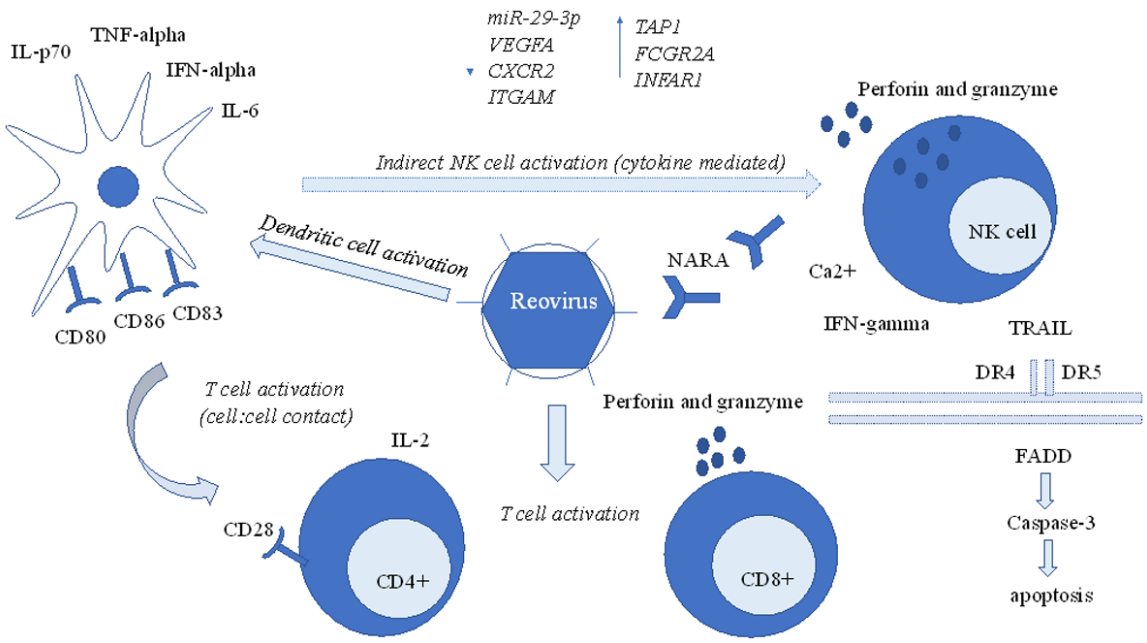

Figure 1. Multimodal immune characteristics of oncolysis by reovirus. Reovirus induces anti-tumor properties through multi-cellular pathways, which include activation and maturation of dendritic cells, which induce pro-inflammatory cytokines such as IL-6, IL-p70, and IFN-alpha, cytokine mediated indirect activation of NK cells through dendritic cells, that lead to perforin and granzyme mediated apoptosis, TRAIL, and direct activation of T-cells. In addition to cell-mediated apoptosis, reovirus has also demonstrated to activate the immune system at a transcriptome level, through downregulation of *miR-29-3p, VEGFA, CXCR2, ITGAM*, and upregulation of *TAP1, FCGR2A, INFAR1*.

peutic agent in *RAS* mutated malignancies. In this review, we highlight the mechanism of oncolysis of reovirus, with an emphasis on its multimodal immune pathways and outline preclinical and clinical applications of reovirus in *KRAS* activated metastatic colorectal cancer.

Immune mechanism of reovirus

The immune system is composed of a complex network of antigen presenting cells and effector cells that work in conjunction to protect the host against tumor antigens. Reovirus exhibits anti-tumor properties through the activation of innate and adaptive host immune systems through dendritic cells, natural killer (NK) cells, and effector T-cells which all play an active role in tumor control (**Figure 1**) [25, 27].

Reovirus mediated dendritic cell activation

Antigen presenting dendritic cells have been directly and indirectly involved in reovirus mediated oncolysis. When dendritic cells from healthy individuals were incubated with reovirus, an upregulation of CD-80, CD-86, CD-83, and TAP-1, phenotypic markers of dendritic cell activation and maturation was observed, in addition to an increase in secretion of proinflammatory cytokines, TNF-alpha, IL-6, IL-p70, and INF-alpha, as well as functional markers of dendritic cell activation [25]. This dual phenotypic and functional activation of dendritic cells was also demonstrated in cell lines derived from patients with metastatic colorectal cancer, supporting the virus's modulation of antigen presenting cells in preclinical studies [25].

Dendritic cells have also been observed to play an indirect role in activation of NK cells, a crucial part of innate and adaptive immunity, through reovirus mediated oncolysis. Rather than direct activation of NK cells by reovirus, NK cells were found to be dependent on virus infected cells for activation. When NK cells were incubated with reovirus alone, NK cell activity was not observed, but when incubated with reovirus-infected dendritic cells, an upregulation of interferon-gamma, a marker of NK cell activity, was observed [25]. Dendritic cells communicated with NK cells indirectly through production of pro-inflammatory cytokines, including IFN-alpha, TNF-alpha, IL-15, and IL-12, rather than direct cell to cell contact since

even when reovirus induced NK cells were separated by a transwell, there was continued NK cell activation [25]. It is proposed that NK cells exhibited their cytotoxicity through exocytosis of perforin/granzyme since in the presence of chelating agent like egtazic acid (EGTA), the cytotoxic effect was found considerably reduced indicating a crucial role of Ca2+, an important player in perforin/granzyme mediated cytotoxicity [25].

Reovirus also exhibits its cytotoxicity through apoptotic pathways, notably through the production of proinflammatory cytokines such as tumor necrosis factor (TNF)-associated death-inducing ligand (TRAIL), which induces apoptosis through oligomerization of death receptors 4 and 5, that leads to Fas-associated death domain complex formation, which in turn activates caspase-3, leading to cell death [28]. The virus can also directly induce translocation of Smac (second mitochondria-derived activator of caspases), a mitochondrial protein encoded by the *DIABLO* gene, which activates Bcl-2 (B-cell lymphoma 2) and induces apoptosis [28].

Early phase clinical studies in metastatic colorectal patients have supported preclinical observations on reovirus mediated dendritic cell activation. In a phase I dose escalation trial of 30 patients with oxaliplatin refractory metastatic colorectal cancer treated with FOLFIRI/bevacizumab and reovirus, rapid maturation of dendritic cells (4.5% to 18.6% at 48 hours) was demonstrated among the 5 patients who have been studied in detail regarding their immune response [29].

Reovirus mediated T-cell cytotoxicity

In addition to dendritic cell activation, maturation, and NK cell activation, reovirus has also demonstrated anti-tumor activity through T-cell immunity. In preclinical studies, reovirus induced dendritic cells incubated with T-cells resulted in increased interferon-gamma and IL-2 production, a marker of T-cell activation, and T-cell cytotoxicity was demonstrated regardless of target cell HLA status [25]. Unlike NK cell activation, which did not require cell to cell contact and exhibited communication through proinflammatory cytokines, T-cell activation through reovirus required cell to cell contact through antigen presentation and specific receptor interaction, which suggests that NK cells and T-cells may exhibit differences in tumor microenvironment ideal for cytotoxicity. Like NK cells, cytotoxicity of T-cells was mediated by exocytosis of perforin/granzyme and activation of T-cells occurred in multiple subtypes of T-cells, including CD4+ and CD8+, rather than a subclonal T-cell population [25].

Early phase clinical trials have supported preclinical observations of reovirus mediated T-cell anti-tumor properties. In a phase I dose escalation trial of 21 heavily treated advanced stage cancer patients exposed to reovirus, an increase in CD3+CD4+ T-cells were observed in 10 (47.6%) patients and 5 patients demonstrated an increase in CD8+ perforin/granzyme+ T-cells [30]. An upregulation of T-cell related cytokine expression, including IL-5, IL-8, IL-6, IL-2, and IL-12p40, was observed in all 21 patients [30].

Reovirus and molecular implications

In addition to the cellular level, reovirus may also activate the immune system at the transcriptome level. In a trial of metastatic CRC patients, a reduction in miR-29-3p, which normally functions to express proteins that suppress IFN-gamma, was observed with an increase in IFN-gamma at 72 hours [31]. A change in several other transcription factors were also demonstrated, including an increase in *TAP1,* which expresses a protein essential for MHC Class I expression, *FCGR2A,* which encodes a protein for antibody binding, and *INFAR1,* which encodes a receptor essential for Type I interferon binding [31]. A reduction in *VEGFA* and *CXCR2*, which promote tumorigenic effects, and *ITGAM*, which express an integrin that promotes metastases were also observed, suggesting that reovirus induced anti-tumor properties involve not only the immune system at the cellular level, but also at the more intricate molecular level [31].

Reovirus and NARA

Reovirus has demonstrated to play an additional role in the adaptive immune system through stimulation of neutralizing anti-reoviral antibodies (NARA), present normally in healthy individuals [32]. In a phase I trial of intravenous reovirus, 22 of 31 heavily pretreated patients with advanced cancers demonstrated an initial NARA titer of >1/100, and post reovirus treatment led to significant increase in

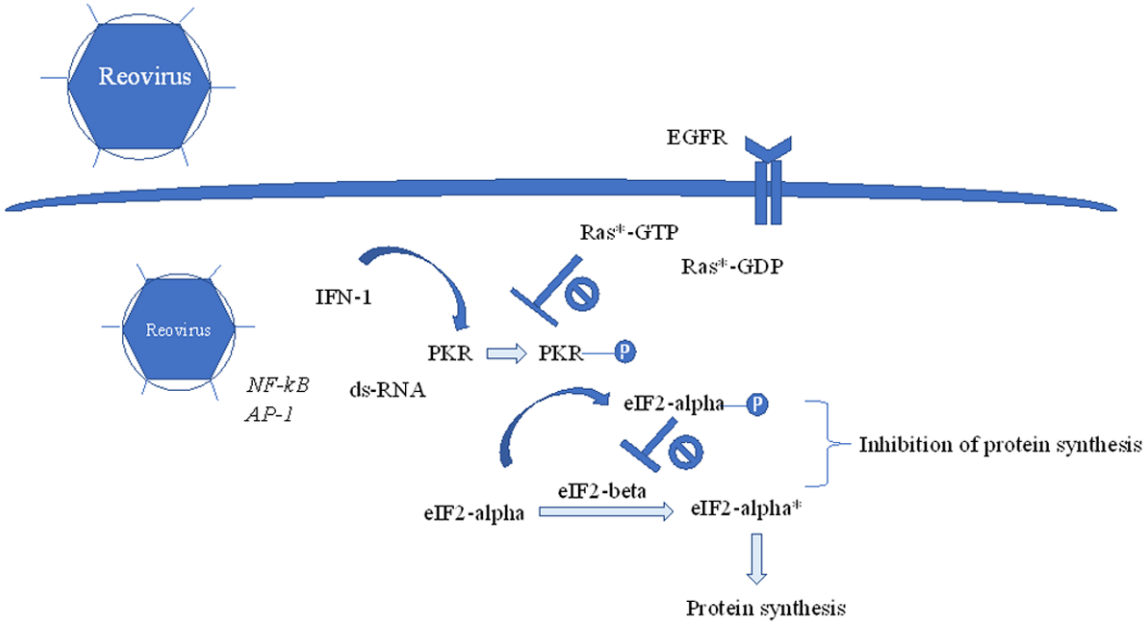

Figure 2. Reovirus-mediated oncolysis is negatively regulated by PKR. Upon reovirus infection, transcription factors such as NF-κB and AP-1 are activated, which leads to increase in interferon-1. Increase in interferon-1 leads to upregulation and autophosphorylation of PKR, which phosphorylates eIF2-alpha. Phosphorylated eIF2-alpha binds to eiF2-beta with high affinity, which in its free form, converts eiF2-alpha from its inactive to active state, leading to protein translation. eiF2-beta, when bound to phosphorylated eIF2-alpha, prevents recycling of active eiF2-alpha as a substrate, thereby blocking protein synthesis. In RAS-mutant cells, phosphorylation of PKR is inhibited, leading to uninhibited protein synthesis and reovirus replication.

NARA to >1/100,000, demonstrating a neutralizing antibody response [30]. Regardless of the NARA response, oncolysis has been observed, suggesting that oncolysis is not impeded with NARA and studies have incorporated immunosuppression or chemotherapy to modulate NARA [30]. For example, gemcitabine induction has demonstrated to blunt the NARA response, which may potentially exacerbate chemotherapy or reoviral adverse effects or increase its therapeutic potential [23, 33]. In combined chemotherapy trials with docetaxel and carboplatin-paclitaxel, however, NARA was not blunted and an increase in toxicity was not observed [34, 35]. While a blunted oncolytic response may be observed with NARA, it has been hypothesized that reovirus may possibly minimize neutralization and be directed at metastatic colorectal cells by being carried within peripheral blood mononuclear cells with prolonged infectivity as up to 10 days from viral induction [36].

Regulation of reovirus through PKR

Oncolysis of reovirus is rather selective and its oncolytic potential is mediated and regulated by activated protein kinase R (PKR) [28]. When reovirus initially affects cells, it is recognized by multiple cellular pattern receptors, such as toll-like receptors, which activate multiple transcription factors including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and activator protein-1 (AP-1) that increase interferon-1 (**Figure 2**) [28, 37]. This increase in interferon-1 upregulates PKR and direct binding of reovirus dsRNA to PKR, leading to dimerization and autophosphorylation of PKR, resulting in phosphorylation of eukaryotic initiation factor-2α (eIF2α), which in its active state, promotes protein synthesis [28]. The phosphorylation of eIF2α leads to a higher affinity and stable complex with eIF2B, which in turn suppresses further viral transcription, resulting in a self-limiting process [28]. In *KRAS* mutant cells, however, the initial phosphorylation of PKR is inhibited, leading to a disruption of the entire process, facilitating viral transcription and replication to continue unabated [26, 38].

Reovirus in clinical application

Reovirus and chemotherapy

Reovirus as monotherapy has demonstrated oncolytic potential in studies in a variety of

cancers, including breast, ovarian, glioma, prostate, carcinoid, melanoma, head and neck cancer, leiomyosarcoma, and non-HIV associated Kaposi's sarcoma, and its efficacy in combination with chemotherapy and radiotherapy has been demonstrated (**Table 1**) [39-43]. The first chemotherapy combination trial studied the safety and tolerability of reovirus with gemcitabine in 16 heavily pretreated cancer patients, including non-small cell lung cancer, colorectal cancer, breast cancer, cervical cancer, cholangiocarcinoma, esophageal adenocarcinoma, and poorly differentiated carcinoma [33]. Of the 10 who had an evaluable response, 1 achieved partial response, 6 stable disease, with a median duration of stable disease to be 72 days [33]. The combination of reovirus and gemcitabine was well tolerated, with most common adverse effects fever, nausea, diarrhea, and vomiting [33]. A reversible rise in liver enzymes was observed, though most also had concomitant acetaminophen use, possibly suggesting an increased possibility of gemcitabine associated liver toxicity with addition of reovirus at high titers [33].

Reovirus has previously exhibited cytotoxic effects synergistically with irinotecan in colorectal cancer cell lines [24]. In a phase I dose escalation trial of FOLFIRI with reolysin in KRAS-mutant metastatic colorectal cancer patients with secondary endpoint of response rate, PFS, and OS, 1 patient achieved partial response, 9 patients stable disease, and 8 patients progression, with median PFS of 7.4 months in FOLFIRI-naïve patients, and not-reached in non-FOLFIRI-naïve patients [44]. Activity of reolysin in KRAS-mutant metastatic colorectal patients was also demonstrated with addition of bevacizumab to irinotecan. In a phase I dose escalation trial of FOLFIRI/bevacizumab with pelareorep in 36 oxaliplatin refractory *KRAS*-mutated metastatic colorectal cancer patients, 20% of patients achieved partial response and 73.3% achieved stable disease [29]. As demonstrated in preclinical studies, an increase in absolute CD-8 count (2.4-fold at 7 days) and absolute CD-4 count (3.5-fold) were observed, demonstrating the activation of T-cells as mechanism of anti-tumor properties of oncolysis in the clinical setting [29]. The addition of reovirus was safely tolerated and resulted in median progression-free survival (PFS) of 65.6 weeks and overall survival (OS) of 25.1 months at the recommended phase II dose [29]. In a prior phase II randomized trial, mixed results were observed, however, between the two cohorts of reovirus and FOLFOLX/bevacizumab versus FOLFOX/bevacizumab alone, where in KRAS-mutated patients, inferior PFS, but a trend towards superior overall response rate (ORR) was observed in the combination arm [45]. This mixed finding may suggest that reovirus may have a synergistic effect with irinotecan, but not with oxaliplatin and it is also possible that reovirus may function as an immune stimulating agent and thus may need time to demonstrate an anti-cancer effect, yielding to early cancer progression. Studies are needed to better evaluate the benefit of reovirus with combination of chemotherapy and to evaluate differential responses based on chemotherapy backbone of irinotecan vs. oxaliplatin.

Reovirus and immunotherapy

Palareorep has also been studied in combination with PD-1 (programmed cell death) checkpoint inhibitors in pre-clinical models and early phase clinical trials. PD-1 receptors are present on B, T cells, and monocytes and inhibit T-cell activation, and tumor cells have adapted to express PD-L1 receptors on their surface to escape immune surveillance [46]. Oncolytic viruses have been demonstrated to work synergistically with checkpoint inhibitors in enhancing immune-mediated tumor cell clearance [47]. In microsatellite stable (MSS) colorectal cancer cell lines, SW620 and HT29, the combined nivolumab and reovirus treated increased cell death compared to nivolumab or reovirus alone [48]. In KRAS-mutant CT26 mouse model, combined anti-PD1 and reovirus treatment significantly suppressed tumor growth and increased overall survival compared to treatment with reovirus or anti-PD-1 alone [48]. The mechanism of increased tumorigenicity in combined reovirus and anti-PD-1 treatment was suggested to be decreased nuclear expression of proliferation marker Ki67, increased expression of apoptosis markers, including cleaved-caspase 3 and TUNEL [48]. In addition to increased apoptotic markers, reovirus was observed to enhance immunogenicity through its impact on the PD-L1/PD-1 axis. CT26 mouse models treated with combined reovirus and anti-PD-1 demonstrated enhanced reduction of PD-1 compared to

Table 1. Clinical trials combining chemotherapy with reovirus and immune parameters

PMID	Cancers	Phase	Reovirus Dose	Anti-cancer agent	Results	Immune parameters
32156785	Refractory metastatic colorectal cancer	I	1×10^{10} $TCID_{50}$-3×10^{10} $TCID_{50}$	FOLFIRI-bevacizumab + reovirus	PR: 3/6 (50%) Median PFS: 65.6 weeks Median OS: 25.1 months	Dendritic cell maturation: 48 hours, 4.5%-18.6% (P=000016) CD4-count increase: 3.5-fold (P=0.00015) CD8-count increase: 2.4-fold (P=0.00015)
18323793	Advanced solid cancers	I	1×10^{8} $TCID_{50}$-3×10^{10} $TCID_{50}$	None	N/A	CD4+: 10/21 (47.6%) CD8+: 7/21 (33%) NK: 6/21 (28.6%) Increase in IL-5, IL-8, IL-6, IL-2, IL-12p40 NARA: increase in 250-fold
29653857	Metastatic colorectal cancer, 1st line	II	3×10^{10} $TCID_{50}$	FOLFOX-6-bevacizumab ± reovirus	Median PFS: 7 vs. 9 months, HR 1.59 (P=0.046) Median OS: HR 1.22; (P=0.38) ORR: 2.52 (P=0.03)	N/A
20926400	Advanced solid cancers	I	3×10^{10} $TCID_{50}$	Docetaxel + reovirus	ORR: 14/16 PR: 4/16 in breast, stomach, gastroesophageal, ocular melanoma Minor response: 3/16 in mesothelioma, prostate, head and neck SD: 7/16 in prostate, melanoma, esophagus, pancreas, unknown	NARA: increase in 729-fold at peak
26709987	KRAS or EGFR mutant metastatic NSCLC	II	3×10^{10} $TCID_{50}$	Carboplatin, paclitaxel + reovirus	PR: 11/37 SD: 20/37 PD: 4/37 Not evaluable: 2/37 Median PFS: 4 months Median OS: 13.1 months	N/A
27039845	Metastatic pancreatic adenocarcinoma	II	3×10^{10} $TCID_{50}$	Carboplatin, paclitaxel ± reovirus	PFS: 4.9 vs. 5.2 (P=0.6)	Reovirus arm with increased pro-inflammatory markers, including fractalkine, IL-10, RANTES, SDF-1, VEGF-A Reovirus arm with increase in CD8+ expressing CD71, CD95, CD45RO
22316603	Relapsed/ metastatic Head and Neck Cancer	I	3×10^{10} $TCID_{50}$	Carboplatin, paclitaxel + reovirus	CR: 1/31 (3.8%) PR: 6/31 (23.1%) SD: 9/31 (34.6%) PD: 8/31 (30.8%) Duration of response: 6 months Median OS: 7.1 months	NARA: increase by 27-729-fold
29748010	NSCLC	II	4.5×10^{10} $TCID_{50}$	Pemetrexed or docetaxel ± reovirus	PFS: 3.0 vs. 2.8 months (P=0.53) STK11 (HR 0.29) and PIK3CA (HR 0.45) mutations with improved PFS	N/A
29799479	Advanced pancreatic adenocarcinoma, 1st line	II	1×10^{10} $TCID_{50}$	Gemcitabine + reovirus	PR: 1/34 SD: 23/34 PD: 5/34 Median OS: 10.2 months	Upregulation of PD-L1 on immunohistochemistry
29027598	Metastatic breast cancer	II	3×10^{10} $TCID_{50}$	Paclitaxel ± reovirus	PFS: 3.78 months vs. 3.38 months (P=0.87) OS: 17.4 months vs. 10.4 months (P=0.1)	N/A
31694832	Advanced pancreatic adenocarcinoma	Ib	4.5×10^{10} $TCID_{50}$	Reovirus and pembrolizumab + 5-fluorouracil, gemcitabine, or irinotecan	Disease control in 3 of 10 patients PR: 1/10 for 17.4 months SD: 2/10 for 9 months and 4 months	Creation of new T-cell clones observed

PR: partial response, SD: stable disease, PFS: progression free survival, OS: overall survival, ORR: overall response rate, NARA: neutralizing anti-reoviral antibodies, NSCLC: non-small cell lung cancer.

anti-PD-1 alone [48]. Tumor infiltrating lymphocytes, specifically CD4+ and CD8+ cells, which play a role in recognition and killing of tumor cells, and granzyme B expression was also observed to be increased in the combination treatment than either treatment alone [48]. An increase in interferon-gamma and TNF-alpha, inflammatory markers of increased cytotoxicity in CD4+, CD8+, and NK-cells, a decrease in TGF-beta, an inhibitor cytokine, and decrease in TOX, a marker of T-cell exhaustion, were observed with combined anti-PD-1 and reovirus treatment, which was not observed with monotherapy [48].

In addition to activation of the adaptive immune system, combination of anti-PD-1 and reovirus was demonstrated to synergistically activate the innate immune system through increase in pattern recognition receptors, including retinoic acid-inducible gene I (RIG-1), melanoma differentiation-associated protein 5 (MDA-5), protein kinase R (PKR), and NOD-like receptor protein 3 (NLRP3) [48].

While there is no published clinical trial combining reovirus with anti-PD1 therapy, palareorep in combination with chemo-immunotherapy has been studied in alternative gastrointestinal-related malignancies. In an open-label phase Ib study, palareorep and pembrolizumab in combination with gemcitabine, irinotecan, or 5-FU, were studied in 11 advanced pancreatic adenocarcinoma who progressed on first-line treatment, until disease progression or unacceptable toxicity [49]. Of the 10 evaluable patients, one patient achieved partial response of 17.4 months and 2 achieved stable disease of 9 months and 4 months without significant toxicity [49]. Peripheral blood samples of most patients demonstrated decreased T-cell clonality, but increased T-cell diversity, with increase in T-cell population turnover as well as statistically significant increases in CXCL10 and CXCL11, which attract CD8+ cells, without significant changes in cytokines attracting Treg [49]. It is hypothesized that the addition of checkpoint inhibitor would demonstrate a positive outcome in metastatic colorectal cancer patients based on preclinical trials. However support through clinical trials are not yet established, especially since prior trial with pancreatic adenocarcinoma comprised a substantial number of patients (6 of 11) who received gemcitabine but only 2 of 11 patients received irinotecan, which serves as a backbone in colorectal cancer [49]. Current multiple early phase clinical trials, namely NCT03206073, NCT03866525, and NCT04301011, are underway to evaluate the outcome of combined oncolytic viral therapy and checkpoint inhibitors in refractory colorectal cancer.

Conclusion

Reovirus is a multipotent immune agent with oncolytic properties that has demonstrated clinical efficacy in early phase trials in metastatic *KRAS* mutant colorectal cancer. Its mechanisms of anti-tumor action involve direct dendritic cell activation, by production of proinflammatory cytokines, dendritic cell mediated NK cell activation, which lead to exocytosis of perforin/granzyme and Fas-associated death domain, and activation of T-cells. Early phase clinical trials have demonstrated in prior chemotherapy refractory metastatic colorectal patients the safety, tolerability, and relatively robust response to the addition of reovirus to FOLFIRI. Further research with randomized trials is needed for to evaluate the efficacy of reovirus in treatment refractory metastatic colorectal cancer patients.

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Disclosure of conflict of interest

None.

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