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REVIEW

Clinical development of reovirus for cancer therapy: An oncolytic virus with immune-mediated antitumor activity

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Abstract

Reovirus is a double-stranded RNA virus with demonstrated oncolysis or preferential replication in cancer cells. The oncolytic properties of reovirus appear to be dependent, in part, on activated Ras signaling. In addition, *Ras*-transformation promotes reovirus oncolysis by affecting several steps of the viral life cycle. Reovirus-mediated immune responses can present barriers to tumor targeting, serve protective functions against reovirus systemic toxicity, and contribute to therapeutic efficacy through antitumor immune-mediated effects *via* innate and adaptive responses. Preclinical studies have demonstrated the broad anticancer activity of wild-type, unmodified type 3 Dearing strain reovirus (Reolysin®) across a spectrum of malignancies. The development of reovirus as an anticancer agent and available clinical data reported from 22 clinical trials will be reviewed.

Key words: Reovirus; Type 3 Dearing; Oncolytic virus; Ras; Epidermal growth factor receptor; Clinical trial; Preclinical; Immune modulation

Core tip: Reovirus has demonstrated oncolysis or preferential replication in cancer cells. The anticancer activity of reovirus has been demonstrated across a spectrum of malignancies in the preclinical setting. The relatively tolerable toxicity profile of reovirus renders it an attractive agent as part of combination therapy in cancer treatment. Reovirus-mediated immune modulation contributes to its antitumor activity *via* innate and adaptive immune responses and renders it an attractive component of immunotherapy. Here we compile the most extensive list of clinical trials investigating the anticancer efficacy of reovirus to date.

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INTRODUCTION

Reovirus and mechanism of oncolysis

The *Reoviridae* family of viruses consists of six genera, three of which including rotavirus, orbivirus, and reovirus are known to infect animals and humans, while the other three are known to infect plants and insects[1,2]. In 1959, the name reovirus was given to a virus commonly isolated from the respiratory and enteric tract that seldom caused few, if any, clinical symptoms (orphan virus)[3]. However, when symptomatic, reovirus infection is characterized by mild enteric and respiratory symptoms in humans[1-5]. Wild-type reovirus is ubiquitous throughout the environment with seropositivity having been documented in as many as 70%-100% of subjects[3]. There exists several serotypes of reovirus [type 1 Lang, type 2 Jones, type 3 Abney, and type 3 Dearing (T3D)] that have been identified by antibody hemagglutination-inhibition and neutralization studies[2,3,5].

Reovirus is approximately 80 nm in diameter and comprised of a protein shell with outer and inner components that altogether create an icosahedral capsid housing ten segments of double-stranded RNA (dsRNA)[1,2,4-7]. It has been more than 30 years since wild-type reovirus was demonstrated to replicate preferentially in transformed cell lines but not in normal cells[8,9]. The means by which reovirus oncolysis occurred remained elusive until rodent cell lines transformed with genes encoding the epidermal growth factor receptor (EGFR) and a truncated form of the EGFR, possessing constitutive tyrosine kinase activity but lacking the extracellular ligand-binding domain, demonstrated increased susceptibility to reovirus infection and thereby proposing that EGFR-mediated pathways facilitated reovirus infection[10,11]. Indeed, transfection with constitutively activated *Ras* oncogenes or son of sevenless in NIH-3T3 fibroblasts resulted in increased vulnerability to reovirus infection and elucidated the involvement of activated Ras signaling pathways in reovirus oncolysis[12,13].

Given that approximately 30% of all cancers in humans have been linked to activating *Ras* mutations, subsequent studies investigated prospective downstream mediators of Ras that may be critical to reovirus oncolysis and implicated, in particular, the Ras/RalGEF/p38 pathway in promoting preferential reovirus replication[14,15]. Additionally, it was determined that dsRNA-activated protein kinase (PKR), which is normally activated in the presence of viral transcripts and inactivates eukaryotic initiation factor 2α (eIF-2α), protein synthesis, and viral replication, is kept inactivated in *Ras*-transformed cells thereby providing the link between PKR and an activated Ras signaling pathway in reovirus oncolysis[13,16]. Aside from viral translation, *Ras*-transformation has been shown to promote oncolysis by affecting other steps of the reovirus infectious life cycle including viral disassembly or uncoating, production of viral progeny with boosted infectivity, progeny release through increased apoptosis, and spread of virus in later cycles of infection[17-19].

PRECLINICAL DEVELOPMENT OF REOVIRUS

Monotherapy

Given the wide-reaching implications of activated *Ras* mutations in human cancers, the first proof-of-concept preclinical studies involved tumors established from v-erbB-transformed murine NIH-3T3 fibroblasts and human U87 glioblastoma cells implanted in severe combined immune deficient (SCID) mice that demonstrated marked tumor regression in approximately 80% of mice following single intratumoral injections of reovirus by day 12 and week 4, respectively[20]. However, SCID mice represented a non-ideal model for reovirus antitumor studies given that approximately 50%-60% of reovirus-treated animals experienced limb necrosis and death[20]. The "Black Foot" syndrome has been characterized by infection with live reovirus of venule endothelial cells and myocardial and musculoskeletal myocytes leading to vasculitis, localized hemorrhage, and/or thrombosis in the extremities of SCID mice[21]. Activated Ras signaling pathways are present in a majority of malignant gliomas, and accordingly, reovirus demonstrated antitumor activity in 83% of malignant glioma cells *in vitro*, in 2 subcutaneous and 2 intracerebral human malignant glioma models *in vivo*, and in 100% of glioma specimens *ex vivo*[22]. In medulloblastoma cell lines, reovirus translation was restricted to cell lines with higher levels of activated Ras, and intratumoral injections of reovirus prolonged survival in orthotopic *in vivo* animal models of medulloblastoma with spinal and leptomeningeal metastases[23].

The incidence of activated *Ras* mutations in colon cancer is approximately 50%[15]. The significance of *Ras* transformation in reovirus oncolysis of colon cancer cells has also been highlighted in K-*Ras* knockdown murine colorectal cancer cells that demonstrated complete nullification of reovirus-induced apoptosis compared to control[24]. Indeed, treatment with reovirus exhibited significant antitumor effects in human colorectal cancer *in vitro* and *in vivo* characterized by elevated Ras activity in colon cancer cell lines and restriction of reovirus infection to tumor cells when compared to controls[25]. Other studies also demonstrated the antitumor efficacy of reovirus *in vitro* in colon cancer cell lines, *in vivo* in rodent models of colorectal liver metastases, and notably, in fresh human colorectal tissue isolates that required the processing of virions to infectious subvirion particles (ISVPs) and proper localization and quantity of junctional adhesion molecule-1 on tumor cells for productive lysis[26-28]. Furthermore, colon cancer cell lines HEK293 and HCT116 demonstrated sensitization to reovirus-induced apoptosis by downregulation of nuclear

factor-kappa B (NF-κB) through inhibition of glycogen synthase kinase-3β[29].

In adenocarcinomas of the pancreas, the incidence of K-*Ras* mutations is among the highest in human cancer (approximately 90%)[15]. Not surprisingly, reovirus demonstrated potent cytotoxicity in 100% of pancreatic cancer cell lines *in vitro* and induced regression in 100% of subcutaneous tumor mouse models *in vivo*[30]. Interestingly, antitumor activity was seen in BxPC3 pancreatic cancer cells, which are known to have normal K-*Ras* oncogenes, treated with reovirus *in vitro* and *in vivo* though the reovirus-induced cytotoxicity observed in these cells was attributed to overall increased Ras activity, a concept reintroduced below[30]. Administration of reovirus also induced regression in immunocompetent hamster models of pancreatic cancer with liver and peritoneal metastases compared to controls[31,32].

Although the incidence of H-*Ras* mutations has been reported as high as 17% in cases of bladder carcinoma, activated EGFR-mediated pathways are present in up to 50% of cases of transitional cell carcinoma (TCC) of the bladder[15,33]. Treatment of co-cultured spheroids established by culturing TCC of the bladder cell lines and fibroblasts with reovirus demonstrated selective killing of tumor cells by lysis or induction of apoptosis *in vitro*[33]. Additionally, intravesical administration of reovirus resulted in significantly higher tumor-free survival in an orthotopic rat model of bladder cancer compared to control[34]. Along similar lines of thought, the incidence of N-*Ras* mutations in melanoma is relatively lower (approximately 8%-19%) compared to those found in colon and pancreatic cancers[15]. Nevertheless, human melanoma cell lines and murine xenograft models of melanoma were susceptible to tumor killing by reovirus with implications towards the role of the immune system in reovirus oncolysis (which will be further discussed later)[35].

Interestingly, activating *Ras* mutations in breast cancer are relatively rare though unregulated stimulation of Ras signaling pathways through mediators such as human EGFR 2 (Her-2 or ErbB-2) and its homologue Neu, both tyrosine kinases of the EGFR family, and the Src family of nonreceptor tyrosine kinases can occur highlighting the concept that activated Ras signaling rather than mutations in the Ras protein itself can be important to disease pathogenesis[3-5,36]. Accordingly, reovirus demonstrated significant antitumor effects *in vitro* in breast cancer cell lines characterized by resistance to infection in normal cell lines, *in vitro* in breast cancer stem cells, and *in vivo* in animal tumor models including models of brain and leptomeningeal metastases[36-39]. Furthermore, the presence of replicating reovirus was confirmed in *ex vivo* surgical breast cancer specimens[36]. Notably, there was no observed relationship between susceptibility to reovirus infection and HER2 expression, *in vitro*, though levels of Ras activity were higher in breast cancer cell lines when compared to control[37]. Ovarian cancer represents another example in which activating *Ras* mutations are rare but increased Ras signaling *via* increased activation of Her-2/Neu and/or Src likely contribute to pathogenesis[4,5,25]. Treatment with reovirus resulted in potent antitumor activity, when compared to controls, in ovarian cancer cell lines *in vitro* highlighted by increased reovirus protein synthesis in tumor cell lines but not in normal cells, in a human ovarian SKOV3 cell line implanted in the flanks of mice *in vivo*, and in a murine ascites model of human ovarian cancer highlighted by prolonged survival in those treated with intraperitoneal injections of live virus every 2 wk[25]. All 3 *ex vivo* human ovarian tumor surgical biopsy specimens also demonstrated susceptibility to reovirus infection[25].

Similarly, marked cytopathic effects and inhibition of tumor growth were observed with reovirus treatment, *in vitro* and *in vivo*, in cancers where relatively little has been known, historically, about the involvement of *Ras* mutations in transformation such as head and neck cancer, prostate cancer, and sarcomas[40-42]. Intriguingly, although reovirus-induced cytotoxicity was observed in several head and neck carcinoma cell lines, correlative analyses revealed no associations between phosphorylated eIF-2α or EGFR levels and cytopathic effects suggesting that reovirus oncolysis appears to occur independently of PKR, Ras signaling, and EGFR signaling pathways[43,44].

Hematologic malignancies posed a perplexing dilemma regarding their susceptibility to reovirus infection given the near absence of N-*Ras* mutations particularly in chronic lymphocytic leukemia (CLL) and non-Hodgkin lymphomas (NHLs) such as follicular lymphoma (FL) and diffuse large B-cell lymphoma (DLBCL)[15,45]. Nevertheless, it was hypothesized that certain hematologic malignancies may still be amenable to reovirus therapy from knowledge that the break point cluster-Abelson (Bcr-Abl) nonreceptor tyrosine kinase present in 95% of chronic myelogenous leukemia is dependent on *Ras* activation, *Myc* oncogenes coordinate with *Ras* in B-cell transformation, specific ligand-receptor interactions in CLL lymphoid cells stimulate Ras signaling, and mutations in a proto-oncogene member of the *Ras* superfamily is present in up to 46% of DLBCLs[3,45]. Indeed, reovirus treatment of human lymphoma cells produced antitumor effects in all 4 DLBCL cell lines and 2 out of 5 Burkitt lymphoma cell lines *in vitro* highlighted by increased reovirus protein synthesis and progeny production in sensitive cell lines compared to resistant cell lines and *in vivo* in a Burkitt cell line sensitive to reovirus implanted in mice but not in a xenograft model of a previously determined resistant Burkitt cell line[45]. Furthermore, all *ex vivo* human primary CLL samples and a majority of NHL samples including Burkitt lymphoma, mantle cell lymphoma, and DLBCL were susceptible to reovirus oncolysis while a majority of FL specimens were resistant[45].

Treatment of acute myeloid leukemia (AML) with reovirus showed marked antitumor responses in 2 out 4 AML cell lines *in vitro* and in 8 out of 10 peripheral blood primary AML specimens *ex vivo*[46]. Concordant

with prior findings, a FL cell line was resistant to reovirus therapy *in vitro* and *in vivo* while mantle cell lymphoma cell lines displayed a heterogenous response to reovirus that correlated with levels of activated Ras and proteolytic disassembly of reovirus into ISVPs *in vitro*[47]. The discrepancies in sensitivity to reovirus infection between various hematologic malignancies have been attributed, in part, to differential *Ras* activation and interferon sensitivities[4,5,45,47]. Reovirus induced cell death *via* apoptotic and autophagic pathways in a majority of multiple myeloma cell lines *in vitro* with sensitivity conferred to *ex vivo* tumor specimens as well[48]. Reovirus also showed meaningful inhibition of tumor growth in *in vivo* multiple myeloma models compared to control, and treatment with reovirus did not abrogate human stem cell repopulation and differentiation *in vivo*[48]. Earlier studies revealed that reovirus did not affect hematopoietic progenitor stem cells, and the mixture of reovirus with human monocytic and myeloma cancer cell lines *in vitro* and *ex vivo* tumor cells of DLBCL, CLL, Waldenström macroglobulinemia, and small lymphocytic lymphoma showed complete purging of disease in patient products of apheresis[49]. The use of reovirus as a purging strategy for autologous stem cell transplantations has since been an emerging concept with demonstrated efficacy in breast cancer and multiple myeloma[50,51].

Combination therapy

The earliest preclinical studies involving reovirus in combination therapy entailed L1210 murine leukemia cells and EL4 murine lymphoma cells treated with the chemotherapeutic agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) followed by treatment with reovirus that increased survival in ascites tumor mouse models when compared to controls and were among the first to illustrate that resistance of surviving animals to challenges with homologous tumor was orchestrated by an immune-mediated process[52-54]. Reovirus in combination with radiation therapy, when compared to controls, produced enhanced apoptosis across head and neck, colorectal, and breast cancer cell lines *in vitro* (independent of treatment sequence or schedule and without affecting viral replication at clinically relevant radiation doses) and delayed tumor growth in colorectal cancer and melanoma models *in vivo*[55]. Criteria for therapeutic enhancement were met for ewing sarcoma (ES) and osteosarcoma murine xenografts and rhabdomyosarcoma and ES murine xenografts treated with reovirus in combination with cisplatin and reovirus in combination with radiotherapy [4 gray (Gy) daily × 5 fractions], respectively[40].

In murine melanoma xenografts, metronomic dosing of high-dose cyclophosphamide with reovirus permitted access to tumors by therapeutically high levels of virus while reducing serious toxicities associated with ablation of neutralizing antibody titers, and cisplatin with reovirus significantly inhibited tumor growth compared to controls without affecting neutralizing antibody response though cisplatin reduced the inflammatory cytokine response to reovirus[56,57]. Treatment with reovirus and cyclosporin A significantly inhibited tumor growth in a murine *Ras*-transformed fibroblastic xenograft while reovirus with cyclosporin A or T-cell depletion significantly improved survival in a murine metastatic lung cancer model compared to controls[58]. Although reovirus alone demonstrated potent cytotoxicity in 7 of 9 non-small cell lung cancer (NSCLC) cell lines *in vitro*, heterogenous synergistic effects on cell killing were observed with reovirus in combination with cisplatin, gemcitabine, or vinblastine on NSCLC cancer cell lines *in vitro*[59]. The reovirus and paclitaxel combination, however, showed synergistic cell killing in all NSCLC cell lines *in vitro* characterized by enhanced apoptosis[59].

More recently, although trastuzumab and reovirus monotherapy both inhibited tumor growth *in vitro*, treatment with reovirus was found to sensitize gastric cancer cells that overexpressed HER2 to apoptosis when combined with trastuzumab[60]. However, in HER2 low expressing cells, reovirus monotherapy or in combination with trastuzumab increased apoptosis *in vitro*, but there was no reduction in growth when treated with trastuzumab alone[60]. Further analysis showed that reovirus induced expression of TRAIL, a protein implicated in promoting apoptosis, without upregulating TRAIL receptors. TRAIL expression was increased with both trastuzumab and reovirus therapy, but this effect was enhanced by combination therapy[60].

Similar synergistic antitumor effects have been established, when compared to controls, in combination regimens involving: (1) reovirus with cisplatin and paclitaxel in head and neck cancer *in vitro* characterized by enhanced apoptosis and cell cycle disruption (though without enhancing reovirus replication) and *in vivo*; (2) reovirus with bortezomib in pancreatic cancer *in vitro* and *in vivo* characterized by enhanced levels of ER stress and apoptosis; (3) reovirus with cyclosporin A in a murine model of colorectal liver metastases; (4) reovirus and gemcitabine in human colon cancer *in vitro* and *in vivo*; and (5) reovirus with paclitaxel, vincristine, cisplatin, doxorubicin, or docetaxel in prostate cancer *in vitro* highlighted by the greatest synergism in the reovirus and docetaxel combination with enhanced apoptosis and microtubule stabilization[27,61-64]. Reovirus and docetaxel also produced significant tumor growth retardation in a murine prostate cancer xenograft[63]. Interestingly, reovirus in combination with Newcastle disease virus or parvovirus resulted in significant synergistic antitumor responses in glioblastoma cell lines *in vitro* with an efficient rate of co-infection and without affecting the kinetics of viral replication among the viruses[65]. Furthermore, reovirus with Newcastle disease virus significantly inhibited tumor growth in a murine glioblastoma xenograft compared to control without significant toxicity though the experiments were terminated 12 d after virus injection[65].

In sum, preclinical studies have demonstrated the broad anticancer activity of reovirus across a spectrum

of malignancies including colon, breast, ovarian, lung, skin (melanoma), neurological, hematological, prostate, bladder, and head and neck cancer which have ultimately provided the basis for human clinical trials[1,5,6,66]. The three serotypes of reovirus including type 1 Lang, type 2 Jones, type 3 Abney, and T3D all have demonstrated oncolytic properties, but the T3D strain has been most extensively studied as an anticancer agent and is the only therapeutic wild-type reovirus in clinical development under its proprietary formulation, Reolysin®, developed by Oncolytics Biotech Inc. (Calgary, Canada)[2-4,67]. Thus far, there are a total of 34 clinical trials involving reovirus in the treatment of a variety of cancers that are both completed and ongoing (http://www.oncolyticsbiotech.com/clinical-trials). Clinical data available and reported from 22 clinical trials will now be discussed (Tables 1 and 2).

CLINICAL DEVELOPMENT OF REOVIRUS

Phase Ⅰ trials

The first phase Ⅰ trial (REO 001) involved administration of intralesional reovirus in patients with advanced solid tumors and histologically confirmed cutaneous lesions[68]. In a dose-escalation design, doses of 1×10^7 plaque forming units (PFU) once weekly up to maximum doses of 1×10^{10} PFU once weekly were used[68]. Out of 19 patients, dose-limiting toxicities (DLTs) and a maximum-tolerated dose (MTD) were not observed even at maximum dose[68]. The most common treatment-related adverse events (AEs) included nausea (79%), vomiting (58%), and local erythema of injection site (42%) while fevers/chills and transient flu-like symptoms accounted for 37% and 32%, respectively[68]. The best overall response ≥ 6 wk was complete response (CR) in 1 (5.3%), partial response (PR) in 2 (10.5%), stable disease (SD) in (21.1%)[68].

REO 002 enrolled 6 patients with localized prostate cancer who received a single intratumoral injection of 1×10^7 PFU of reovirus 3 wk prior to planned prostatectomy as definitive cancer treatment[42]. There were no DLTs or grade 3 or higher toxicities observed and the most common AE included mild flu-like illness in 4 out of 6 patients[42]. In all patients, prostate-specific antigen (PSA) levels did not significantly fluctuate from baseline, and pathologic specimens showed moderate to strong staining for reovirus proteins localized to areas of cancer but sparing of adjacent benign areas and remote areas of cancer in 5 patients[42].

Another phase Ⅰ study (REO 003) involved single stereotactic intralesional injection of reovirus at doses ranging from 1×10^7 tissue culture infectious dose-50 ($TCID_{50}$) to 1×10^9 $TCID_{50}$ in 12 patients with progressive or recurrent malignant gliomas[69]. The MTD was not reached even at maximum doses and there were no DLTs observed with the only grade 3 or higher treatment-related AE being an elevation in γ-glutamyl transpeptidase[69]. The median time to disease progression (TTP) was 4.3 wk (range 2.6-39 wk), median overall survival (OS) was 21 wk (range 6-234 wk), and best overall response was SD in 1 patient with TTP of 39 wk[69]. REO 007, a multicenter phase Ⅰ study, aimed to determine DLTs, MTD, and target lesion response rate after administering reovirus *via* intratumoral infusion in 15 patients with recurrent malignant gliomas[70]. Similarly to REO 003, the MTD was not achieved at maximum doses. Only three patients suffered from convulsions, a grade 3 AE, which does occur commonly in patients with intracranial tumors[70]. Additionally, only one of these three grade 3 AEs was possibly related to infusion of reovirus[70]. During the study period of 24 wk, ten patients were reported to have stable disease, four with progressive disease, and one with partial response[70]. However, ultimately 12 out of the 15 patients did have progressive disease with the median time to progression being 61 d (range 29-150) and the median survival being 140 d (range 97-989)[70]. The one patient that did achieve a partial response did receive the maximum dose[70].

REO 004 included 18 patients with advanced solid tumors treated with intravenous (*IV*) reovirus from 1×10^8 $TCID_{50}$ to 3×10^{10} $TCID_{50}$ once every 28 d in which the latter dose was declared the MTD due to protocol termination once the protocol-defined highest dose was reached[71]. No DLTs were observed and the most common AEs included myalgia, fatigue, and fever[71]. Out of 18 patients, the best overall response was PR > 5 cycles in 1 patient (5.6%) with taxane and anthracycline refractory breast cancer (whose post-treatment chest wall biopsy showed viral replication and extensive necrosis consistent with reovirus activity) and SD > 1 cycle in 7 patients (38.9%) for a clinical benefit rate (CBR) of about 45% (combined CR, PR, and SD)[71]. Of note, 5 patients had *Ras* mutations and 1 patient had a *Braf* mutation, and the formation of neutralizing anti-reovirus antibodies (NARAs) bore no relationship to clinical benefit while those with detectable viral shedding appeared to have greater benefit[71]. One phase Ⅰ study (REO 005) pitted *IV* reovirus against various refractory or metastatic cancers, and a MTD was reached at a dose of 3×10^{10} $TCID_{50}$ once daily for 5 d every 28 d by virtue of being the highest dose available for administration (this subsequently also became the recommended phase Ⅱ dose)[72]. No DLTs were observed and the most common AEs were fever, fatigue, and headache[72]. Out of 33 enrolled patients, the best overall response was SD > 7 wk in 10 patients, and no relationships between SD to dose or duration of reovirus therapy were established[72].

REO 006 enrolled 25 patients with various refractory or progressive solid cancers in a two-stage dose-escalation design where phase Ia treated patients with 1×10^8 $TCID_{50}$ to 1×10^{10} $TCID_{50}$ intratumoral injection of reovirus on days 2 and 4 with 20 Gy local irradiation daily × 5 fractions while phase 1b treated patients with 1×10^{10} $TCID_{50}$ intratumoral injection of reovirus twice weekly for 1-3 wk with 36 Gy local irradiation × 12 fractions over 16 d[73]. There were no DLTs observed, a MTD was not reached, and the most common treatment-related AEs

Table 1 Phase Ⅰ trials involving reovirus

Phase	Malignancy	Dosing regimen	Clinical response
Ⅰ (REO 001)	Various advanced or refractory solid malignancies	1×10^7 PFU to 1×10^{10} PFU intralesional injection once or 3 × weekly (dose escalation)	Out of 19 patients, best overall response ≥ 6 wk was CR in 1 with Klatskin (5.3%), PR in 2 with head and neck cancer (10.5%), SD in 4 1 with head and neck, 1 with melanoma, 1 with breast cancer, 1 with Kaposi's (21.1%)
Ⅰ / translational (REO 002)	Localized prostate cancer	1×10^7 PFU single intratumoral injection 3 wk prior to planned prostatectomy	Out of 6 patients, all did not exhibit significant fluctuations in PSA from baseline. Five of 6 patients showed staining for reovirus proteins localized to cancer areas but sparing of adjacent benign and remote cancer areas. Pathologic specimens showed peritumoral inflammation in 4 patients, apoptosis in 4 patients, and necrosis in 2 patients
Ⅰ (REO 003)	Advanced or recurrent malignant gliomas	1×10^7 $TCID_{50}$ to 1×10^9 $TCID_{50}$ single stereotactic intralesional injection (dose escalation)	Out of 12 patients, best overall response was SD in 1 patient with oligo-astrocytoma with a TTP of 39 wk. The overall median TTP was 4.3 wk (range 2.6-39 wk), and median OS was 21 wk (range 6-234 wk)
Ⅰ (REO 004)	Various advanced or refractory solid malignancies	60-min *IV* infusion from 1×10^8 $TCID_{50}$ to 3×10^{10} $TCID_{50}$ once every 28 d (dose escalation)	Out of 18 patients, best overall response was PR > 5 cycles in 1 patient with breast cancer (5.6%) and SD > 1 cycle in 7 (5 with ovarian cancer, 1 with carcinoid, 1 with STS, 38.9%); CBR of about 45%
Ⅰ (REO 005)	Various advanced or refractory solid malignancies	60-min *IV* infusion from 1×10^8 $TCID_{50}$ once every 28 d to 3×10^{10} $TCID_{50}$ once daily for 5 d every 28 d (dose escalation); *IV* reovirus 3×10^{10} $TCID_{50}$ once daily for 5 d every 28 d became recommended phase Ⅱ dose	Out of 33 enrolled patients, best overall response was SD > 7 wk in 10 patients (2 with colon cancer, 2 with prostate cancer, 2 with STS, 1 with lung cancer, 1 with TCC of the bladder, 1 with melanoma, 1 with endometrial cancer)
Ⅰ (REO 006)	Various advanced or refractory solid malignancies	1×10^8 $TCID_{50}$ to 1×10^{10} $TCID_{50}$ intratumoral injection on days 2 and 4 with 20 Gy local irradiation daily × 5 fractions *vs* phase 1b: 1×10^{10} $TCID_{50}$ intratumoral injection twice weekly from 1-3 wk with 36 Gy local irradiation × 12 fractions over 16 d (two-stage dose escalation); intratumoral 3×10^{10} $TCID_{50}$ × 2 injections with 20 Gy × 5 fractions and intratumoral 1×10^{10} TCID50 × 6 injections with 36 Gy × 12 fractions became recommended phase Ⅱ doses for short and prolonged palliative regimens, respectively	Out of 7 patients in phase 1a, best overall response was PR in 2 (esophageal adenocarcinoma and SCC of skin), SD in 5 (melanoma, pancreatic adenocarcinoma, SCC of larynx, and 2 with SCC of skin); out of 7 patients in phase 1b, 5 had PR (lung adenocarcinoma, colorectal cancer, ovarian adenocarcinoma, 2 with melanoma) and 2 had SD (melanoma) up to 3 mo post-treatment
Ⅰ (REO 007)	Recurrent malignant gliomas	72-h intratumoral infusion from 1×10^8 $TCID_{50}$ to 1×10^{10} $TCID_{50}$ (dose escalation)	Out of 15 patients enrolled, best overall response was SD in 10 patients during the study period of 24 wk. The median TTP was 61 d (range 29-150 d), and median survival was 140 d (range 97-989)
Ⅰ (REO 009)	Various advanced or refractory solid malignancies	60-min *IV* infusion from 1×10^9 $TCID_{50}$ to 3×10^{10} $TCID_{50}$ on day 1 (dose escalation) with 30-min *IV* infusion of gemcitabine 1000 mg/m^2 days 1 and 8 every 21 d (1×10^{10} $TCID_{50}$ reovirus on day 1 became recommended phase Ⅱ dose with gemcitabine)	Out of 10 patients, best overall response was PR after 4 cycles in 2 patients (1 with nasopharyngeal carcinoma, 1 with breast cancer) and SD for 4-8 cycles in 5 patients (median SD 72 d, range 36-112 d); CBR of 80%
Ⅰ (REO 010)	Various advanced or refractory solid malignancies	60-min *IV* infusion from 3×10^9 $TCID_{50}$ to 3×10^{10} $TCID_{50}$ days 1-5 (dose escalation) with 60-min *IV* infusion of docetaxel 75 mg/m^2 day 1 every 21 d (3×10^{10} $TCID_{50}$ reovirus days 1-5 every 21 d became recommended phase Ⅱ dose with docetaxel)	Out of 16 patients, best overall response was PR ≥ 2 cycles in 4 patients (1 with breast cancer who experienced CR in liver lesion, 1 with gastric cancer, 1 with gastroesophageal cancer, 1 with ocular melanoma) and SD ≥ 2 cycles in 10 patients (cancers included prostate, mesothelioma, SCC of head and neck, unknown primary, melanoma, esophageal cancer, pancreatic cancer); CBR of 88%
Ⅰ / translational (REO 013)	Colorectal cancer metastatic to the liver	60-min *IV* infusion of 1×10^{10} $TCID_{50}$ daily × 5 d between 6-28 d prior to planned radical resection of liver metastases	Out of 10 patients, 9 patients with resected tumor specimens demonstrated positive staining for reovirus that was greatest in tumor metastases compared to surrounding tumor stroma or adjacent normal liver. In addition, tissue analysis in 4 patients showed findings consistent with reovirus-associated apoptosis
Ⅰ (REO 022)	Metastatic colorectal cancer	60-min *IV* infusion from 1×10^{10} $TCID_{50}$ to 3×10^{10} $TCID_{50}$ days 1-5 every 28 d (dose escalation) with standard FOLFIRI doses (recommended phase Ⅱ dose was irinotecan 150 mg/m^2 with 3×10^{10} $TCID_{50}$ *IV* reovirus days 1-5 every 28 d)	Out of 18 patients, best overall response was PR in 1 patient (5%) and SD in 9 (50%) with median PFS in FOLFIRI-naïve patients of 7.4 mo (95%CI: 1.9-12.9 mo) and overall median PFS of 7.4 mo (95%CI: 0.6-14.1 mo)
Ⅰ (OSU-11148, NCI trial)	Refractory or relapsed multiple myeloma	60-min *IV* infusion from 3×10^9 $TCID_{50}$ to 3×10^{10} $TCID_{50}$ days 1-5 every 28 d (dose escalation)	Out of 12 patients, best overall response was SD with longest duration being 8 cycles. During cycle 1, 5 patients had decreased myeloma proteins, 3 had minimal increases, and 4 had progressive disease

PFU: Plaque forming units; CR: Complete response; PR: Partial response; SD: Stable disease; PSA: Prostate-specific antigen; $TCID_{50}$: Tissue culture infectious dose-50; TTP: Time to disease progression; OS: Overall survival; *IV*: Intravenous; STS: Soft tissue sarcoma; CBR: Clinical benefit rate; TCC: Transitional cell carcinoma; Gy: Gray; SCC: Squamous cell carcinoma; FOLFIRI: Irinotecan/fluorouracil/leucovorin; PFS: Progression-free survival.

Table 2 Phase Ⅰ/Ⅱ, Ⅱ, and Ⅲ trials involving reovirus

Phase	Malignancy	Dosing regimen	Clinical response
Ⅰ/Ⅱ (REO 011)	Various advanced or refractory solid malignancies	60-min *IV* infusion from 3×10^9 $TCID_{50}$ to 3×10^{10} $TCID_{50}$ days 1-5 (dose escalation) with *IV* paclitaxel 175 mg/m^2 over 3 h and *IV* carboplatin AUC_5 (over 30 min) on day 1 every 21 d (3×10^{10} $TCID_{50}$ *IV* reovirus days 1-5 every 21 d became recommended phase Ⅱ dose with paclitaxel and carboplatin)	Out of 26 patients, best overall response was CR in 1 patient (3.8%,head and neck cancer), PR in 6 patients (23.1%, 3 each with SCC of head and neck and head and neck cancer), major clinical response not evaluable by RECIST criteria in 2 patients (7.7%, SCC of head and neck), and SD in 9 patients (34.6%, 3 with SCC of head and neck, 3 with head and neck cancer, 1 with gynecological cancer, 1 with melanoma, 1 with sarcoma) with median duration of SD and PR of 6 mo (range 3-10 mo). Of the 24 patients with head and neck cancer, median OS was 7.1 mo (CI: 4.2-11.5 mo)
Ⅰ/Ⅱ (OSU-07022, NCI trial)	Recurrent or refractory ovarian, peritoneal, and fallopian tube carcinomas	60-min *IV* infusion 3×10^{10} $TCID_{50}$ days 1-5 with daily IP administration days 2-3 beginning cycle 2 every 28 d (dose escalation with IP dosing)	Thus far 8 patients have received treatment. Biopsied ovarian and peritoneal tumor samples reveal detection of viral proteins in tumor tissues compared to control after systemic (*IV*) administration of reovirus and presence of reovirus replication in tumors due to overlap of reovirus protein and microtubules
Ⅱ (REO 008)	Various advanced or refractory solid malignancies	Open-label, single-arm, multicenter: 1×10^{10} $TCID_{50}$ intratumoral injection on days 2 and 4 with 4 Gy local irradiation daily × 5 (total 20 Gy) every cycle	Out of 16 patients enrolled (5 with melanoma, 4 colorectal, 1 gastric, 1 ovarian, 1 pancreatic, 1 lung, 1 cholangiocarcinoma, 1 sinus, 1 thyroid), 14 were evaluable and best overall response was SD or better in 13 patients (93%). Of these patients, 4 had PR (2 with melanoma, 1 lung, 1 gastric) and 2 had minor responses (1 thyroid and 1 ovarian)
Ⅱ (MAYO-MC0672, NCI trial)	Metastatic melanoma	Open-label, single-arm, multicenter: 60-min *IV* infusion 3×10^{10} $TCID_{50}$ days 1-5 every 28 d	Out of 21 evaluable patients, best overall response was SD > 8 wk in 6 patients. The median TTP was 45 d (range 13-96 d) and median OS was 165 d (range 15 d-15.8 mo). Trial was closed as did not meet previously defined efficacy criteria to proceed to second stage of accrual
Ⅱ (REO 014)	Advanced or refractory sarcomas metastatic to lung	Open-label, single-arm, multicenter: 60-min *IV* infusion 3×10^{10} $TCID_{50}$ days 1-5 every 28 d	Out of 53 enrolled patients, best overall response was SD ≥ 12 wk in 18 patients (34%) with a subgroup of 12 patients (3 with synovial sarcoma, 2 with leiomyosarcoma, 2 with MFH, 1 with ES, 1 with non-specified spindle cell sarcoma, 1 with chordoma, 1 with ASPS), 1 with myxoid liposarcoma) having prolonged SD > 16 wk. Three of these patients demonstrated SD > 1 yr (1 with MFH, 1 with synovial sarcoma, 1 with ES). The median TTP was 58.0 d (95%CI, 54-110, range 8-726 d). The prolonged SD demonstrated fulfilled the study criteria for consideration as an active agent
Ⅱ (REO 015)	Refractory, recurrent, or metastatic SCC of the head and neck	Open-label, single-arm: 60-min *IV* infusion 3×10^{10} $TCID_{50}$ days 1-5 with *IV* paclitaxel 175 mg/m^2 over 3 h and *IV* carboplatin AUC_5 (over 30 min) on day 1 every 21 d	Out of 13 evaluable patients (sites included 3 larynx, 6 oral cavity, 4 pharynx, 1 other), 4 had PR (31%) and 2 had SD ≥ 12 wk for a CBR of 46%
Ⅱ (REO 016)	Recurrent or metastatic NSCLC	60-min *IV* infusion 3×10^{10} $TCID_{50}$ days 1-5 with *IV* paclitaxel 175 mg/m^2 over 3 h and *IV* carboplatin AUC_5 (over 30 min) on day 1 every 21 d	Out of 37 patients enrolled, 20 patients had detected K-Ras mutations, 3 patients had EGFR mutations, 10 patients had EGFR amplifications alone, and 4 patients had BRAF V600E mutations. Median PFS was 4 mo (95%CI: 2.9-6.1), median OS was 13.1 mo (95%CI: 9.2-21.6), and 1-yr OS rate was 57% (95%CI: 39%-72%)
Ⅱ (REO 017)	Advanced or unresectable pancreatic cancer	60-min *IV* infusion 1×10^{10} $TCID_{50}$ on days 1, 2, 8 and 9 with *IV* infusion of gemcitabine 800 mg/m^2 days 1 and 8 every 21 d	Out of 34 enrolled patients, median PFS was 4 mo and OS was 10.2 mo. One- and 2-yr survival rates were 45% and 24%, respectively
Ⅱ (REO 021)	Recurrent or metastatic SCC of the lung	Open-label, single-arm: 60-min *IV* infusion 3×10^{10} $TCID_{50}$ days 1-5 with *IV* paclitaxel 200 mg/m^2 over 3 h and *IV* carboplatin AUC_6 every 21 d	Out of 25 patients who received more than 1 cycle of therapy, best overall response was PR in 12 patients (48%) and SD in 10 patients (40%) for a CBR of 88%. Of 21 patients with > 6 mo follow-up 7 had PFS ≥ 6 mo (33.3%)
Ⅲ (REO 018)	Advanced or metastatic head and neck cancer	Randomized, double-arm, double-blinded, multicenter: 60-min *IV* infusion 3×10^{10} $TCID_{50}$ days 1-5 with standard doses of *IV* paclitaxel and *IV* carboplatin on day 1 only every 21 d (treatment arm) *vs* standard doses of *IV* paclitaxel and *IV* carboplatin alone (control arm)	Out of 167 enrolled patients, 118 patients were segregated into an intent-to-treat basis group with loco-regional head and neck cancer (with or without metastases). In this group, median PFS was 94 d (13.4 wk, n = 62) in the test arm *vs* 50 d (7.1 wk, n = 56) in control arm maintained through 5 cycles. In the 88 patients discontinued from the study from this group, median OS was 150 d (21.4 wk, n = 50) in the test arm *vs* 115 d (16.4 wk, n = 38) in the control arm. Survival analysis in the other group (distal metastases-only) has not been conducted

IV: Intravenous; $TCID_{50}$: Tissue culture infectious dose-50; $AUC_{5/6}$: Area under curve-5/-6; CR: Complete response; PR: Partial response; SCC: Squamous cell carcinoma; RECIST: Response evaluation criteria in solid tumors; SD: Stable disease; OS: Overall survival; IP: Intraperitoneal; Gy: Gray; TTP: Time to disease progression; PFS: Progression-free survival; MFH: Malignant fibrous histiocytoma; ES: Ewing sarcoma; ASPS: Alveolar soft part sarcoma; CBR: Clinical benefit rate; NSCLC: Non-small cell lung cancer; EGFR: Epidermal growth factor receptor.

included pyrexia (43.5%), lymphopenia (26.1%), and influenza-like symptoms (17.4%)[73]. The best overall response was PR in 2 patients and SD in 5 patients (out of 7 patients in phase Ⅰa) and PR in 5 patients and

SD in 2 patients (out of 7 patients in phase Ⅰb) up to 3 mo post-treatment[73]. The recommended phase II doses were 1 × 10^{10} $TCID_{50}$ of reovirus × 2 intratumoral injections with 20 Gy of radiation × 5 fractions and 1 × 10^{10} $TCID_{50}$ of reovirus × 6 intratumoral injections with 36 Gy of radiation × 12 fractions for short and prolonged palliative regimens, respectively[73]. Another phase Ⅰ study (REO 009) originally used 3 × 10^9 $TCID_{50}$ days 1-5 of *IV* reovirus with gemcitabine in the treatment of advanced solid tumors but the dosing of reovirus was amended to 1 × 10^9 $TCID_{50}$ to 3 × 10^{10} $TCID_{50}$ *IV* reovirus on day 1 only with 30-min *IV* infusion of gemcitabine 1000 mg/m^2 on days 1 and 8 every 21 d when DLTs of grade 3 transaminitis and grade 3 elevation in troponin Ⅰ occurred[74]. A MTD was not reached, but a third DLT of grade 3 transaminitis also occurred at the amended 3 × 10^{10} $TCID_{50}$ day 1 dose[74]. Interestingly, the elevation in liver enzymes was associated with concomitant acetaminophen use and prompted the recommendation of avoidance of acetaminophen during reovirus clinical trials[74]. The most common treatment-related AEs were pyrexia (68.8%), nausea (43.8%), and diarrhea (37.5%), and 1 × 10^{10} $TCID_{50}$ *IV* reovirus on day 1 became the recommended phase Ⅱ dose in combination with gemcitabine[74]. Out of 10 patients, the best overall response was PR after 4 cycles in 2 patients and SD from 4-8 cycles in 5 patients (median SD of 72 d, range 36-112 d) for a CBR of 80%[74].

In REO 010, a MTD was not reached though a DLT of grade 4 neutropenia resulted in a 20% reduction of the docetaxel dose in refractory or metastatic solid cancers treated with 3 × 10^9 $TCID_{50}$ to 3 × 10^{10} $TCID_{50}$ days 1-5 of *IV* reovirus (the last being the recommended phase Ⅱ dose with docetaxel) with *IV* docetaxel 75 mg/m^2 on day 1 every 21 d[75]. Four AEs of grade 4 neutropenia were felt to be due to docetaxel alone and an additional grade 4 lymphopenia also occurred; the most common AEs were flu-like symptoms, diarrhea, and fatigue[75]. Out of 16 patients, the best overall response was PR ⩾ 2 cycles in 4 patients and SD ⩾ 2 cycles in 10 patients for a CBR of 88%[75]. REO 013 enrolled 10 patients with metastatic colorectal cancer to the liver to be treated with 1 × 10^{10} $TCID_{50}$ of *IV* reovirus daily × 5 d between 6-28 d prior to planned radical resection of liver metastases[76]. There were no grade 3 or higher toxicities and the most common AEs were flu-like symptoms[76]. Resected tumor specimens from 9 patients showed staining for reovirus protein greatest in tumor when compared to surrounding tumor stroma and normal liver[76].

Preliminary results of REO 022 included PR in 1 patient (5%) and SD in 9 patients (50%) with a median progression-free survival (PFS) in irinotecan/fluorouracil/leucovorin (FOLFIRI)-naïve patients of 7.4 mo (95%CI: 1.9-12.9 mo) and overall median PFS of 7.4 mo (95%CI: 0.6-14.1 mo) in 18 patients with metastatic colorectal cancer treated with 60-min *IV* infusion of reovirus from 1 × 10^{10} $TCID_{50}$ to 3 × 10^{10} $TCID_{50}$ days 1-5 every 28 d with standard FOLFIRI[77]. Irinotecan 150 mg/m^2 with 3 × 10^{10} $TCID_{50}$ *IV* reovirus days 1-5 every 28 d became the recommended phase Ⅱ dose[77]. The most common (> 10%) grade 3 or higher toxicities were neutropenia, anemia, and thrombocytopenia, and DLTs of neutropenia were observed[77].

Results from a National Cancer Institute (NCI)-sponsored phase Ⅰ study (OSU-11148) included SD in 5 of 12 patients (42%) with relapsed multiple myeloma treated with 60-min *IV* infusion of reovirus from 3 × 10^9 $TCID_{50}$ to 3 × 10^{10} $TCID_{50}$ days 1-5 every 28 d[78]. A MTD was not reached, no DLTs were observed, and grade 3 toxicities included neutropenia, leukopenia, thrombocytopenia, and hypophosphatemia (Table 1)[78]. From this study, combination therapy is presumed to be more beneficial than oncolytic reovirus therapy alone in patients with multiple myeloma. Overall, phase Ⅰ trials did demonstrate that treatment with reovirus *via* various methods of administration was well tolerated by patients with minimal adverse effects.

Phase Ⅰ/Ⅱ trials

A phase Ⅰ/Ⅱ trial (REO 011) involved 60-min *IV* infusion of reovirus from 3 × 10^9 $TCID_{50}$ to 3 × 10^{10} $TCID_{50}$ days 1-5 (the latter being the recommended phase Ⅱ dose) with *IV* paclitaxel 175 mg/m^2 over 3 h and *IV* carboplatin area under curve-5 (AUC_5) over 30 min on day 1 every 21 d in untreatable, relapsed, or metastatic solid cancers[79]. A MTD was not reached even at ceiling doses and there were no DLTs observed though a total of 8 patients required dose reductions in paclitaxel and carboplatin[79]. The most common treatment-related AEs were alopecia (64.5%), fever (58.1%), and fatigue (58.1%); no relationships between reovirus dose and incidence or grade of symptoms were observed[79]. Out of 26 patients, the best overall response was CR in 1 patient (3.8%), PR in 6 patients (23.1%), major clinical response not evaluable by standard criteria in 2 patients (7.7%), and SD in 9 patients (34.6%) with a median duration of SD and PR of 6 mo (range 3-10 mo)[79]. Of the 24 patients with head and neck cancer, the median OS was 7.1 mo (CI: 4.2-11.5 mo)[79]. Preliminary results from a NCI-sponsored trial (OSU-07022) showed penetration and detection of replicating reovirus in tumor tissues thus far in 8 patients with recurrent or refractory ovarian, peritoneal, and fallopian tube carcinomas treated with *IV* reovirus at a fixed dose of 3 × 10^{10} $TCID_{50}$ days 1-5 with dose-escalation of daily intraperitoneal (IP) reovirus every 28 d (Table 2)[80,81].

Pharmacokinetics and pharmacodynamics

In keeping with the wide range of historically observed seropositivities to reovirus, baseline seropositivity for NARAs was 37% in one phase Ⅰ study and more than 90% in another phase I trial[68,82]. In general, phase Ⅰ trials demonstrated a wide time to induction and time to peak levels of NARA titers from baseline though both more or less occurred within 1-4 wk with a median time to induction of 1.4 wk (range 1-3 wk) and median time to peak of 3.8 wk (range 1-10 wk) in one study[42,68,69,71-76,78,79]. Maximum NARA titers also

varied considerably from 1/512 in one study to greater than 1/531441 in another (expressed as last dilution causing < 80% cytotoxicity) with a median increase from baseline of 250-fold (range 9- to 6437-fold)[42,72,82]. The neutralizing antibody response appeared to be blunted in cohorts with leukopenia from high-dose systemic reovirus therapy and myelosuppression from prior lumbosacral or pelvic radiotherapy[82]. Interestingly, reovirus in combination with gemcitabine or paclitaxel/carboplatin resulted in an attenuation in the time to induction and peak levels of NARA titers compared to prior phase Ⅰ results while co-administration with docetaxel had no such effects[74,75,79]. Pharmacokinetic parameters, however, of gemcitabine, docetaxel, or paclitaxel/carboplatin, when co-administered with reovirus, were not appreciably different compared to receiving those agents alone[74,75,79]. Phase Ⅰ data also illustrated that reverse transcription-polymerase chain reaction (RT-PCR) analysis of specimens including serum, stool, urine, saliva, and sputum for post-treatment viral shedding were negative in a majority of cases highlighting that reovirus administration in the outpatient setting is relatively safe[42,68,69,71-73,75,79]. When post-treatment viral shedding RT-PCR analyses were positive, they generally occurred within a few weeks (range 1-149 d) with some exceptions[42,68,69,71-73,75,79]. REO 013 showed that viral genome, though replication-incompetent, was present in plasma in 80% of patients at 1 h after the first dose of reovirus[76]. However, replication-competent reovirus was detected in peripheral blood mononuclear cells (PBMCs), granulocytes, and platelets, but not in plasma and red blood cells, at 1-h post-infusion and as late as 5-d post-infusion in PBMCs highlighting the idea of reovirus "hitchhiking" on such cells to evade the NARA response[76].

With respect to pharmacodynamics, available pathologic specimens have demonstrated positive detection of reovirus proteins localized to areas of cancer (occasionally with less involvement of surrounding tumor stroma and adjacent areas of normal tissue) with evidence of reovirus replication, apoptosis, and necrosis consistent with cytopathic effects[42,71-73,75,76,78,80,81]. In REO 005, 3 patients had reductions in cancer markers (carcinoembryonic antigen and PSA) consistent with clinical benefit, and 3 patients with biopsies showed the presence of viable reovirus post-treatment whose recovered titers correlated with doses of reovirus administered[72]. Similarly, in REO 013, replicating virus was recovered from lysates from surgical specimens in all 4 patients tested[76]. Interestingly, patients with 100% co-expression of reovirus RNA and CD138 showed greatest reductions in percent of myeloma cells with treatment in a NCI-sponsored phase Ⅰ study (OSU-11148)[78].

Pathologic specimens in REO 002 showed peritumoral inflammation in 4 patients while REO 003 demonstrated focal collections of plasma cells not present previously during pathologic tumor examination in 3 of 6 patients[42,69]. Indeed, these observations have been somewhat corroborated in a separate phase Ⅰ trial (REO 005) that revealed increases in $CD3^+CD4^+$ T-lymphocytes in 47.6% of patients, $CD3^+CD8^+$ T-lymphocytes in 33% of patients, $CD8^+$ perforin/granzyme$^+$ T-lymphocytes in 23.8% of patients, $CD3^-CD56^+$ natural killer (NK) cells in 28.6% of patients, and combined T-cell helper 1 and 2 (Th1 and Th2) cytokines in 38% of patients after reovirus therapy highlighting the potential significance of immune-mediated responses as a facilitator of reovirus anticancer efficacy[82]. Of note, there were no clear relationships between immune responses and reovirus dose, clinical response, or toxicity[82].

Phase Ⅱ and Ⅲ trials

An early multicenter, single-arm, open-label, phase Ⅱ trial (REO 008) involved 1×10^{10} $TCID_{50}$ intratumoral injections of reovirus on days 2 and 4 with 4 Gy of local irradiation daily × 5 fractions (total 20 Gy per cycle) in the treatment of refractory or metastatic solid tumors[83]. Out of 14 evaluable patients, the best overall response was SD or better in 13 patients (93%)[83]. Of these 13 patients, 4 experienced PR (2 with melanoma, 1 with lung cancer, and 1 with gastric cancer) and 2 experienced minor response (1 with thyroid cancer and 1 with ovarian cancer)[83]. The most common treatment-related AEs were grade 1 or 2 chills, pyrexia, headache, lethargy, anorexia, vomiting, shivering, nausea, and mild pain at injection site[83]. The NCI-sponsored MAYO-MC0672 was a multicenter, single-arm, open-label, phase Ⅱ trial pitting 60-min *IV* infusion of reovirus 3×10^{10} $TCID_{50}$ days 1-5 every 28 d against metastatic melanomas[84]. Out of 21 evaluable patients, the best overall response was SD > 8 wk in 6 patients with a median TTP of 45 d (range 13-96 d) and median OS of 165 d (range 15 d-15.8 mo)[84]. The study was ultimately closed due to failure to meet previously defined efficacy criteria to proceed to second stage of accrual, but 1 patient with 2 surgically removed metastatic cutaneous lesions demonstrated treatment effect as 75%-90% necrosis of these lesions were present on sampling[84]. Of note, out of 13 biopsies with metastatic tumor, productive reovirus replication was detected in 2 patients who had longer PFS of 80 and 87 d, respectively[84]. No dose reductions occurred, and the most common treatment-related grade 1 or 2 AEs were fatigue (66.7%), nausea (57.1%), and fever (52.4%)[84]. The most common treatment-related grade 3 or 4 AEs were fatigue (9.5%), hyponatremia (9.5%), and lymphopenia (9.5%)[84].

REO 014 enrolled 53 patients with refractory or untreatable soft tissue and bone sarcomas metastatic to the lung in a multicenter, single-arm, open-label phase Ⅱ trial with 60-min *IV* infusions of reovirus 3×10^{10} $TCID_{50}$ administered on days 1-5 every and given 28 d (personal communication). The best overall response was SD ≥ 12 wk in 18 patients (34%) with a subgroup of 12 patients having prolonged SD > 16 wk. Of these 12 patients, 3 patients demonstrated SD > 1 year (1 with malignant fibrous histiocytoma, 1 with synovial sarcoma, and 1 with ES). The median TTP was 58.0 d

(95%CI: 54-110, range 8-726 d). The prolonged SD demonstrated fulfilled study criteria for consideration as an active agent. No dose reductions occurred, and the most common treatment-related AEs were pyrexia (81.1%), chills (66.4%), fatigue (47.2%), myalgia (37.7%), and nausea (37.7%). Of note, the first case of optic neuritis related to reovirus therapy was reported as a serious AE. Results from a single-arm, open-label, phase Ⅱ study (REO 015) were PR in 4 patients (31%) and SD ≥ 12 wk in 2 patients for a CBR of 46% in 13 patients with refractory, recurrent, or metastatic SCC of the head and neck treated with 60-min *IV* infusion of reovirus 3×10^{10} $TCID_{50}$ days 1-5 with IV paclitaxel 175 mg/m^2 over 3 h and IV carboplatin AUC_5 over 30 min on day 1 every 21 d[85]. Grade 1 or 2 AEs included fevers, chills, fatigue while grade 3 or 4 AEs were hypokalemia, fatigue, nausea, aspartate aminotransferase elevation, neutropenia, and anemia[85].

REO 016 enrolled 37 patients with recurrent or metastatic NSCLC originally treated with *IV* reovirus in combination with *IV* paclitaxel 200 mg/m^2 and *IV* carboplatin AUC_6, but due to grade 3 diarrhea and febrile neutropenia (1 each), the dosing regimen was amended to 60-min *IV* infusion of reovirus 3×10^{10} $TCID_{50}$ days 1-5 with *IV* paclitaxel 175 mg/m^2 over 3 h and *IV* carboplatin AUC_5 (over 30 min) on day 1 every 21 d[86]. Of note, 20 patients had detected K-*Ras* mutations, 3 patients had EGFR mutations, 10 patients had EGFR amplifications alone, and 4 patients had BRAF V600E mutations[86]. Updated results have shown a median PFS of 4 mo (95%CI: 2.9-6.1), median OS of 13.1 mo (95%CI: 9.2-21.6), and 1-year OS rate of 57% (95%CI: 39%-72%)[86]. The most common AEs were fatigue, diarrhea, nausea, arthralgia/myalgia, and anorexia[86]. Results from REO 017 have thus far included a median PFS of 4 mo and OS of 10.2 mo in 34 enrolled patients with advanced or unresectable pancreatic cancer treated with 60-min *IV* infusion of reovirus 1×10^{10} $TCID_{50}$ on days 1, 2, 8 and 9 with IV gemcitabine 800 mg/m^2 days 1 and 8 every 21 d[87]. Treatment was well tolerated with manageable non-hematologic toxicities including grade 3-4 asthenia (38%), fever (12%), diarrhea (9%), chills (3%), flu-like syndrome (3%), and nausea/vomiting (3%). Intriguingly, upregulation of immune checkpoint markers including programmed death-ligand 1 (PD-L1) on immunohistochemistry (IHC) was demonstrated following treatment with reovirus[87].

The open-label, single-arm phase Ⅱ trial (REO 021) involved 60-min *IV* infusion of reovirus 3×10^{10} $TCID_{50}$ days 1-5 with *IV* paclitaxel 200 mg/m^2 over 3 h and *IV* carboplatin AUC_6 every 21 d in the treatment of recurrent or metastatic SCC of the lung[88]. Out of 25 patients who received more than 1 cycle of therapy, the best overall response was PR in 12 patients (48%) and SD in 10 patients (40%) for a CBR of 88%[88]. Of the 21 patients with > 6 mo follow-up, seven patients experienced PFS ≥ 6 mo (33.3%)[88]. The most common AEs were those expected of paclitaxel/carboplatin including neutropenia and thrombocytopenia and those expected of reovirus such as fever and fatigue[88]. The only treatment-related serious AE was reversible grade 2 elevation in creatinine and blood urea nitrogen[88].

On November 21, 2013, Oncolytics Biotech® Inc. reported preliminary top-line data from the randomized, double-arm, double-blinded, multicenter phase Ⅲ trial involving 60-min *IV* infusion of reovirus 3×10^{10} $TCID_{50}$ days 1-5 with standard doses of *IV* paclitaxel and IV carboplatin on day 1 only every 21 d (test arm) *vs* standard doses of *IV* paclitaxel and *IV* carboplatin alone (control arm) in the treatment of advanced or metastatic head and neck cancers (http://www.oncolyticsbiotech.com/clinical-trials). Per their report, 167 patients were enrolled and divided into an intent-to-treat group of 118 patients with loco-regional head and neck cancer (with or without metastases) and another group with distal metastases only. In the group of 118 patients, the median PFS was 94 d (13.4 wk, *n* = 62) in the test arm *vs* 50 d (7.1 wk, *n* = 56) in control arm maintained through 5 cycles. In the 88 patients discontinued from the study from this group, median OS was 150 d (21.4 wk, *n* = 50) in the test arm *vs* 115 d (16.4 wk, *n* = 38) in the control arm. Survival analysis in the distal metastases-only group has not been conducted. Of note, at the time of the first post-treatment scan (post-cycle 2 of therapy), 62 patients in the test arm experienced PD (32.3%) *vs* 56 patients on the control arm (51.8%, *P* = 0.04), and of the 86 patients with measurable disease at the first post-treatment scan, 48 patients demonstrated tumor reduction in the test arm *vs* 38 patients in the control arm (*P* = 0.049). There was a statistically significant increase in AEs of fever, chills, nausea, and diarrhea in the test arm *vs* control arm though there were no statistical differences in hematologic parameters in both arms. Nine patients in each arm experienced serious AEs of neutropenia with or without demonstrated infection. Interestingly, there were no dose reductions of paclitaxel for neuropathy or neurotoxicity in the test arm *vs* 6 dose reductions in the control arm (*P* = 0.028, Table 2).

IMMUNE RESPONSES TO REOVIRUS

Neutralization by the host immune system

Early preclinical evidence showed that prior exposure to reovirus did not significantly limit the antitumor activity of locally administered (intratumoral) reovirus in immune-competent C3H mice implanted with *Ras*-transformed fibroblasts and previously challenged with intramuscular injection of reovirus (detection of reovirus antibodies occurred after 2 wk in all challenged animals)[20]. Neutralizing antibodies similarly did not affect the efficacy of intratumoral reovirus in immune-competent rodent models of subcutaneous and intracranial glioblastoma[89]. However, systemic administration (*IV*) of reovirus is of therapeutic importance in advanced cancers, and phase Ⅰ data illustrated that even heavily pretreated patients were capable of mounting brisk and dynamic immune responses to *IV* reovirus characterized by peak

NARA titers reached by day 7 in 37.5% of patients and by day 14 in 62.5% of patients[82]. Indeed, neutralization of systemic reovirus by the host immune system was demonstrated when immune-competent C3H mice bearing *Ras*-transformed fibroblastic tumor allografts treated with *IV* reovirus (*via* tail vein injections) exhibited significant inhibition of tumor growth compared to controls at first, but tumor regrowth occured by 3 wk of *IV* reovirus therapy which coincided with rising serum NARA titers[58]. The ability of systemic reovirus to suppress tumor growth in immunized mice, however, was restored when co-administered with immunosuppressive agents such as cyclosporin A or cyclophosphamide which correlated with significantly decreased production of NARAs comparable to levels in mice without previous exposure to reovirus[58].

Systemic reovirus carries an innate ability to evade the NARA response by "hitchhiking" in PBMCs, granulocytes, and platelets; this process is detectable within a few hours post-infusion[82]. However, to further counteract the significant barrier to efficacy imposed by the neutralizing antibody response, it has been recommended that systemic reovirus be administered in rapid, repeated, and high doses within the first week of treatment when the NARA response has yet to become amplified[82]. Another strategy has involved the combination of reovirus with chemotherapeutic, particularly immunosuppressive, agents that attenuate the NARA response and therefore enhance tumor seeding of the virus, as previously suggested and described[66]. Importantly, early phase clinical trials have demonstrated that reovirus in combination with gemcitabine or paclitaxel/carboplatin resulted in attenuation of the NARA response while co-administration with docetaxel had no such effects though this finding was inconsistent with preclinical data[66,74,75,79]. All 3 combination regimens, however, have produced promising findings of clinical efficacy in various advanced malignancies and await further investigation in later trials[74,75,79].

Protective function against reovirus toxicities

High-dose systemic reovirus therapy is not without inherent risks as mice killed by viral overdose showed pathologic changes among several organs including liver and heart[58]. The role of the immune system in protecting against reovirus toxicity was highlighted when reovirus co-administered with high-dose cyclophosphamide resulted in both undetectable levels of NARA titers and severe systemic toxicities characterized by myocarditis, liver necrosis, tail detachment, and death compared to controls[56]. Furthermore, reovirus has been associated with limb necrosis and death in approximately 50%-60% of reovirus-treated SCID mice[20]. Upon metronomic dosing of high-dose cyclophosphamide with reovirus, however, systemic toxicities were markedly reduced in the presence of detectable NARA titers while preserving high levels of tumor access to virus and antitumor efficacy[56].

In a phase Ⅰ, dose escalation trial cyclophosphamide was co-administered with reovirus in 36 patients with various solid tumors that had received prior therapies. The dose of cyclophosphamide ranged from 25-1000 mg/m^2 with at least 3 patients per cohort with a consistent dose of reovirus dose of 3×10^{10} $TCID_{50}$/d[90]. The combination of cyclophosphamide and reovirus was well tolerated with few grade 3 toxicities including fever, diarrhea, neutropenia, and anemia[90]. However, cyclophosphamide did not have an effect at stimulating an antiviral response as NARA titers rose > 50 fold in all but one patient[90]. Interestingly, reoviral RNA was detected *via* RT-PCR in PBMCs despite the significant rise in NARA titers, suggesting that PBMCs play a role in viral delivery to tumor cells[90].

Immune-mediated antitumor activity of reovirus

It has long been postulated that oncolytic virotherapy stimulates antitumor immune responses through innate and adaptive pathways[91]. Accordingly, several investigations have shown that reovirus infection: (1) induces the release of a host of pro-inflammatory mediators including interleukin (IL)-1α, IL-1β, IL-2, IL-6, IL-8, IL-12p40/70, IL-17, regulated on activation, normal T cell expressed and secreted, macrophage inflammatory protein-1α/β, granulocyte macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-α, IFN-γ, and tumor necrosis factor-α; (2) suppresses the release of the immunosuppressive IL-10; and (3) increases activation of dendritic cells (DCs) and recruits effectors from both innate and adaptive immunity including cytotoxic CD8$^+$ T-lymphocytes (CTLs) and NK cells to facilitate tumor cell killing[35,92,93]. Furthermore, reovirus-infected melanoma cells released eotaxin, interferon gamma-induced protein 10, and IFN-β, in a NF-κB and PKR-dependent manner, and recruited NK cells, DCs, and CTLs to altogether promote bystander immune-mediated cytotoxicity in the tumor microenvironment[94].

Although reovirus infection has been shown to induce DC maturation in a dose-dependent manner, the immune-mediated antitumor activity of reovirus appears to occur independent of direct viral oncolysis or replication[95,96]. Nevertheless, reovirus therapy is capable of stimulating pro-inflammatory responses, enhancing tumor antigen presentation and exposing inaccessible tumor antigens for processing by DCs and CTLs, overcoming tumor evasion strategies and priming adaptive tumor-specific T-cells *in vitro* and *in vivo*, and initiating antitumor immunity to protect against subsequent tumor challenges in an antigen-dependent but reovirus-independent manner[92,96]. These processes that orchestrate reovirus-mediated antitumor immune responses have been demonstrated, in part, across several cancers including melanoma, lung cancer, AML, and prostate cancer[46,92,97]. Importantly, further support has been offered in early clinical trials when phase Ⅰ data showed increases in CD3$^+$CD4$^+$ T-lymphocytes in 47.6% of patients, CD3$^+$CD8$^+$ T-lymphocytes in 33% of patients, CD8$^+$perforin/granzyme$^+$ T-lymphocytes in 23.8% of patients, CD3-CD56$^+$ NK cells in 28.6% of

patients, and combined Th1 and Th2 cytokines in 38% of patients after reovirus therapy[82].

Interestingly, administration of reovirus with tumor-specific DCs or OT-1 T-cells in melanoma-bearing mice resulted in significantly higher survival rates compared to controls and highlighted the synergistic potential of reovirus with immunotherapy[92]. Intratumoral reovirus co-administered with intraperitoneal genetically modified cells expressing IL-2, IL-12, or GM-CSF in mice inoculated with TC-1 cancer cells failed to demonstrate significant synergistic effects with respect to tumor suppression though the combination of reovirus with cyclophosphamide (administered at specific time points) produced synergistic inhibition of tumor growth[98]. Preconditioning of mice bearing subcutaneous melanomas with regulatory T-cell (Treg) depletion and IL-2 significantly enhanced the delivery of *IV* reovirus to tumors and increased antitumor efficacy compared to controls though with severe systemic toxicities such as shortness of breath, inactivity, and tail necrosis/detachment[99]. Instead, preconditioning with cyclophosphamide and IL-2, which mimicked Treg depletion, induced "hyperactivated" NK cells and similarly enhanced antitumor efficacy with *IV* reovirus though without detectable toxicities[99]. Alternatively, reovirus in combination with gemcitabine in mice implanted with ovarian cancer cells demonstrated greater survival and postponement of peritoneal carcinomatosis by inhibiting myeloid-derived suppressor cells (MDSCs), downregulating pro-MDSC factors, and accelerating tumor-specific T-cell responses[100].

Also of relevance, recent phase Ⅱ trials have identified prolonged OS with reovirus in combination with conventional chemotherapy in advanced NSCLC and pancreatic cancer suggestive of an immunomodulatory influence on outcomes[86,87]. Upregulation of the immune checkpoint marker PD-L1 on IHC was observed following treatment with reovirus in REO 017. Although immune checkpoint inhibition and boosting of the immune response may be counterintuitive and detrimental to the efficacy of oncolytic reovirus by restricting viral replication, reovirus therapy in combination with anti-PD-1 therapy demonstrated improved survival in mouse models of melanoma, *in vivo*, compared to reovirus or anti-PD-1 therapy alone[101]. Checkpoint inhibition improved the ability of NK cells to kill reovirus-infected tumor cells and enhanced the $CD8^+$ Th1 antitumor response primed by reovirus therapy *in vitro*. Furthermore, PD-1 blockade enhanced antiviral immune responses but through mechanisms that may differ from those affecting the antitumor response and thus offering a novel platform for combining immune modulation and reovirus in anticancer therapy.

DISCUSSION, PERSPECTIVES AND THE FUTURE

At the time of this review, there are a total of 34 clinical trials (both ongoing and completed) involving wild-type, unmodified T3D reovirus (Reolysin®) in the treatment of a variety of cancers (http://www.oncolyticsbiotech.com/clinical-trials). Nineteen of these clinical trials are early phase trials (phase Ⅰ and Ⅰ/Ⅱ) or translational studies, and 10 of these 19 trials (53%) have investigated reovirus as monotherapy. Although not the primary objectives of these early trials, several phase Ⅰ trials investigating single-agent reovirus produced promising results with a CBR as high as 45% in one study (though with a smaller and limited cohort of patients) when antitumor responses were evaluated by conventional criteria and reported (Table 1)[68,69,71,72,78]. However, of the remaining 15 clinical trials (phase Ⅱ and Ⅲ), only 2 of these investigated reovirus as monotherapy (13%). In an attempt to carry over the clinical efficacy observed in earlier trials, one phase Ⅱ trial investigating single-agent reovirus in metastatic melanomas (the NCI-sponsored MAYO-MC0672) failed to meet previously defined efficacy criteria to advance to second stage of accrual and was ultimately closed[84]. However, REO 014 is the only phase Ⅱ trial in which single-agent reovirus fulfilled study criteria for consideration as an active agent in untreatable, refractory, or metastatic sarcomas; further trials involving reovirus as montherapy in advanced sarcomas are warranted (personal communication). Nevertheless, this trend is likely a reflection of a growing consensus that single-agent reovirus is unlikely to have sufficient clinical efficacy to be used alone as an anticancer agent[2,4,6,7].

The delivery of viruses to target tissues in sufficient numbers to produce a meaningful therapeutic effect has been a longstanding tenet of virotherapy[2]. Early investigations into the anticancer potential of reovirus demonstrated that the neutralizing antibody response to the virus may pose a dilemma to its therapeutic efficacy given its ubiquitous nature and high seropositivity within the population. The effect of the neutralizing antibody response was most profound with systemic (*IV*) reovirus, which is of therapeutic importance in advanced cancers, when repeated *IV* delivery of reovirus in immune-competent mice bearing *Ras*-transformed tumor allografts demonstrated tumor regrowth within a few weeks that coincided with rising NARA titers[58]. Furthermore, phase Ⅰ data showed that even heavily pretreated patients experienced a brisk induction of NARA titers from baseline with a time to peak levels within a few weeks after systemic reovirus.

In an attempt to circumvent this barrier to efficacy, systemic reovirus has been administered in rapid, repeated, high doses within the first week of treatment before the NARA response is boosted. Another strategy involves improving tumor cell killing by including reovirus in combination with other anticancer therapies; this appears to be the avenue in which the majority of ongoing and future trials involving reovirus are headed. Reovirus offers an excellent toxicity profile with the most common treatment-related AEs being mild respiratory/enteric and constitutional symptoms characteristic of its viral pathophysiology. As a result, reovirus becomes

an attractive agent to use in combination with other therapies and, overall, makes combination clinical trials much more feasible. Furthermore, the mechanism of reovirus oncolysis offers synergistic potential when used with other agents due to differing pathways of inducing cancer cell death. These reasons have formed, in part, the rationale for late phase clinical trials, and so far, very promising preliminary results have been produced in several phase Ⅱ trials involving reovirus in combination with chemotherapy and radiotherapy with CBRs achieved as high as greater than 90% in one study (Table 2). Recently, updated results from REO 016 and 017 have demonstrated discordance between PFS and OS in advanced NSCLC and pancreatic cancer treated with reovirus in combination with conventional chemotherapy. The reported PFS in these trials are comparable to historical controls, but OS is substantially longer than what has ever been reported in the literature for both cancers[86,87]. The clear OS benefit in the face of apparently limited impact on PFS is often characteristic of immune involvement in outcomes and may suggest further immunomodulatory anticancer effects from reovirus therapy (see below). Preliminary results from the phase Ⅲ trial involving reovirus with paclitaxel and carboplatin in advanced head and neck cancer are also promising with improved median PFS and median OS when compared to control arms; the results of this trial are highly anticipated.

Independent of direct viral oncolysis and replication, reovirus offers further anticancer potential by promoting antitumor immune-mediated responses characterized by stimulation of pro-inflammatory cascades, activation of DCs, and recruitment of NK cells and CTLs that altogether contribute to bystander cytotoxicity within the tumor microenvironment. In addition, reovirus infection primes adaptive tumor-specific T-cell responses that can provide further tumor immunity and protection against subsequent challenges with tumor. Aside from the added cytotoxic effects offered with chemotherapy, certain agents may also enhance tumor seeding of reovirus due to attenuation of the NARA response as shown by gemcitabine, paclitaxel, and carboplatin. Immunomodulation with immunosuppressive agents such as cyclosporin A and cyclophosphamide also enhanced reovirus antitumor efficacy by attenuating NARAs but consequently revealed the protective function of the NARA response against reovirus systemic toxicity. The immune responses to reovirus, therefore, represent a double-edged sword in that they can pose a significant barrier to tumor seeding of virus and antitumor efficacy but also serve to protect against severe reovirus toxicity and promote antitumor cytotoxicity through innate and adaptive responses.

Despite the promising development of reovirus as an anticancer agent, there remain several key areas warranting further investigation in order to maximize the anticancer potential of reovirus. Firstly, a few reports have argued that reovirus oncolysis can occur independently of activated Ras and EGFR signaling pathways[43,44]. Despite the coordination between *Ras*-transformation, PKR, and viral translational inhibition, which remains one of the best characterized hypotheses in explaining the mechanism of reovirus oncolysis, greater understanding of the infectious life cycle of reovirus has uncovered that multiple steps of the oncolytic cycle including viral uncoating, production of viral progeny, progeny release through increased apoptosis, and spread of virus in later rounds of infection are influenced by *Ras*-transformation. Other studies have demonstrated potential ties between reovirus oncolysis and cell cycle phase[102]. These new insights have presented potential opportunities to enhance reovirus antitumor efficacy such as adding exogenous proteases to enhance reovirus infectivity, using Nutlin-3a to enhance reovirus-induced apoptosis and virus spread through p53-dependent NF-κB activation, using hydroxyurea to affect cell cycle synchronization and enhance sensitivity to reovirus, and avoiding agents that inhibit microtubules as functional microtubules are required for reovirus endocytic processing and infectivity[102-105]. Recent studies demonstrated that cancer-upregulated gene 2 inhibits PKR activation but is still dependent on p38 and Ras activation for permissiveness to reovirus replication, which highlights the increasing complexity and degree of crosstalk evident between mediators in coordinating sensitivity to reovirus oncolysis[106]. Undoubtedly, the mechanism of reovirus oncolysis in relation to EGFR/Ras activated signaling pathways (both upstream and downstream), PKR, the reovirus life cycle, cell cycle phase, and pathways of cell death warrant further investigation.

Future studies will also need to elucidate methods to promote antitumor immune responses while suppressing immune responses against tumor seeding of reovirus without severe systemic toxicities. Immunomodulation with preconditioning with cyclophosphamide and IL-2 has shown to enhance systemic delivery of reovirus and antitumor efficacy with reduced toxicities[99]. Future trials involving reovirus in combination with immunotherapy are warranted and likely to grow in number. Phase Ⅰ data involving reovirus and cyclophosphamide in advanced malignancies will likely provide greater insight in how to safely maximize reovirus-mediated antitumor immune responses while minimizing the immune responses against tumor targeting. Checkpoint inhibition represents an alternative, but increasingly popular, means for combining immunomodulation with reovirus as anti-cancer therapy. Preclinical studies have demonstrated improved anticancer efficacy with the combination of PD-1 blockade and reovirus therapy compared to either therapy alone. Although antiviral responses were enhanced with the addition of anti-PD-1 therapy, they appear to occur through pathways that may differ from those affecting the antitumor response. Furthermore, checkpoint inhibition improved T-cell antitumor responses primed by reovirus therapy and the ability to locally clear reovirus-infected tumor cells. Indeed, with the growing popularity of checkpoint inhibitors in the treatment of

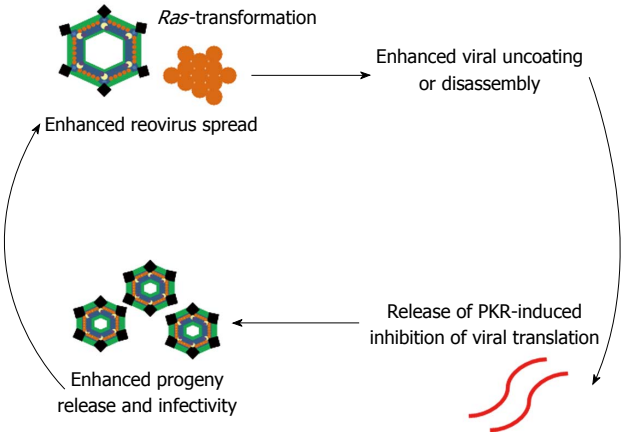

Figure 1 ***Ras*-transformation promotes reovirus preferential replication in cancer cells or oncolysis by affecting several key steps of the viral infectious life cycle.** *Ras*-transformation enhances viral uncoating or dissembly. dsRNA-activated protein kinase (PKR), which in the presence of viral transcripts, normally phosphorylates eukaryotic initiation factor 2α rendering it inactive and thereby leading to the inhibition of protein synthesis and viral replication, remains inactivated in *Ras*-transformed cells. Lastly, *Ras*-transformation enhances generation of viral progeny with increased infectivity, enhances release of progeny through apoptosis, and enhances viral spread in subsequent rounds of infection.

advanced cancers, clinical trials with immunomodulation and reovirus should be a focus of future studies. Upregulation of the immune checkpoint marker PD-L1 on IHC has also been observed following treatment with reovirus in REO 017. Whether increased levels of PD-L1 affect response to checkpoint inhibitors and reovirus therapy represents another issue in need of further investigation.

Recent developments highlight that reverse genetics and classical genetics have allowed for the engineering of genetically modified variants of reovirus that maintain or even enhance selective oncolytic potency while reducing toxicity[107-110]. Lastly, immune resistance to one particular oncolytic virus may not necessarily confer resistance to others, and combination therapies including multiple oncolytic viruses are possible as exemplified by the preclinical success of reovirus in combination with Newcastle disease virus or parvovirus in glioblastomas[65].

CONCLUSION

Reovirus is a dsRNA virus with demonstrated preferential replication in cancer cells, or oncolysis. The mechanism of reovirus oncolysis is still poorly understood though *Ras*-transformation and activated Ras signaling, appears central for sensitivity to reovirus replication. *Ras*-transformation modulates several steps of the viral life cycle in promoting reovirus oncolysis: (1) virus disassembly and uncoating; (2) releasing translational inhibition by PKR; (3) generation of infectious progeny; (4) enhanced apoptosis and progeny release; and (5) spread of virus in subsequent cycles of infection (Figure 1). The antitumor efficacy of reovirus is also largely dependent on immune-mediated antitumor effects involving both innate and adaptive responses. Wild-type, unmodified, replication-competent T3D reovirus (Reolysin®) has demonstrated anticancer activity across a spectrum of malignancies. Early clinical trials have shown a safe and tolerable toxicity profile of reovirus with a predictable NARA response, minimal viral shedding, and localization, replication, and cytotoxic effects in pathologic specimens consistent with activity. Phase Ⅱ and Ⅲ trials involving reovirus have demonstrated promising results of clinical efficacy and reinforce its potential as an anticancer agent. Future trials will likely take advantage of its excellent toxicity profile in combination therapies for synergistic tumor cell killing.

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