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REVIEW

# Oncolytic viruses against cancer stem cells: A promising approach for gastrointestinal cancer

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## Abstract

Gastrointestinal cancer has been one of the five most commonly diagnosed and leading causes of cancer mortality over the past few decades. Great progress in traditional therapies has been made, which prolonged survival in patients with early cancer, yet tumor relapse and drug resistance still occurred, which is explained by the cancer stem cell (CSC) theory. Oncolytic virotherapy has attracted increasing interest in cancer because of its ability to infect and lyse CSCs. This paper reviews the basic knowledge, CSC markers and therapeutics of gastrointestinal cancer (liver, gastric, colon and pancreatic cancer), as well as research advances and possible molecular mechanisms of various oncolytic viruses against gastrointestinal CSCs. This paper also summarizes the existing obstacles to oncolytic virotherapy and proposes several alternative suggestions to overcome the therapeutic limitations.

**Key words:** Cancer stem cells; Gastrointestinal cancer; Oncolytic virotherapy; Molecular mechanism



**Core tip:** Cancer stem cells (CSCs) are derived from tumor cells, which are responsible for tumor relapse and drug resistance. The high incidence, lethality, relapse and drug resistance of gastrointestinal cancer requires a novel therapeutic strategy against CSCs. Oncolytic viruses hold much promise because they kill tumor cells but are minimally toxic to normal cells. Isolation and identification of CSC markers for

treatment of gastrointestinal cancer will benefit the engineering of oncolytic viruses and targeting anti-tumor effects. This paper reviews research on oncolytic viruses against gastrointestinal CSCs, and toxicity and immunological barriers to oncolytic virotherapy, and proposes alternative strategies.

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## INTRODUCTION

Cancers of the genital, digestive and respiratory systems have the highest incidence and mortality[1]. Stomach, liver and colon cancers have been among the five most commonly diagnosed and leading causes of cancer mortality over the past few decades[1]. However, since the early 1990s, the cancer mortality rate has declined due to improvements in health care[2].

Traditional therapies for tumors (surgery, chemotherapy and radiotherapy) have made great progress in most patients with early cancer. Especially in recent years, novel targeted anti-cancer agents have been utilized clinically and have largely improved the survival rate of many cancer patients. Unfortunately, relapse still occurs months or years later when cancer patients are treated with the above approaches and they cannot be treated successfully again. Apart from suboptimal surgical debulking, drug resistance of tumor cells, and inability of chemotherapy or radiotherapy to target all cancer cells within a given patient[3], the cancer stem cell (CSC) theory can explain cancer relapse, which has been confirmed by many studies and accepted by an increasing number of scientists.

CSCs, also named cancer-initiating cells, are a small population of tumor cells and a subclass of stem-cell-like tumor cells. The term CSC was originally coined to describe features of these cells that are similar to *bona fide* normal stem cells that share basic properties of self-renewal and pluripotency[4]. Since a subclass of CSCs, $CD34^{+}CD38^{-}$ cells, derived from the blood of patients with acute myeloid leukemia, was reported in 1994, the presence of CSCs has been verified in a variety of primary tumor tissues and tumor cell lines, including gastrointestinal tract cancer[5]. The hypothesis that CSCs originate from normal stem cells is still uncertain, but their origin is likely to differ among human cancers. CSCs are tumorigenic and responsible for cancer relapse and metastasis, which implies that their role in producing daughter cells that constitute a new tumor bulk is similar to the role of normal stem cells in generating a bulk organ, such as blood from bone marrow stem cells. Moreover, both normal stem cells and CSCs express drug resistance genes, such as the ATP-binding cassette protein efflux pump ABCG2, which endows these cells with resistance to environmental toxins and chemotherapy or radiotherapy[6]. Nevertheless, CSCs also have many other features dissimilar to normal stem cells as well as their different or uncertain origin. Thus, it is urgent to isolate and characterize the CSCs, and exploit targeting treatment to reduce relapse and improve survival rate in patients with gastrointestinal tract cancer[7].

In the past two decades, researches have discovered a promising biological therapy for cancer, namely, oncolytic virotherapy. Oncolytic viruses are natural or modified viruses that can effectively and specifically infect cancer cells and kill them in preclinical models and clinical trials[8]. Oncolytic virotherapy has attracted increasing attention in cancer research as an emerging therapeutic approach because of its multiple anti-cancer pathways. For example, oncolytic viruses can infect highly proliferative cells (non-CSCs) and quiescent cells (CSCs), and directly lyse them, but they are not pumped out of infected cells by ABCG2 like chemotherapeutic drugs[9-11]. Other mechanisms include indirect killing of uninfected cancer cells, such as destruction of tumor vessels, and induction of anti-tumor immunity[12]. More importantly, oncolytic viruses exhibit targeted anti-tumor activity against CSCs, which is responsible for resistance to traditional treatments and tumor recurrence[11].

This review focuses on recent studies using oncolytic viruses against gastrointestinal cancer and highlights the novel approach to eradicate CSCs.

## GASTROINTESTINAL CANCER, CSCs AND THERAPY

### *Gastric cancer*

Gastric cancer (GC) is a heterogeneous chronic disease characterized by epidemiological and histopathological differences among countries. GC is one of the leading causes of cancer-related death worldwide. The origin of gastric carcinogenesis is still controversial. The past most popular model involved several initiators and continuator agents that provide a multifactorial and multistep pathogenesis for GC formation[13]. *Helicobacter pylori* (*H. pylori*) infection is recognized as a necessary but insufficient cause of GC[14].

Recent advances consider that GC essentially is a stem cell disease and GC stem cells (GCSCs) are the basis for gastric carcinogenesis[15]. GCSCs may be derived from resident stem cells in gastric tissues with a chronically inflamed environment in the case of *H. pylori*-induced gastritis[16]. Alternatively, due to exhaustion of the native gastric stem cells from their niches induced by chronic inflammatory stress, GCSCs are recruited from bone-marrow-derived stem

cells into the gastric epithelium. Further studies have found high expression of drug-resistance genes such as *ALDH* and *MDR* and specific molecular markers such as CD44, CD133, leucine-rich repeat-containing G-protein coupled receptor (Lgr)5, signal transducer and activator of transcription 3, and aquaporin 3[15,17,18]. These form the basis of drug resistance in GC and provide a molecular target for identification and isolation of GCSCs, and GCSC-targeted therapy. Treatment for GC patients is currently suboptimal, due to patients being commonly treated in a uniform fashion irrespective of disease subtype[19].

### *Liver cancer*

Liver cancer is the sixth most common cancer and third leading cause of cancer mortality worldwide. Liver cancer mainly falls into three types: hepatocellular carcinoma (HCC) (90%), intrahepatic cholangiocarcinoma, and mixed cell carcinoma[20]. Besides, there are many metastatic liver cancers from other malignant diseases, such as lung cancer. In Asia, especially in China, liver cancer is common; mainly because of the escalating epidemic of chronic hepatitis B or C infections[21]. Therefore, exploring optimal therapy for liver cancer is an important area of research. Liver cancer stem cells (LCSCs) have been isolated from liver cancer tissues. This has resulted in progress in liver cancer diagnosis and evaluation of prognosis and pathogenesis, despite constant debate about the new surface markers of LCSCs[22]. The reported major LCSC markers include CD133, CD90, epithelial cell adhesion molecule (EpCAM), OV6, CD44 and Nanog[22]. Although some of the markers are also expressed on the surface of other CSCs and normal stem cells, detection of LCSC-specific molecules is beneficial for diagnosis and evaluating and monitoring treatment of liver cancer.

### *Pancreatic cancer*

Pancreatic cancer (PC) is considered to be one of the deadliest cancers, with almost uniform lethality despite aggressive treatment[23]. However, resistance to conventional therapy and early distant metastasis are still cause for unsatisfactory prognosis of PC patients, even though there has been important progress in the molecular, pathological and biological understanding of PC. Thus, there is an urgency to explore the mechanisms of pancreatic carcinogenesis and tumor recurrence and metastasis, and develop novel, targeted therapeutic strategies. Recently, a small population of tumor-initiating cancer cells, termed PC stem cells (PCSCs), has been identified in many PC patients and cell lines, and is responsible for tumor initiation, progression and metastasis[24]. Identification of PCSC surface markers is crucial to isolate and characterize PCSCs. So far, the identified molecular markers for PCSCs include CD133, CD24, CD44, EpCAM, epithelial specific antigen, c-Met, aldehyde dehydrogenase (ALDH)1, and more recently, doublecortin-like kinase 1 and Lgr5[24,25], which are well recognized in xenograft models and some PC tissues. Further studies have shown that these markers are often co-expressed at metastatic sites or invading margins of PCSCs and pancreatic ductal cancers[23], such as CD133/CXCR4 receptor, CD24/CD44/EpCAM and CD133. Although the populations of PCSCs account for $\leqslant$ 1% of all PC cells, they are involved in cancer relapse and resistance to chemo- or radiotherapy[26]. Thus, our ultimate goal is to understand PCSCs further and explore potential therapeutic targets for PC.

### *Colorectal cancer*

Colorectal cancer (CRC) is one of the most common cancers worldwide, and affects > 1 million people, resulting in about 715000 deaths in 2010[27,28]. In China, CRC is the fifth most common form. Incidence of CRC in China is lower than that in western countries, but it has increased in recent years to become a substantial burden, particularly in more-developed areas[29]. Treatment options for CRC are based largely on cancer stage. Patients without distant metastasis usually receive surgery as initial treatment. In patients with advanced disease, CRC is rarely cured completely due to drug resistance and recurrence, and patients are not eligible for surgery[30]. Therefore, understanding of CRC formation and progression is urgently needed. In addition to accumulation of genetic abnormalities and dysregulation of gene expression, CRC stem cells (CCSCs) also play important roles in CRC carcinogenesis, promotion, metastasis and recurrence. CCSCs share the same molecular signaling features with normal stem cells, such as Wnt, Notch and transforming growth factor-β, and differ in tumorigenic potential[31]. More importantly, isolation of CCSCs can be achieved by screening subpopulations of CRC cells based on one or more cell surface markers, including CD133, CD166, CD44, CD24, β1 integrin-CD29, Lgr5, EpCAM, ALDH1, Musashi RNA binding protein-1, doublecortin-like and CAM kinase-like 1 (DCAMLK1) or ephrin B receptors[32-34] (Table 1), which largely contribute to the better stratification of prognosis and treatment response, as well as the development of new targeting strategies.

## ONCOLYTIC VIROTHERAPY

The issue of which oncolytic viruses are to be engineered to eliminate CSCs, and their mechanisms of action have begun to be addressed. Current viruses have a broad range of sources and categories, including wild-type animal viruses, live virus vaccines, and human virus mutants in which critical genes for virus replication that are dispensable in cancer cells have been deleted or mutated. These attenuated live virus vaccines or modified viruses hold much promise because they have been proved to be efficient against

**Table 1 Cancer stem cell markers of different gastrointestinal cancers**

| Cancer types | CSC markers | Ref. |
|---|---|---|
| Gastric cancer | CD44, CD133, Lgr5, STAT3, Aquaporin 3 | [15,17,18] |
| Liver cancer | CD133, CD90, EpCAM, OV6, CD44, Nanog | [22] |
| Pancreatic cancer | CD133, CD24, CD44, EpCAM, ESA, c-Met, Aldh1, DclK1, Lgr5 | [24,25] |
| Colorectal cancer | CD133, CD166, CD44, CD24, b1 integrin-CD29, Lgr5, EpCAM, ALDH1, Msi-1, EphB | [32-34] |

CSC: Cancer stem cell; Lgr5: Leucine-rich repeat-containing G-protein coupled receptor 5; STAT3: Signal transducer and activator of transcription 3; EpCAM: Epithelial cell adhesion molecule; ESA: Epithelial-specific antigen; Alkh1: Aldehyde dehydrogenase 1; DclK1: Doublecortin-like kinase-1; Msi-1: Musashi-1; EphB: Ephrin-B.

tumor tissues, yet minimally toxic to normal cells and tissues[3]. Oncolytic viruses have also been armed to deliver anti-cancer genes with different functions, thereby increasing their anti-tumor effects.

### *Oncolytic adenovirus*

In the past two decades, oncolytic adenovirus (OncoAd) has become a promising agent for treatment of many cancers including gastrointestinal cancer. The cancer targeting gene-virotherapy (CTGVT) strategy, which was proposed by our group through combining virotherapy and gene therapy, showed greater anti-tumor effects when compared with monotherapy[35,36]. The representative modified mutant adenovirus, ZD55, was designed by deleting the immediate-early protein E1B (55 kDa) based on the CTGVT strategy to target the p53 dysfunction pathway or nuclear export of viral RNA in tumor cells[10]. Other than E1B detection, another common mutant of adenoviruses is the 24 bp deletion of the E1A retinoblastoma (Rb) binding site (Δ24). Mutant adenoviruses show obvious tumor selectivity because viral replication is promoted in tumor cells with a defective Rb/p16 pathway and abolished in normal cells with intact Rb/p16[37]. Most cancer cells and CSCs harbor defects in the Rb and/or p53 pathway, which makes it possible to use OncoAd to eradicate the gastrointestinal cancer cells. Besides, the transcription-targeted strategy has been a common approach through using cancer or tissue-specific promoter to control the expression of viral early essential genes for replication.

Recent studies have shown that the Golgi glycoprotein GOLPH2, usually named GP73, is an excellent HCC marker candidate, and even its promoter activity and specificity are better than the most common liver cancer marker α-fetoprotein[38,39]. Our group constructed a novel dual-regulated oncolytic adenovirus GD55 targeting HCC, using the GP73 promoter to regulate E1A expression and deletion of E1B based on the CTGVT strategy[10]. The novel GOLPH2-regulated GD55 conferred higher adenovirus replication and infectivity for liver cancer cells than did ZD55. We also confirmed that ZD55 eliminated LCSCs (data unpublished). The LCSC-like cells were enriched with suspension culture and the properties of acquired LCSCs were validated through detecting expression of CSC-related transcription factors and receptors (*e.g.*, Nanog, octamer-binding transcription factor 4, EpCAM and DR5). Oncolytic virus ZD55 resulted in obvious cytotoxicity and killing (the minimum cell viability for Huh7 spheres is 26.7%) of LCSC-like cells, and induced significant apoptosis (the maximum apoptosis rate for Huh7 spheres is 60%)[40]. We proceeded to verify whether GD55 could also destroy LCSCs as well as non-CSCs. Our results indicated that GD55 significantly elicited cytotoxicity and oncolysis in LCSC-like cells enriched in suspension culture, and exhibited more obvious killing than ZD55. GD55 induced marked apoptosis of LCSC-like cells *in vitro* and *in vivo*, and inhibited propagation of cells and angiogenesis in xenograft tumor tissues[40]. Thus, GD55 may represent an attractive therapeutic agent for targeting LCSCs with better clinical outcomes for HCC patients.

Studies of other targeting strategies for OncoAd have also been pursued. Adenovirus tropism modification by constructing chimeric virus capsid has been used to overcome the lack of the host cell surface coxsackie-adenovirus receptor (CAR) in tumor cells, because most common adenovirus serotypes such as Ad5 infect and enter cells through the fiber knob of the viral capsid binding to CAR[41]. Yu *et al*[42] reported that a new OncoAd, Ad5PTDf35, which is an Ad5 vector with Tat-PTD modified hexon and 35 serotype fiber, showed greatly enhanced transduction of primary human cell cultures, including pancreatic islets and tumor-initiating cells, compared to unmodified Ad5. Therefore, this modified Ad5PTDf35 may be further developed as an oncolytic agent for targeted CSC therapy.

Xu *et al*[43] reported that oncolytic adenovirus ZD55-mediated acetylcholinesterase (AChE) overexpression exhibited a potent anti-tumor effect on GC. The results showed that the constructed adenoviral vector ZD55-AChE inhibited GC cell and GCSC growth, and low doses of ZD55-AChE induced the mitochondrial pathway of apoptosis. ZD55-AChE repressed tumor growth *in vivo*, and the anti-tumor efficacy was greater than that of the replication-deficient adenoviral vector (Ad-AChE). ZD55-AChE represents a potential therapeutic agent for human GC. Yano *et al*[44] investigated the efficacy of a genetically engineered, telomerase-specific oncolytic adenovirus, OBP-301, to mobilize the cell cycle and kill quiescent $CD133^+$ CSC-like cells in human GC cells. They found that OBP-301 efficiently killed $CD133^+$ GCSCs resistant to chemoradiotherapy. OBP-301 induced cell-cycle mobilization from G0/G1 to S/G2/M phases and subsequent cell death in quiescent GCSCs by mobilizing cell-cycle-related proteins. OBP-301

mobilized quiescent CSC-like cells in tumor spheres and xenografts into S/G2/M phases where they lost viability and CSC-like cell properties and became chemosensitive.

### *Oncolytic herpes simplex viruses*

As a neurotropic virus, oncolytic herpes simplex virus (OncoHSV) has been investigated widely in preclinical and clinical trials for patients with neurological malignancies like glioblastoma and neuroblastoma, and melanoma. In particular, the first-in-class oncolytic virus agent talimogene laherparepvec (T-VEC) is a genetically modified, attenuated recombinant HSV expressing granulocyte-macrophage colony-stimulating factor (GM-CSF). It was authorized by the US Food and Drug Administration and European Food Safety Authority because T-VEC improved durable response rate in patients with advanced melanoma in a phase Ⅲ trial[45]. T-VEC is also being tested in several other cancers, such as digestive tract cancers, alone and in combination with standard cancer therapeutics and other immunotherapy agents[46].

Although OncoHSV was broadly utilized in clinical trials on nervous system tumors, some studies have shown its potential for killing gastrointestinal cancer cells and CSCs. Yang *et al*[47] reported that OncoHSV is an effective agent for colon cancer and exhibits significant killing efficacy in colon cancer cells and colon CSC-like cells *in vitro* and *in vivo*. Miao *et al*[48] designed a transcriptionally regulated OncoHSV, YE-PC8, in which a cell-cycle-regulatable luciferase transgene cassette was replaced with the infected cell protein (ICP)6 coding region of the HSV-1 genome, and found that intratumoral injection of YE-PC8 resulted in 77% and 80% tumor regression in human glioma and human HCC xenografts, respectively. Thus, YE-PC8 viruses confer tumor selectivity in proliferating cells and may be developed further as a feasible approach to treat human cancers. A report by Fong *et al*[49] showed a phase Ⅰ trial of another multimutated OncoHSV, NV1020, in patients with metastatic CRC who had failed first-line chemotherapy *via* hepatic arterial administration. The tumor size decreased, median survival rate was prolonged, and levels of the tumor marker carcinoembryonic antigen decreased in patients after HSV infection, which suggested that genetically engineered HSV can be delivered safely into the human bloodstream to produce selective infection of tumor tissues and biological effects. In an earlier phase Ⅰ clinical trial, OncoVEXGM-CSF, a second-generation OncoHSV, was administered by intratumoral injection in patients with gastrointestinal cancer who had failed prior therapy. The results showed that OncoVEXGM-CSF was well tolerated, with the main adverse effects being local inflammation, erythema and febrile responses, and exhibited an anti-tumor effect after delivery *via* a safe protocol[50].

Efficacy of OncoHSV has been demonstrated preclinically and clinically in LC, CRC and glioma. A recent study has examined the ability of OncoHSV to kill CSCs mainly from neural tumors. Kambara *et al*[51] developed an oncolytic HSV-1 mutant rQNestin34.5 which expresses ICP-34.5 under control of a synthetic nestin promoter. Nestin is expressed in embryonic neuroglial cells and has been used as a CSC marker in several cancers including brain tumors, and rQNestin34.5 showed significantly more potent inhibition of tumor growth compared with control virus *in vivo*[52]. Further studies found that rQNestin34.5 can infect and kill neuroblastoma CSCs[9], implying that OncoHSV efficiently targets CSCs from gastrointestinal cancer.

### *Oncolytic vaccinia virus*

Vaccinia virus (VV) belongs to the poxvirus family, and is famous because it was first utilized as a vaccine against smallpox until its eradication worldwide. Recently, oncolytic vaccinia virus (OncoVV) showed potential as it was attenuated for use as a transfer vector for therapy of human cancers. Two main mutated OncoVVs were designed by deleting the thymidine kinase (TK) gene or B18R gene[53]. The TK-deleted OncoVV undergoes preferential replication in dividing cells and shows tumor cell specificity, and the DNA synthesis of mutant virus requires TTP, which is only provided by dividing cells. The B18R-deleted mutant virus has oncolytic capacity because it causes interferon (IFN)-mediated enhanced virus inactivation in normal cells, based on the effect of B18R gene against type Ⅰ IFNs[53]. Our group previously constructed a tumor-targeted VV carrying *SMAC/DIABLO* gene, which was knocked out in the region of the TK gene (VV-SMAC). We found that VV-SMAC efficiently infected and destroyed HCC cells *via* triggering both caspase-dependent apoptosis and necroptosis[54]. Our data suggest that VV-SMAC is a potential candidate, and combination of VV-SMAC and vinblastine may provide a new avenue for treatment of HCC[54]. To date, several genetically modified OncoVVs delivering various therapeutic genes have exerted obvious anti-tumor effects in clinical trials, through targeting cancer-specific antigens and inducing anti-tumor immunity[55].

Recently, Yoo *et al*[56] reported that a cancer-favoring OncoVV (CVV) shows enhanced suppression of stem cell-like colon cancer (SCC). The engineered CVV is an evolved Wyeth strain of VV lacking TK, and can successfully override drug resistance and suppress SCC, with improved survival rates and complete eradication of tumor mass. This can be synergistically enhanced by simultaneous treatment with the anticancer drug 5-fluorouracil[56]. Chard *et al*[57] investigated the anti-tumor efficacy of interleukin (IL)-10-armed VV (VVLΔTK-IL10) in PC cell lines, mice bearing PC xenografts, and a PC transgenic mouse model. They found that VVLΔTK-IL10 has strong potential as an anti-tumor therapeutic agent for PC.

**Table 2 Oncolytic viruses against gastrointestinal cancer stem cell**

| Oncolytic viruses | Cancer types | CSC source | Effect | Description | Ref. |
|---|---|---|---|---|---|
| Adenovirus | Liver | CL | Susceptible | GP73 needed | [40] |
| | Pancrease, prostate | PC | Susceptible | Tat-PTD modified hexon and Ad5/35 | [42] |
| | Gastric | CL | Mixed | AChE | [43] |
| | Gastric | CL | Susceptible | OBP-301, telomerase-specific | [44] |
| HSV2 | Colon | CL | Susceptible | No virus modification or co-therapies | [47] |
| Vaccinia | Colon | CL | Mixed | Viral TK deficiency | [56] |
| Measles virus | HCC, colon | PX | Susceptible | Retargeted to CD133 | [68] |

CL: Cell line-derived spheres or cell lines sorted by marker expression; GP73: Golgi protein 73; HCC: Hepatocellular carcinoma; HSV: Herpes simplex virus; PX: Primary xenograft; PC: Primary cancer cells sorted by marker expression; Tat-PTD: Protein transduction domain (PTD) from the HIV-1 Tat protein; AChE: Acetylcholinesterase; TK: Thymidine kinase; NDV: Newcastle disease virus.

Another preclinical trial was performed using hamsters as a PC model to assess the anti-tumor immunity of another OncoVV armed with human GM-CSF, which was selective for epidermal growth factor receptor pathway mutation and tumor-associated hypermetabolism[58]. However, their cytocidal effect on PCSCs needs further study. Although there are few reports about OncoVV inhibiting gastrointestinal CSCs, many OncoVV constructs exhibit excellent anti-tumor effects targeting CSCs from ovarian cancer, glioma and lymphoma[59].

OncoVV JX-594, derived from Wyeth strain VV and genetically modified to delete the TK gene and express human GM-CSF gene, has entered into phase Ⅲ clinical trials because of its excellent anti-tumor efficacy. JX-594 was first modified to augment the intrinsic targeting and oncolytic potential of VV and to enhance anti-tumor immunity by GM-CSF expression[60]. Several clinical trials using JX-594 have shown functional anti-cancer immunity, tumor necrosis, and improved survival through multiple mechanisms and injection pathways in primary HCC and other metastatic gastrointestinal cancers[61,62].

### *Newcastle disease virus*

Newcastle disease virus (NDV), an avian paramyxovirus type 1, is an attractive oncolytic agent for cancer virotherapy. The mechanisms of NDV-mediated cytotoxicity in cancer cells include the dominant role of apoptosis induction by caspase pathway activation, and indirect anti-cancer activity by activation of both innate and adaptive immune responses[63]. Although no specific studies have reported the effect of NDV on CSCs, there are several completed and ongoing clinical trials using NDV-based tumor vaccines and direct administration of naturally occurring NDV to patients with gastrointestinal tumors[64]. For most treatment of CRC, attenuated NDV Ulster strain exerted obvious prolonged survival with 97.9% 2-year survival compared to 73.8% in the control group[65]. Liang *et al*[66] reported a clinical study of an autologous NDV-modified tumor cell vaccine in a phase Ⅲ study of stage Ⅰ-Ⅳ CRC patients and found significant improvement of median overall survival in the vaccine group. In addition, the clinical benefit was shown in patients with unresectable colorectal, stomach, liver and pancreatic cancers after treatment with NDV vaccine, suggesting its promising future.

### *Measles virus*

The attenuated strains of measles virus (MV) have been shown to infect and kill a large variety of tumor cells specifically but not normal cells in phase Ⅰ clinical trials. The most common Edmonston strain of MV has shown clinical benefits for treating diverse solid cancer types, including lymphoma and myeloma[67]. Bach *et al*[68] designed oncolytic MV retargeted to CD133, termed MV-141.7 and MV-AC133, which infected and selectively eliminated $CD133^+$ cells from tumor tissue, and showed strong anti-tumor effects and prolonged survival in mouse models of human HCC and colon cancer. This virus is currently being assessed as an oncolytic agent in clinical trials (Table 2). Another study armed and retargeted MV through the prostate stem-cell antigen expressed on PC but not on non-neoplastic tissue, and obtained beneficial therapeutic effects in a PC xenograft model and PC cells, including gemcitabine-resistant pancreatic adenocarcinoma cells[69].

### *Myxoma virus*

Similar to VV, myxoma virus (MYXV) is a double-stranded DNA virus from the Poxviridae family. The natural host of MYXV is rabbits, which makes MYXV only pathogenic to European rabbits and it does not cause any human diseases. MYXV has been shown to infect human cancer cells and result in cytotoxicity through Akt activation *via* interaction with a viral ankyrin-repeat host range factor[70]. Akt is the key factor of the PI3K/Akt pathway, which plays a critical role in cancer development and regulating the survival of CSCs in medulloblastoma following radiation[71], suggesting that MYXV is a potential therapeutic agent for eradication of CSCs. To date, several preclinical studies have proven that MYXV is an attractive candidate oncolytic virus that could be developed as a promising oncolytic agent for PC, where activated Akt signaling is often up-regulated[72,73]. In PC cell lines and

disseminated PC models, MYXV was shown to inhibit tumor growth, prolong survival and act synergistically with gemcitabine therapy[74,75]. However, further evaluation of MYXV in other gastrointestinal cancers and CSCs is warranted.

### *Reovirus*

Reovirus is a double-stranded RNA virus, and is considered an orphan virus due to its ubiquitous nature and absence of severe pathophysiology. Reovirus infects the respiratory or gastrointestinal tract, but infection is asymptomatic and considered benign, implying that reovirus exhibits cytopathic effects and oncolytic potential in cancer cells[76]. Furthermore, activated Ras signaling contributes to tumor-specific viral replication and oncolysis of reovirus[77]. Currently, oncolytic reovirus is used widely to treat Ras-activated gastrointestinal cancers *in vitro* and *in vivo*, which causes apoptosis of TRAIL-resistant GC cells by down-regulation of Akt and inhibition of peritoneal metastasis[78,79]. In particular, reolysin, a novel reovirus-based agent, induced endoplasmic reticulum stress-mediated apoptosis in PC[80] and prolonged overall survival in a phase I trial of recurrent glioma[81,82]. Although there are few reports of reovirus in gastrointestinal CSCs, research in breast CSCs and glioblastoma stem-like cells has yielded promising results[83,84].

### *Vesicular stomatitis virus*

Vesicular stomatitis virus (VSV) is a negative-sense, single-stranded RNA rhabdovirus. VSV mainly infects livestock as an animal pathogen and is usually asymptomatic in humans and only rarely causes a flu-like syndrome. VSV is highly sensitive to IFN response, which makes VSV as an ideal naturally oncolytic agent for cancer cells with a deregulated IFN response, but having no effect in normal cells[59,85]. Another study reported that VSV exhibits effective oncolytic activity and apoptosis induction in tumor cells with aberrant p53, Ras, or myc function[86]. This indicates that VSV is an optimal candidate as an oncolytic agent because most gastrointestinal cancers have the above aberrant signaling pathways.

Metastasis of CRC is incurable with currently available treatments. Recombinant VSV-GFP is able to replicate extensively in CRC cells and lyse hepatic metastasis of CRC in immunocompetent mice[87]. Recombinant VSV (rVSV) vectors expressing a mutant (L289A) NDV fusion protein, rVSV-NDV/F(L289A), was effective in treating a multifocal CRC liver metastasis model through repeated hepatic arterial administration[88]. The results indicate that VSV can be an effective and safe oncolytic agent against hepatic CRC metastasis and may be developed for the treatment of cancer patients in the future. However, oncolytic VSV is toxic in animals when administered systemically at high doses. Its safety can be improved by an MΔ51 deletion in the viral genome. A mutant attenuated form of the virus, rVSV(MΔ51), which has a single amino acid deletion of methionine-51 of the matrix protein to provide additional protection for normal cells by restoring IFN-mediated responses, exerts robust cellular inflammatory responses and cytotoxicity in HCC lesions[89]. The safety of oncolytic VSV delivering IFN β gene was further demonstrated by intrahepatic or intratumoral injection in rodents and non-human primates[90]. This implies that VSV can be developed as an effective and safe oncolytic agent to treat advanced HCC patients in the future. Although numerous studies have convincingly shown the ability of rVSV to inhibit tumor growth in CRC, HCC and PC[91], whether rVSV is able to target and kill CSCs remains unknown. Reports that an engineered VSV variant could target Her2/neu-overexpressing breast CSCs[3,92] bring greater understanding of the biology and molecular mechanisms of VSV.

### *Alphavirus M1*

Alphavirus M1 virus is a naturally occurring alphavirus and an arthropod-borne togavirus, which was isolated from culicine mosquitoes by the Yan group on Hainan Island, China[93]. The novel alphavirus M1 possesses the features of oncolytic viruses and can induce apoptosis of malignant glioma cells *via* down-regulation and nucleolar translocation of p21WAF1/cyclin-dependent kinase inhibitor 1 or CDK-interacting protein 1 (CIP1)[93]. It was recently found that M1 can target cancer cells deficient in zinc-finger antiviral protein and has potent oncolytic efficacy and high tumor tropism in LC *in vitro*, *in vivo* and *ex vivo*[94]. The studies provide a novel insight into potentially unknown oncolytic viruses for further cancer therapy.

## IMMUNOGENIC EFFECTS OF VIROTHERAPY AND POTENTIAL FOR COMBINATION WITH IMMUNOTHERAPY

Although the mechanisms of carcinogenesis and cancer development and their relationship with CSCs have not been clarified, the CSC theory in diverse cancer types, including gastrointestinal cancer, is supported by increasing evidence. Studies have testified that a few subpopulations of CSCs derived from tumor tissues are tumorigenic and able to generate the bulk of non-tumorigenic tumor cells. With the isolation, identification and characterization of CSCs, many new targeting therapy strategies have been shown to target CSCs to prevent cancer recurrence and metastasis to secondary organs[3]. Oncolytic viruses are considered to have therapeutic potential because they can eradicate CSCs through broadening the permissiveness for viral replication to CSCs, and their unique molecular mechanisms.

There are some limitations hampering the efficacy of oncolytic virotherapy for CSCs when it is performed by

intravenous administration. These drawbacks include liver or spleen trapping, clearance of viral particles by neutralizing antibodies, impact of tumor microenvironment or niche on viral replication, activated cellular immune response against viral infection, and virus-induced inflammatory response. To overcome these obstacles, recent efforts have been made towards: (1) isolation and identification of gastrointestinal CSC-specific markers and design of new engineered viruses to enhance potency; (2) PEGylation of oncolytic viruses, and use of cells or nanoparticles as potent vectors for oncolytic virus delivery[95]; (3) modification and disruption of the tumor vasculature to suppress the pernicious environmental conditions[96]; (4) combinatorial strategies with viruses, therapeutic genes and chemo- or radiotherapy with a mechanistic rationale[97]; (5) transient immunosuppression to improve the efficacy of oncolytic virotherapy[98]; and (6) use of gastrointestinal CSC-derived models in oncolytic virus evaluation[97].

With the advent of a new era of cancer immunotherapy, the checkpoint inhibitors such as cytotoxic T-lymphocyte antigen (CTLA)-4, programmed cell death protein-1 and programmed death-ligand (PD-L)1 have shown promising results in gastrointestinal cancer patients with tumor regression, and have prolonged survival[99]. In particular, chimeric antigen receptor therapy, a personalized therapeutic approach that involves genetically modifying patients' T cells with tumor antigen receptor to target tumor cells, has yielded encouraging results in leukemia, up to complete remission[100]. Besides tumor cytolysis and growth inhibition, oncolytic virotherapy also promotes an immune response against distant niduses due to production of cytokines and release of tumor antigens[101]. Therefore, the combinatorial strategy of oncolytic virotherapy and cancer immunotherapy may synergistically boost the anti-tumor response as well. Actually, the practice of combining oncolytic viruses and immunotherapy is still ongoing in the following two aspects. The common strategy is the design of oncolytic virus vectors encoding immuno-related genes such as antibodies against CTLA-4, PD-L1 and GM-CSF, which show therapeutic benefits[102]. Another hopeful approach is combined therapy with oncolytic viruses and immune cells such as cytokine-induced killer cells and dendritic cells[103].

Current preliminary data support the rationale that oncolytic virotherapy has outstanding potential in targeting CSCs in patients with gastrointestinal cancer. The clinical benefit of the first OncoHSV T-VEC in melanoma has spread to other oncolytic viruses, such as OncoVV JX-594, and other types of cancer, including gastrointestinal cancers[104]. With the discovery of new tumor antigens and CSC markers, new engineered viruses can be developed to target entry receptors specific to tumors and limit CSC function. The goals of combination of oncolytic viruses and other therapeutic methods (chemotherapy, radiotherapy, and especially immunotherapy) are to eradicate tumor progress and CSCs and avoid systemic side effects in cancer patients.

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