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# Journal of General Virology

## Progress in Clinical Oncolytic Virus-based Therapy for Hepatocellular Carcinoma --Manuscript Draft--

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<b>Abstract:</b>	<p>Hepatocellular carcinoma (HCC) carries a dismal prognosis, with advanced disease being resistant to both radiotherapy and conventional cytotoxic drugs, whilst anti-angiogenic drugs are marginally efficacious. Oncolytic viruses (OV) offer the promise of selective cancer therapy through direct and immune-mediated mechanisms. The premise of OV lies in their preferential genomic replication, protein expression and productive infection of malignant cells. Numerous oncolytic viruses are being tested in pre-clinical models of HCC, with good evidence of direct and immune-mediated anti-tumour efficacy. Efforts to enhance the performance of these agents have concentrated on engineering OV cellular specificity, immune evasion, enhancing anti-tumour potency and improving delivery. The lead agent in HCC clinical trials, JX-594, a recombinant Wyeth strain Vaccinia virus has demonstrated evidence for significant benefit and earned orphan drug status. Thus, JX-594 appears to be transcending the barrier between novel laboratory science and credible clinical therapy. Otherwise, relatively few other OV have entered clinical testing, a hurdle that must be overcome if significant progress is to be made in this field.</p> <p>This review summarises the pre-clinical and clinical experience of OV therapy in the difficult-to-treat area of HCC.</p>

1     **Progress in Clinical Oncolytic Virus-based Therapy for**  
2                     **Hepatocellular Carcinoma**

3     Review article

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## 16 **Abstract**

17 Hepatocellular carcinoma (HCC) carries a dismal prognosis, with advanced disease being resistant to  
18 both radiotherapy and conventional cytotoxic drugs, whilst anti-angiogenic drugs are marginally  
19 efficacious. Oncolytic viruses (OV) offer the promise of selective cancer therapy through direct and  
20 immune-mediated mechanisms. The premise of OV lies in their preferential genomic replication,  
21 protein expression and productive infection of malignant cells. Numerous oncolytic viruses are being  
22 tested in pre-clinical models of HCC, with good evidence of direct and immune-mediated anti-tumour  
23 efficacy. Efforts to enhance the performance of these agents have concentrated on engineering OV  
24 cellular specificity, immune evasion, enhancing anti-tumour potency and improving delivery. The  
25 lead agent in HCC clinical trials, JX-594, a recombinant Wyeth strain Vaccinia virus has  
26 demonstrated evidence for significant benefit and earned orphan drug status. Thus, JX-594 appears to  
27 be transcending the barrier between novel laboratory science and credible clinical therapy. Otherwise,  
28 relatively few other OV have entered clinical testing, a hurdle that must be overcome if significant  
29 progress is to be made in this field.

30 This review summarises the pre-clinical and clinical experience of OV therapy in the difficult-to-treat  
31 area of HCC.

## 32 **Introduction**

33 HCC is a malignancy of hepatocytes with an annual incidence over 500,000 (Boyle *et al.*, 2008). The  
34 majority of HCC cases can be attributed to defined environmental risks; worldwide, the proportion of  
35 HCC caused by chronic hepatitis B virus (HBV) infection is approximately 54%, with 31% being  
36 attributed to hepatitis C virus (HCV) (Boyle *et al.*, 2008). A safe and effective vaccine exists for HBV  
37 with good global coverage (“WHO | Immunization coverage,” 2014). In contrast, the highly  
38 heterogeneous nature of HCV has hampered attempts at vaccine development. In the UK, HCC  
39 caused by alcohol and non-alcohol-related fatty liver disease is on the rise (CRUK, 2013). The  
40 majority of patients present with advanced incurable disease and the overall 5 year survival rate is  
41 between 5 and 9% (Boyle *et al.*, 2008). Early diagnosis is critical, as patients with localised HCC and  
42 a good performance status may be offered potentially curative liver resection or transplantation. The  
43 management of patients with advanced HCC is complicated by underlying liver disease and the need  
44 to avoid undue toxicity in patients with a poor prognosis. For patients with inoperable predominantly  
45 hepatic disease, locoregional therapies offer the potential for disease control, symptomatic relief and  
46 improved survival times (Cammà *et al.*, 2002) (Bouza *et al.*, 2009) (Memon *et al.*, 2011). Clinical  
47 studies evaluating the use of cytotoxic chemotherapy have typically reported low response rates, with  
48 no impact on overall survival (Wrzesinski *et al.*, 2011). The current standard of care for patients with  
49 metastatic HCC is sorafenib, an oral multi-kinase inhibitor with anti-proliferative and anti-angiogenic

50 properties. It confers a mean survival advantage of 3 months in comparison to best supportive care  
51 (BSC), but rarely induces radiological responses, and is associated with significant toxicity (Cheng *et al.*, 2009) (Llovet *et al.*, 2008).

53 Immunotherapies are a promising class of drugs that include OV, therapeutically useful viruses that  
54 preferentially replicate in, and kill cancerous cells. Growing evidence suggests that effective oncolytic  
55 virotherapy is unlikely to be achieved merely by direct infection and cell lysis, but rather through  
56 efficient stimulation of an anti-cancer immune-response as reviewed by Melcher *et al.*, 2011. To date,  
57 hundreds of patients with HCC have been treated using OV in phase 1 and 2 clinical trials. The  
58 emerging data is encouraging both in terms of the relatively favourable side-effect profiles and early  
59 signs of efficacy. The current lead agent, JX-594 also known as Pexa-Vec (pexastimogene  
60 devacirepvec) was granted orphan drug status in HCC by the U.S. Food and Drug Administration in  
61 2013 and by the European Medicines Agency in 2009 (France, 2013). Orphan drug designation  
62 (ODD) is approved for drugs that seek to treat rare diseases for which there may be few adequate  
63 therapies, and comes with incentives that include marketing exclusivity, grant funding for clinical  
64 trials and tax credits for clinical research expenses. Whilst these incentives assert the dominance of  
65 JX-594 in the field, they have not perturbed the translational development of other OV for HCC  
66 therapy. In addition to JX-594, three other OV have been or are currently being tested in HCC-  
67 directed clinical trials, including two based on type 5 adenoviruses, *dl1520* (ONYX-015) (Habib *et al.*, 2002) and H101 (Oncorine) [NCT01869088] as well as a vesicular stomatitis virus (VSV)  
68 encoding the human interferon (IFN)- $\beta$  gene (VSV-hIFN- $\beta$ ) [NCT01628640].

70 This review summarises the pre-clinical and clinical progress of oncolytic virotherapy in HCC,  
71 focussing on the molecular methods employed to improve virus targeting to malignant hepatocytes,  
72 the use of virus-encoded therapeutic genes, and methods to improve viral survival. We also  
73 summarise the completed and ongoing clinical trials, routes of clinical viral delivery, and published  
74 clinical safety and efficacy data.

## 75 **Engineering Oncolytic Viruses for HCC Therapy**

76 Although the first wave of OV clinical trials took place in the 1950s and 1960s, it was not until the  
77 1990s that engineered OV blossomed alongside advances in DNA manipulation and molecular  
78 biology techniques.

### 79 **Targeting Malignant Hepatocytes**

80 The specificity of any given drug determines its side-effect profile and greatly influences its efficacy.  
81 JX-594, *dl1520*, H101 and VSV-hIFN- $\beta$  have all been engineered for pan-cancer specificity, targeting  
82 hallmark cancer characteristics, such as TP53 deletion and upregulated thymidine kinase (TK)

83 expression. Other engineered pan-cancer specific OV have also shown efficacy in pre-clinical HCC  
84 models, including those whose genome expression is driven by survivin, an inhibitor of apoptosis  
85 protein family that is overexpressed in the majority of HCC cases (survivin promoter-regulated  
86 oncolytic adenovirus vector carrying TP53 gene, AdSurp-P53) and human telomerase reverse  
87 transcriptase (hTERT), expressed in up to 90% of HCCs, but only some 20% of non-malignant liver  
88 cells (hTERT promoter-regulated replicative adenovirus, SG300) (Kannangai *et al.*, 2005) (He *et al.*,  
89 2012) (Nagao *et al.*, 1999) (Liu *et al.*, 2011). More recently, OV that preferentially target tumour  
90 initiating cells have been engineered, including oncolytic measles virus retargeted to CD133 positive  
91 cells (Bach *et al.*, 2013). In the liver, CD133 expression is limited to cancerous tissue, and is  
92 associated with colony formation and high proliferative capacity (Kohga *et al.*, 2010) (Zhu *et al.*,  
93 2010).

94 In contrast to pan-cancer specific OV, numerous pre-clinical OV have been engineered to specifically  
95 target HCC (table 1). Commonly, HCC-specific viral promoters are inserted into the viral genomes  
96 that restrict the transcription of viral genes to HCC cells and hence limit the destruction of healthy  
97 cells (Ohguchi *et al.*, 1998) (Foka *et al.*, 2010). Viral gene expression in these systems can be further  
98 increased by the insertion of an insulator element upstream of the HCC specific promoter, to shield  
99 from viral silencers, while retaining specific gene expression in hepatoma cells (Ye *et al.*, 2003).

100 A further approach to specifically target malignant hepatocytes is to exploit the differential expression  
101 of micro-RNA (miRNA) transcripts; recently, a 30 miRNA signature consisting of 10 down-regulated  
102 and 20 up-regulated miRNAs was established for distinguishing HCC from non-cancerous liver  
103 tissues (Wei *et al.*, 2013). Complementary sequences to miRNA transcripts that are specifically down-  
104 regulated in HCC e.g. mir-122 and mir-199 have been inserted into the 3' untranslated regions of OV  
105 including oncolytic type 5 adenovirus and HSV (Cawood *et al.*, 2009) (Fu *et al.*, 2012) (Khalid  
106 Elamin Elhag, 2012). The resulting selective viral RNA degradation in normal hepatocytes led to  
107 decreased hepatotoxicity, whilst retaining anti-HCC potency in animal models.

108 These methods are not without their problems as shown in table 1, and the protein or miRNA-binding  
109 site to be engineered into the OV genome must be chosen wisely.

## 110 **Enhancing Anti-Cancer Efficacy**

111 Numerous therapeutic anti-cancer genes have been engineered into oncolytic viruses in a bid to  
112 enhance efficacy. In particular, replication competent adenovirus vectors have been extensively  
113 modified and tested in pre-clinical models of HCC as illustrated in Fig. 1. The engineered therapeutic  
114 genes fall under one of two broad categories: those that modify the tumour microenvironment  
115 including stimulation of anti-HCC immune responses, and those acting directly on HCC cells to  
116 induce apoptosis and reduce cell growth and survival. In addition to the examples shown in figure 1,

117 both oncolytic measles and Newcastle disease viruses have been engineered to express enzymes that  
118 convert the prodrug 5-fluorocytosine into the active chemotherapeutic 5-fluorouracil, enabling OV-  
119 mediated targeted chemotherapy, significantly enhancing OV efficacy (Lv *et al.*, 2013) (Lampe *et al.*,  
120 2013). The majority of approaches to arming OV can be equally applied in the treatment of any solid  
121 malignancy. Exceptions include recombinant human erythropoietin (rhEPO), which was recently  
122 engineered into an oncolytic Lister strain Vaccinia virus (GLV-1h210) and tested in lung cancer  
123 xenografts (Nguyen *et al.*, 2013). rhEPO is essential for erythropoiesis and significantly improves  
124 quality of life in anaemic cancer patients, but is associated with angiogenesis, limiting its use in  
125 highly vascularised tumours, such as HCC (Yasuda *et al.*, 2003) (Crawford *et al.*, 2002).

126 A large body of evidence gathered both from pre-clinical and clinical studies in various cancer types  
127 points to the potential of OV to stimulate both innate and adaptive anti-cancer immune responses  
128 (Melcher *et al.*, 2011) (Prestwich *et al.*, 2008a) (Prestwich *et al.*, 2009). This could also be important  
129 for OV therapy in HCC. However, the liver is an immunologically privileged organ, skewed towards  
130 an environment of immunological tolerance rather than immunity, as evidenced, for example, by  
131 reports of the acceptance of liver allografts across major histocompatibility barriers without  
132 immunosuppressive therapy (Seyfert-Margolis & Turka, 2008). This immunosuppressive  
133 microenvironment is further compounded in HCCs that frequently harbour enriched regulatory T-  
134 cells, elevated immunosuppressive cytokines such as transforming growth factor (TGF)- $\beta$  and  
135 interleukin (IL)-10, and decreased immunostimulatory cytokines such as IL-2 and IFN- $\gamma$  (Shirabe *et al.*  
136 *et al.*, 2010). In addition, frequently impaired functional activities of NK cells in HCC are associated  
137 with poor prognosis (Wada *et al.*, 1998) (Wu *et al.*, 2013).

138 Encouragingly, HCCs with a more favourable immune microenvironment, including NK cell  
139 accumulation are associated with improved survival, and pre-clinical evidence exists for the  
140 infiltration of HCC by NK cells following OV therapy, whilst the depletion of NK cells inhibits OV-  
141 mediated anti-HCC effects (Chew *et al.*, 2009) (Gentschev *et al.*, 2011) (Tsuchiyama *et al.*, 2007)  
142 (Kwon *et al.*, 2001). Several cytokines that have the potential to stimulate anti-cancer NK cell  
143 responses have been engineered into OV; IL-12 induces the proliferation and activation of NK cells,  
144 in addition to the differentiation of naïve CD4<sup>+</sup> T-cells into Th1 cells (Hamza *et al.*, 2010). Similarly,  
145 chemokine (C-C motif) ligand 5 (CCL5) also drives the cytolytic activity of NK cells, and induces  
146 NK cell proliferation through T-cell mediated IL-2 secretion (Taub *et al.*, 1995) (Maghazachi *et al.*,  
147 1996). Whilst IL-12 and CCL5 have shown promising anti-HCC effects in pre-clinical models, others  
148 including IFN- $\beta$ , a powerful stimulator of NK cell activation, are currently being tested in patients  
149 with advanced HCC (NCT01628640).

150 Key to priming successful T-cell anti-HCC responses are antigen presenting cells (APCs), of which  
151 dendritic cells (DCs) are of utmost importance. It is known that DCs from HCC patients have

152 significantly lower capacity to stimulate T-cells than DCs from patients with liver cirrhosis or normal  
153 controls (Ninomiya *et al.*, 1999). Furthermore in chronic viral hepatitis, there are decreased DC liver  
154 populations and impairment in DC capacity to prime naïve T-cells, contributing to the inadequate  
155 adaptive immune responses observed (Kanto *et al.*, 2004) (Averill *et al.*, 2007). OV are capable of  
156 driving successful T-cell anti-cancer therapy as shown in melanoma models utilising oncolytic wild-  
157 type reovirus and VSV-GFP (Prestwich *et al.*, 2008b) (Wongthida *et al.*, 2011). In HCC pre-clinical  
158 models, the oncolytic Vaccinia virus GLV-1h68, encoding several biomarker genes only (see table 3),  
159 has been shown to promote the intense infiltration of DCs into both HBV positive and hepatitis virus  
160 negative xenografts, whilst VSV-GFP promoted the infiltration of DCs into HCC tumours in an  
161 orthotopic immunocompetent animal model (Gentshev *et al.*, 2011) (Shinozaki *et al.*, 2005).  
162 Although not a prerequisite for successful T-cell therapy, the OV-mediated expression of engineered  
163 immunostimulatory genes has the potential to greatly improve efficacy. Several approaches to  
164 enhance DC maturation/activation have been tested in pre-clinical HCC models, and include arming  
165 viruses with granulocyte macrophage colony-stimulating factor (GM-CSF) or CpG-rich sequences,  
166 the latter of which has been shown to increase IFN- $\gamma$  and DC activation in draining lymph nodes,  
167 resulting in improved therapy against hepatoma lung metastases in comparison to the wild-type virus  
168 (Raykov *et al.*, 2008). Other groups have shown enhanced DC and CD4+ T-cell tumour infiltration  
169 using Vaccinia viruses encoding CCL5 or a secretory bispecific T-cell engager consisting of two  
170 single- chain variable fragments specific for CD3 and the tumour cell surface antigen EphA2 (Li *et*  
171 *al.*, 2011) (Yu *et al.*, 2014).

172 Immune cell recruitment and activation also plays a prominent role in the OV-induced disruption of  
173 tumour-associated vasculature. Indeed, inflammation-mediated disruption of vasculature is a well-  
174 documented phenomenon (Bryant *et al.*, 2005) (Lee & Slutsky, 2010). VSV infection of subcutaneous  
175 tumours resulted in transcriptional activation of the neutrophil chemoattractants CXCL1 and CXCL5,  
176 inducing tumour infiltration by neutrophils, vascular shutdown and the apoptosis of uninfected tumour  
177 cells (Breitbach *et al.*, 2007). The depletion of neutrophils prior to VSV infection abrogated these  
178 effects. In addition to the role played by OV-induced inflammation, JX-594 has been shown to  
179 directly infect and kill tumour-associated vascular endothelial cells in mice following intravenous  
180 delivery (Breitbach *et al.*, 2013). These findings have been confirmed in human HCC trials,  
181 demonstrating disruption of tumour perfusion following JX-594 therapy (Liu *et al.*, 2008) (Heo *et al.*,  
182 2011).

183 The effects of OV on the wider HCC microenvironment is complex and has recently been reviewed  
184 elsewhere (Altomonte & Ebert, 2014).

## 185 **Enhancing viral delivery and viral survival**

186 Perhaps the biggest challenge to successful oncolytic virotherapy in HCC is the ability to infect  
187 sufficient numbers of malignant hepatocytes with a sufficiently high multiplicity of infection and to  
188 maintain viral propagation. It is well established that the immune response to OV is likely to play a  
189 dual role; simultaneously clearing the virus and hence limiting efficacy, whilst at the same time  
190 becoming more activated and primed to attack malignant cells (Melcher *et al.*, 2011). It is known that  
191 adenovirus is rapidly removed following IV delivery by Kupffer cells, liver resident macrophages,  
192 and the same may be true of other viruses (Tao *et al.*, 2001). A number of novel methods have been  
193 employed to enhance systemic viral delivery to the desired target including Kupffer cell depletion  
194 using replication-defective adenovirus, prior to replication-competent adenovirus therapy, and  
195 warfarinisation to block coagulation factor and complement dependent binding of adenovirus to  
196 hepatocytes (Shashkova *et al.*, 2008). Combined Kupffer cell depletion and warfarinisation resulted in  
197 decreased hepatotoxicity and increased anti-tumour potency, albeit in subcutaneous xenografts  
198 (Shashkova *et al.*, 2008). A different approach that has been tested in pre-clinical models of HCC is to  
199 engineer OV to evade immune inactivation (table 2). These engineered OV are yet to be tested in  
200 clinical trials and it remains to be seen whether they paradoxically result in reduced immune-mediated  
201 anti-cancer efficacy.

## 202 **Engineered OV Tested in HCC-Directed Clinical Trials**

203 In addition to the plethora of engineered oncolytic adenoviruses, a large number of wild-type and  
204 recombinant OV have been investigated in pre-clinical models of HCC, but are yet to enter HCC-  
205 directed clinical trials (table 3). Some of these viruses are clinical-grade agents that have been  
206 employed in other anti-cancer clinical trials, and are hence the more likely to proceed to HCC-  
207 directed trials.

208 The following sections describe the OV that have entered HCC-directed clinical trials to date:

### 209 **JX-594**

210 JX-594 was first filed for patent in 2005 by Jennerex Biotherapeutics ULC; a company that entered  
211 into a commercialization and development agreement for JX-594 with Transgene in 2010 and was  
212 later acquired by SillaJen Inc. in 2013 (Kirn, 2006) (Transgene, 2010) (Transgene, 2013a). The Wyeth  
213 strain of Vaccinia virus, that forms the backbone of JX-594 was derived from the poorly pathogenic  
214 New York City Board of Health strain. The Wyeth strain was extensively employed as a smallpox  
215 vaccine in the U.S. until routine vaccination was rescinded in 1971 (ODC, 1971). JX-594 has been  
216 genetically modified by the homologous recombination of a pSC65 plasmid with the Vaccinia virus  
217 TK gene. The plasmid sequence contains the human GM-CSF gene under the control of a synthetic

218 early-late promoter and the *LacZ* reporter gene (Mastrangelo *et al.*, 1998). GM-CSF induces direct  
219 anti-tumour effects and importantly influences the immune system through the stimulation,  
220 recruitment and maturation of dendritic cells (Urduingio *et al.*, 2013) (Mach *et al.*, 2000).

221 The expression of TK, an enzyme of the DNA precursor pathway, is strictly regulated during the  
222 normal cellular cycle, but is much higher and permanently expressed in malignant growing cells  
223 (Hengstschlager *et al.*, 1998). Being TK deleted, JX-594 cancer-selectivity was believed to be  
224 dependent on elevated cellular TK levels in cancers. However, recent work has shown JX-594 cancer  
225 specificity to be multi-mechanistic, with replication being dependent on epidermal growth factor  
226 receptor/Ras /mitogen-activated protein kinase pathway signalling, cancer cell resistance to type-I  
227 interferons, as well as cellular TK levels (Parato *et al.*, 2012).

### 228 **VSV-hIFN- $\beta$**

229 VSV is a negative-strand RNA virus that is non-pathogenic to humans. Effective immune defence to  
230 VSV is dependent on the host interferon response, with mice harbouring defective interferon systems  
231 succumbing to normally harmless VSV exposure (Durbin *et al.*, 1996). Insertion of genes between the  
232 viral glycoprotein and polymerase genes does not affect the fitness of the resultant recombinant virus  
233 (Fernandez *et al.*, 2002). Generation of VSV-hIFN- $\beta$  is achieved by insertion of the human (h)IFN- $\beta$   
234 gene into the same position of the full-length viral antigenomic cDNA, pVSV-XN2, using unique  
235 restriction enzyme sites (Obuchi *et al.*, 2003). The expression of hIFN- $\beta$  renders successful virus  
236 propagation dependent on defective cellular interferon response pathways, as found in many cancers  
237 (Barber, 2004). In addition, expression of hIFN- $\beta$  is envisaged to activate NK and T-cells and  
238 facilitate the maturation of DCs for immune-mediated anti-tumour therapy, as well as directly  
239 inhibiting malignant cell proliferation (Odaka *et al.*, 2001) (Ferrantini & Belardelli, 2000) (Kadowaki  
240 *et al.*, 2000). VSV-hIFN- $\beta$  is patented and being developed by the Mayo Foundation for Medical  
241 Education and Research (Federspiel *et al.*, 2010).

### 242 **dl1520 (ONYX-015) and H101 (Oncorine)**

243 The adenovirus type 5 early regions 1A (E1A) and 1B (E1B) can be exploited to engineer cancer  
244 specificity; the protein products of E1A induce cellular DNA synthesis and transformation, but trigger  
245 apoptosis mediated by the mammalian tumour cell suppressor protein p53, with a resultant reduction  
246 in the yield of progeny (Bayley & Mymryk, 1994) (Debbas & White, 1993). The 55-kDa E1B protein  
247 binds to the p53 protein and blocks p53-mediated transcriptional activation, hence limiting p53-  
248 dependent cell cycle arrest and apoptosis (Sarnow *et al.*, 1982) (Yew & Berk, 1992). Early gene-  
249 therapy adenoviral type 5 vectors were modified to disable productive infection by the deletion of  
250 both E1A and E1B. These replication deficient adenovirus vectors were extensively used in cancer  
251 gene therapy trials, however evidence for efficacy was restricted due to self-limiting transgene

252 expression, poor target cell transduction and lack of tumour cell targeting (Vile *et al.*, 2000). On the  
253 other hand, disabling the E1B region alone theoretically leads to selective replication in p53-deficient  
254 cells. One of the first such replication-selective type 5 adenoviruses, *dl1520* has an 827-base pair  
255 deletion in the E1B region and a point mutation at codon 2022 that generates a stop codon preventing  
256 expression of a truncated protein from the deleted gene (Barker & Berk, 1987).

257 Initial data suggested that *dl1520* does indeed selectively replicate in TP53-deficient cells (Bischoff *et al.*  
258 *et al.*, 1996). However, it is now accepted that TP53 status is in fact a poor predictor of the sensitivity of  
259 tumour cells to *dl1520* with tumour specificity being determined by other factors such as the  
260 inhibition of viral RNA export in non-malignant cells (Edwards *et al.*, 2002) (O'Shea *et al.*, 2004). An  
261 incomplete understanding of the mechanisms of OV cancer specificity can hamper clinical progress,  
262 as exemplified by a trial testing *dl1520* in hepatobiliary cancers, where patients with HBV infections  
263 were in hindsight unnecessarily excluded due to theoretical risks that HBV protein X can inactivate  
264 p53 protein in non-malignant hepatocytes, rendering them susceptible to *dl1520* productive infection  
265 (Makower *et al.*, 2003).

266 *dl1520* was clinically developed by Onyx Pharmaceuticals under the name ONYX-015 until 2003  
267 when a promising phase 3 trial in head and neck cancer was suspended. Exclusive rights to ONYX-  
268 015 were sold to Shanghai Sunway Biotech in 2005 (Investis, 2005). In the years preceding this  
269 acquisition, Shanghai Sunway Biotech was simultaneously developing H101 (Oncorine), a  
270 recombinant human adenovirus type 5 similar to ONYX-015. In November 2005, the Chinese State  
271 Food and Drug Administration approved H101 for advanced nasopharyngeal carcinoma in  
272 combination with chemotherapy (Medscape, 2005). Like *dl1520*, H101 is E1B gene deleted, but  
273 unlike *dl1520*, H101 has an additional partial E3 78.3-85.8µm gene segment deletion (Lu *et al.*,  
274 2004). E3 gene products prevent T-cell and NK cell recognition of infected cells by preventing  
275 transport of MHC class I to the plasma membrane and by sequestration of MHC class I-related  
276 molecules A and B respectively (Burgert & Kvist, 1985) (McSharry *et al.*, 2008). The partial E3 gene  
277 deletion in H101 is thought to enhance its safety profile, although this may be at the cost of decreased  
278 anti-cancer potency (Suzuki *et al.*, 2002).

## 279 **Clinical Experience of Oncolytic Virus-Based Therapy in** 280 **HCC**

281 To date only 4 HCC-directed clinical trials using two different OV, JX-594 and *dl1520* have been  
282 undertaken and completed follow-up (table 4). Early phase trials that include a mixed population of  
283 patients with digestive tract tumours, have typically recruited very small numbers of patients with  
284 HCC, making it difficult to adequately characterise the performance of these agents (Park *et al.*, 2008)

285 (Habib *et al.*, 2001). It is also noteworthy that patients with significant chronic infections including  
286 HIV, HBV and HCV infection are frequently excluded from trials of OV that include multiple disease  
287 sites, primarily due to the perceived risk of increased adverse events (Pecora, 2002) (Vidal *et al.*,  
288 2008). Encouragingly, at least 3 other OV trials exclusively for HCC are either underway or nearing  
289 completion (table 5).

## 290 **Route of Delivery**

291 The safety and efficacy of OV therapy is dependent not only on viral specifics, but also on numerous  
292 clinical considerations, including the administered dose of virus, the rate of infusion, the anatomical  
293 distribution of disease and the route of delivery.

## 294 **Intratumoural Injection**

295 Numerous intratumoural (ITu) therapies have been trialled in liver tumours, and it is a popular OV  
296 delivery method in HCC (see tables 4 and 5) (Venook, 2000). The advantages of the ITu route are the  
297 delivery of a high concentration of drug to the target, whilst minimising off-target side-effects, an  
298 important consideration in HCC where the background liver is frequently cirrhotic with reduced  
299 functional capacity. However, direct ITu injection carries significant risks of bleeding, infection,  
300 peritoneal tumour seeding as well as technical challenges. It is frequently impossible to inject all HCC  
301 foci, but this is not necessarily a limitation of the technique; Park *et al* reported that ITu injection of  
302 JX-594 led to the initial release of virus into the bloodstream, that was rapidly cleared (Park *et al.*,  
303 2008). This was then followed by the re-emergence of circulating JX-594 days to weeks later,  
304 consistent with productive infection. In keeping with these observations, replicating JX-594 infection  
305 was found in a non-injected HCC focus metastatic to the neck following ITu liver injection (Park *et*  
306 *al.*, 2008).

## 307 **Intravenous Injection**

308 The IV delivery of OV avoids the local injection-site side-effects associated with invasive ITu  
309 therapy. IV injection is also more likely to be acceptable to both patients and their physicians when  
310 administered at regular intervals as part of a scheduled course of treatment. Intravenous  
311 administration of JX-594 has been shown to result in viral delivery to tumours, with the key  
312 determinant of tumour infection being the administered dose (Breitbach *et al.*, 2011). Of the patients  
313 treated with doses  $\geq 1.5 \times 10^7$  PFU kg<sup>-1</sup> and subsequently biopsied, 87% showed JX-594 positivity in  
314 tumour by IHC or qPCR, whereas those treated with lower doses were negative. All patients on this  
315 trial had a history of vaccination with live Vaccinia virus as children, and delivery was demonstrated  
316 in a patient despite the presence of neutralizing antibodies at baseline. This finding lends support for  
317 the need to establish the maximum tolerated dose in trials of oncolytic virotherapy and to use the  
318 maximum tolerated dose in subsequent phase 2 and 3 trials. The IV route is further supported by a  
319 translational trial where oncolytic reovirus was recovered post-surgery from colorectal cancer liver

320 metastases following IV delivery, and shown to be capable of plaque formation *ex-vivo* (Adair *et al.*,  
321 2012). In the same trial, no replicating reovirus was recovered from normal liver samples, but faint  
322 staining for reovirus sigma 3 protein was seen by IHC, supporting the notion of preferential  
323 productive infection in cancerous tissue.

324 Several trials have employed an initial IV injection of OV followed by ITu injections. The theory  
325 behind this approach is that initial IV injection will prime an immune response that is then amplified  
326 at the target site upon further ITu injections.

### 327 **Hepatic Artery Injection (HAI)**

328 HAI using cytotoxic agents is in routine clinical practice for patients with HCC and warrants further  
329 investigation in oncolytic virotherapy. This is commonly employed in the form of transarterial  
330 chemoembolization (TACE), either as a palliative technique per se, or as a 'bridging' modality before  
331 liver transplantation (Jelic & Sotiropoulos, 2010). The TACE principle employs HAI of cytotoxic  
332 drug combinations followed by lipiodol or degradable microsphere injection for vessel occlusion,  
333 resulting in tumour cell ischaemia and necrosis.

334 It is debateable whether HAI enhances viral delivery to localised targets over the simpler method of  
335 ITu injection. HAI also does not prevent systemic side-effects as was significantly highlighted by the  
336 well-publicised death of the teenager Jesse Gelsinger secondary to systemic inflammatory response  
337 syndrome (SIRS) induced by the hepatic artery injection of  $3.8 \times 10^{13}$  virus particles of replication  
338 incompetent adenovirus type 5 (E1 and E4 deleted) encoding ornithine transcarbamylase cDNA  
339 (Raper *et al.*, 2003). The strength of HAI lies in the opportunity to improve on existing locoregional  
340 therapies in combination with TACE, and encouragingly, a phase 3 trial of H101 in combination with  
341 TACE in patients with HCC is currently recruiting (table 5). Clearly, further trials testing OV by HAI  
342 are warranted and it remains to be seen which route of delivery is preferable in terms of safety,  
343 efficacy and patient acceptability.

### 344 **Clinical Safety Data**

345 As can be seen from table 4, both ITu and IV injections of JX-594 have been tested in patients with  
346 HCC. The most common adverse events are an influenza-like illness comprising headache, nausea,  
347 vomiting and fatigue (Park *et al.*, 2008) (Breitbach *et al.*, 2011) (Heo *et al.*, 2013a). A mild fever  
348 occurs in all patients and is dose-related (Heo *et al.*, 2013a). A maximum tolerated dose was reached  
349 at  $10^9$  PFU due to grade III hyperbilirubinaemia subsequent to transient tumour swelling inducing  
350 biliary obstruction (Park *et al.*, 2008). Peri-tumoural oedema, induced by acute inflammation has been  
351 commonly reported in trials using OV and in fact response after initial tumour flare is a class effect of  
352 immune therapies in general (Pecora, 2002) (Senzer *et al.*, 2009) (Wolchok *et al.*, 2009). The absence

353 of substantial changes in AST and ALT suggest that direct destruction of healthy hepatocytes  
354 following JX-594 injection is mild (Park *et al.*, 2008).

355 Habib *et al* reported safety data from 10 patients with HCC treated with *dl1520*. Following a dose-  
356 escalation study in patients with either primary or secondary liver tumours in which no maximum  
357 tolerated dose was reached, a further small HCC-directed trial was undertaken in Egypt (Habib *et al.*,  
358 2001). In the latter study 10 patients were randomised in a 1:1 ratio to receive either a single IV dose  
359 of  $3 \times 10^{11}$  PFU of *dl1520* followed by 5 ITu doses, or standard of care therapy with 95% ethanol by  
360 ITu injection (Habib *et al.*, 2002). Of the five patients treated with *dl1520*, three suffered from  
361 CTCAE grade I-II fever and rigors, and 2 patients suffered from transient hypotension at the time of  
362 the infusions. Very minor changes in AST and ALT were observed for patients treated with *dl1520*, in  
363 comparison to the much higher levels of serum transaminases observed following ethanol treatment  
364 (Habib *et al.*, 2002).

### 365 **Assessing Efficacy in OV Therapy for HCC**

366 For the approval of new anti-cancer drugs, the FDA accepts improved survival, as well as surrogate  
367 markers that predict clinical benefit. The Response Evaluation Criteria in Solid Tumours (RECIST)  
368 use single linear summation of target lesions to define response to therapy (Therasse *et al.*, 2000).  
369 However, the clinical benefit provided by anti-cancer therapy in HCC correlates poorly with  
370 conventional methods of response assessment (Llovet *et al.*, 2008) (Forner *et al.*, 2009). In 2008, the  
371 American Association for the Study of Liver Diseases (AASLD) developed a set of guidelines, termed  
372 the modified RECIST or mRECIST criteria aimed at providing a common framework for the design  
373 of clinical trials in HCC (Lencioni & Llovet, 2010). These guidelines consider estimation of the  
374 reduction in viable tumour area using contrast-enhanced radiologic imaging to be the optimal method  
375 to assess treatment response in HCC. Nonetheless, both RECIST and mRECIST criteria must be  
376 employed with caution in trials using immunotherapies; in particular, OV may cause transitory  
377 tumour-flare secondary to inflammatory cytokine release, leading to tumour enlargement and  
378 increased contrast enhancement, prior to tumour necrosis and shrinking (Senzer *et al.*, 2009).  
379 Delaying radiologic assessment following OV therapy could potentially avoid this issue (Hales *et al.*,  
380 2010).

### 381 **Clinical Evidence of Anti-Tumour Efficacy**

382 In a recent pivotal study, 30 patients with advanced HCC were randomised to low ( $10^8$  PFU) or high  
383 dose ( $10^9$  PFU) intratumoural JX-594 administered every 2 weeks (Heo *et al.*, 2013a). The majority of  
384 patients in both groups had previously received locoregional therapy, but more patients in the high  
385 dose group had previously failed sorafenib therapy, a poor prognostic factor. Median overall survival  
386 was 14.1 months for the high dose arm and 6.7 months for the low dose arm. Despite the relatively

387 small sample size, a statistically significant survival benefit ( $P=0.020$ ) was demonstrated because of  
388 the large effect size. Both doses were associated with mRECIST responses, decreased tumour  
389 perfusion and decreased tumour contrast enhancement. This is the first study to show a statistically  
390 significant benefit derived from OV therapy in patients with HCC.

391 JX-594 has been tested as second line therapy in two phase 2 HCC trials (see table 4). In the larger of  
392 these studies (TRAVERSE), patients who had previously failed sorafenib therapy were treated with  
393 JX-594 and BSC or BSC alone (Transgene, 2013b). Sadly, the primary endpoint of improved overall  
394 survival was negative. The failure of JX-594 in the TRAVERSE trial following promising randomised  
395 dose-finding trial data remains to be fully explained. Patients recruited to the TRAVERSE trial were  
396 more likely to have sorafenib-resistant cancers. Acquired cellular resistance mechanisms to sorafenib  
397 following long-term exposure include compensatory crosstalk between PI3K/Akt and MAPK  
398 pathways, upregulation of the JAK-STAT pathway and enhanced epithelial-mesenchymal transition  
399 (Zhai & Sun, 2013). These changes could theoretically affect OV infection and anti-cancer efficacy,  
400 although recently, the modified Lister strain Vaccinia virus, GLV-1h68, was shown to effectively  
401 infect and kill sorafenib-resistant HCC cell lines (Ady *et al.*, 2014). Alternatively, the failure of JX-  
402 594 in the TRAVERSE trial could be attributed to more advanced disease in the second line setting;  
403 fitter patients carrying a smaller HCC disease burden are most likely to respond to OV therapy, as has  
404 been the experience with other immunotherapies (Coppin *et al.*, 2005). Furthermore, the relatively  
405 small number of patients included in phase 2 trials presents a challenge when seeking outcomes of  
406 study drug superiority over standard care. Nonetheless, Transgene recently announced a shift in  
407 strategy, moving JX-594 trials away from the second-line setting in HCC. Instead, a phase 3 trial  
408 which is expected to enrol approximately 600 patients and is anticipated to begin recruitment in 2015,  
409 will be testing whether first line IT JX-594 (weeks 0, 2 and 4) followed by sorafenib (week 6  
410 onwards) improves overall survival in comparison to sorafenib alone (Transgene, 2014).

411 In contrast, no meaningful efficacy data can be derived from the *dl1520* trial by Habib *et al.*; one  
412 patient who received *dl1520* experienced a partial response with reduction in tumour volume from  
413 306 to 22.5 cm<sup>3</sup> associated with a concomitant decrease in AFP level from 7604 to 300 ng mL<sup>-1</sup>  
414 (Habib *et al.*, 2002). The remaining four patients demonstrated progressive disease with an increase in  
415 both tumour volume and AFP levels. Larger randomised trials are needed to determine whether  
416 recombinant type 5 adenoviruses are efficacious in HCC.

## 417 **Clinical Evidence of Anti-cancer Immune Stimulation**

418 Anti-cancer immune stimulation could be at least partially responsible for the reported decreases in  
419 the size and contrast enhancement of non-injected tumours following intratumoural JX-594 injection  
420 elsewhere (Park *et al.*, 2008) (Heo *et al.*, 2013a). However, little *ex-vivo* evidence has been gathered  
421 to date from clinical trials for anti-HCC immune responses. In their randomised dose-comparison

422 phase 2 trial, Heo *et al* demonstrated HCC immune infiltration following JX-594 injection by both  
423 radiographic peripheral tumour enhancement and histologically confirmed diffuse lymphocyte  
424 infiltration from biopsied tumours (Heo *et al.*, 2013a). In the same trial, Heo *et al* assessed antibody-  
425 mediated complement-dependent cytotoxicity (CDC) by the addition of serum from JX-594 treated  
426 patients to HCC cell lines, resulting in cytotoxicity from 11 of the 16 subjects tested (Heo *et al.*,  
427 2013a). Indeed, CDC could be of vital importance in OV therapy as evidenced by a recent JX-594  
428 study in patients with a variety of cancer types, where patients with the longest survival duration had  
429 the highest CDC activity (Kim *et al.*, 2013). Evidence was also gathered for antibody development  
430 and T-cell immunity against JX-594 encoded proteins including  $\beta$ -galactosidase, an observation of  
431 likely importance in the elimination of virus-infected tumour cells (Heo *et al.*, 2013a). Whilst  
432 encouraging, these results do not constitute an adaptive anti-HCC immune response. At least 6  
433 different HCC-specific tumour associated antigens (TAA) that are targeted by T-cells have been  
434 identified and future OV trials should assess whether specific T-cell responses against these antigens  
435 are induced (Breous & Thimme, 2011).

436 Other evidence for immune stimulation is similarly encouraging, though sparse; both elevated TNF- $\alpha$   
437 and IFN- $\gamma$  have been observed in the serum of HCC patients treated with JX-594 (Liu *et al.*, 2008)  
438 (Park *et al.*, 2008). These are likely to contribute to DC maturation, cancer growth inhibition and  
439 apoptosis. Of interest, the presence of type I interferons, powerful stimulators of NK cell activity and  
440 DC maturation, has not been reported in JX-594-treated patients, perhaps due to efficient Vaccinia  
441 virus-mediated inhibition of the interferon system (Perdiguero & Esteban, 2009). In contrast, other  
442 viruses e.g. measles, reovirus and VSV, are known to efficiently induce type I interferons, wetting the  
443 appetite for HCC clinical trials in HCC with thorough translational read-outs using such agents  
444 (Steele *et al.*, 2011) (Diaz *et al.*, 2007) (Donnelly *et al.*, 2013). One potential concern is that co-  
445 infection of HCV infected hepatocytes with OV will not lead to robust interferon induction due to the  
446 interferon evasion mechanisms employed by HCV. For example, HCV NS3/NS4a protease disrupts  
447 pattern recognition receptor signalling by cleaving the RIG-I and TLR3 downstream adaptors, MAVS  
448 and TRIF respectively (Foy *et al.*, 2005) (Li *et al.*, 2005b) (Ferreon *et al.*, 2005). NS3/NS4A also  
449 perturbs RIG-I downstream signalling through disruption of virus-induced NF- $\kappa$ B binding to the DNA  
450 PRDII element, hence limiting IFN- $\beta$  gene expression (Foy *et al.*, 2005) (Li *et al.*, 2005c).

451 Realistically however, the scenario of reovirus co-infection with HCV is unlikely to be a major factor  
452 in HCC patients, as the majority of patients only have detectable HCV proteins or genomes in a  
453 minority of clustered hepatocytes (Stiffler *et al.*, 2009) A further concern is that HCV and HBV could  
454 suppress OV-mediated adaptive anti-tumour immune responses, however, no clinical evidence for this  
455 yet exists, and future HCC-directed trials cannot afford to exclude the majority of HCC patients, with  
456 a viral aetiology.

## 457 **Future Perspectives**

458 The clinical progress of JX-594 in HCC therapy provides much optimism in the field. This agent  
459 appears to be transcending the barrier between novel laboratory science and credible clinical therapy.  
460 From this clinical progress have come clues to support existing laboratory research into the  
461 mechanisms of OV-mediated anti-HCC efficacy including the direct, immune and anti-vascular  
462 effects. However, much remains to be discovered in terms of the differential response to OV therapy  
463 in subsets of patients, the optimal route of delivery and combinations with other anti-cancer therapies.  
464 Furthermore, biomarkers predictive of treatment response are greatly needed, as are continued efforts  
465 to establish early diagnoses of cirrhosis and HCC using technologies such as the non-invasive  
466 enhanced liver fibrosis test (Lichtinghagen *et al.*, 2013).

467 The combination of OV with sorafenib warrants particular mention. These drug combinations have  
468 non-overlapping toxicities, and potentially synergistic mechanisms of action, hence forming the focus  
469 of past and future trials. For JX-594, the sequence of this combination is of paramount importance;  
470 upfront JX-594 therapy is thought to induce acute vascular disruption, sensitising tumours to the anti-  
471 angiogenic effects of subsequent sorafenib treatment. In murine tumour models, sequential JX-594  
472 followed by sorafenib therapy was superior to either simultaneous therapy or sorafenib followed by  
473 JX-594 (Heo *et al.*, 2011). *In vitro*, sorafenib, a multi-kinase inhibitor, perturbs JX-594 productive  
474 infection of HCC cell lines, a result that can be predicted as sorafenib inhibits a wide range of cellular  
475 kinases in addition to its principal targets, whereas Vaccinia viruses are known to encode kinases,  
476 including B1R and TK that are essential for productive infection (Rempel & Traktman, 1992) (Parato  
477 *et al.*, 2012) (Kitagawa *et al.*, 2013). The very fact that the cancer specificity of JX-594 is partially  
478 dependent on elevated TK levels in malignant cells highlights the reliance of this OV on functional  
479 viral and cellular kinases. Hence, sequential scheduling works best for this OV, as was employed in  
480 the second line trial using JX-594 followed by sorafenib therapy, and a similar schedule is planned for  
481 the first line phase 3 trial (Heo *et al.*, 2011) (Transgene, 2014).

482 The combination of other OV that are less reliant on cellular kinase functions with sorafenib should  
483 form the focus of future studies. The precise scheduling should be determined by preclinical studies in  
484 immunocompetent animal models. Kottke *et al.*, showed that tumours treated *in vivo* with VEGF  
485 inhibitors became highly susceptible to systemic treatment with reovirus, but only if the drugs were  
486 withdrawn 24–48 hours before virus delivery. The authors concluded that the rebound of VEGF  
487 signalling upon drug withdrawal conditions tumour-associated endothelium for productive infection  
488 of reovirus (Kottke *et al.*, 2010).

489 The complex immunomodulatory effects of sorafenib are also likely to be critical determinants of  
490 success. One report cited that sorafenib significantly reduced the number of NK cells and inhibited

491 their reactivity against tumour targets in animal models, whilst a contradictory report stated that  
492 sorafenib enhances IL-12 secretion from human liver-derived macrophages, hence activating NK cells  
493 (Sprinzl *et al.*, 2013) (Zhang *et al.*, 2013). The efficacy of OV in combination with sorafenib will  
494 therefore be partially dependent on the stimulation or suppression of immune responses. Sorafenib  
495 could theoretically enhance OV therapy through a number of mechanisms including the synergistic  
496 activation of NK cells, and inhibition of the OV-directed humoral response, thus enhancing IV  
497 delivery, as has been the experience with chemotherapy (Lolkema *et al.*, 2011). Alternatively,  
498 sorafenib-induced immunosuppression could limit the immune-mediated efficacy of OV, whilst  
499 immune stimulation could limit virus propagation, both resulting in reduced efficacy. Orthotopic  
500 immunocompetent animal models could begin to answer these questions, but the lack of concordance  
501 between animal models and human research highlights the need to pursue early phase clinical trials  
502 using sorafenib-OV combinations.

503 In addition to sorafenib, numerous successful preclinical studies have been conducted, using OV in  
504 combination with cytotoxic agents, radiotherapy and targeted biotherapies including other pre-clinical  
505 OV (Mao *et al.*, 2009) (Zheng *et al.*, 2009) (Chung *et al.*, 2002). More recently, antibodies targeting  
506 the immune checkpoint molecules, CTLA-4 and PD-1/PD-L1 have been tested in early-phase HCC-  
507 directed clinical trials (Sangro *et al.*, 2013a) (Sangro *et al.*, 2013b). CTLA-4 is expressed on T-cells  
508 and inhibits T-cell activation, whilst PD-1/PD-L1 interactions limit the activation of NK, B- and T-  
509 cells (Pardoll, 2012). Combinations of OV with immune checkpoint inhibitors are being explored in  
510 solid and haematological malignancies and should also be tested in HCC, with the premise that OV-  
511 mediated tumour vaccination, followed by immune activation through checkpoint inhibition may  
512 prove highly beneficial (Engeland *et al.*, 2014) (Minev *et al.*, 2014). As with all combination  
513 regimens, overlapping side effects are of concern, especially severe immune-related toxicity. HCC  
514 therapy provides the opportunity to limit systemic side-effects by HAI, a delivery method that is  
515 likely to become increasingly important in future trials.

516 Taking these combinations one step further, future studies should assess the efficacy of OV carrying  
517 cDNA libraries, in combination with checkpoint inhibitors. Effective cancer immunotherapy requires  
518 the release of TAA in the context of potent immune activation. Kottke *et al.*, showed that a cDNA  
519 library of normal tissue, expressed from oncolytic VSV, acting as an immune adjuvant, cured  
520 established tumours of the same histological type from which the cDNA library was derived (Kottke  
521 *et al.*, 2011). In HCC therapy, such broad antigenic stimulation can potentially lead to the attack of  
522 healthy hepatocytes. This problem can be avoided by engineering OV to express specific TAA  
523 including AFP, EpCAM and SSX-2. Clues to indicate the likely efficacy of the latter approach can be  
524 found in patients with HCC who have a better prognosis, associated with the expression of such TAA  
525 (Liang *et al.*, 2013). Unleashing specific T-cell responses against OV-expressed TAA through  
526 combination with checkpoint inhibitors could prove to be a very valuable strategy.

527 Other than JX-594, a large number of clinically active and pre-clinical oncolytic viruses have been  
528 tested in HCC models, yet precious few of these agents have progressed into HCC-directed clinical  
529 trials. As in other fields, OV laboratory science races well ahead of clinical practice, and in this  
530 respect, anti-HCC oncolytic virotherapy is no different. The potential exists for the medicines  
531 regulatory authorities to approve multiple efficacious OV in HCC clinical practice, paving the way for  
532 stratified therapy. In order to realise this potential and reap the rewards, we must first push these pre-  
533 clinical agents into the clinic.

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**Table 1 – HCC-specific oncolytic viruses; mechanisms of targeting**

Targeting principle	Example	Description	Issues	Reference
Liver specific viral promoter	Transthyretin-promoter driven adenovirus	Transthyretin is a thyroid hormone transport protein, secreted into serum by hepatocytes.	Requires additional cancer specificity	(Hsieh <i>et al.</i> , 2009)
HCC specific viral promoter	AFP-promoter-driven adenovirus	AFP is produced in high levels from the foetal liver and yolk sac, but not normally in adults.	AFP is frequently only expressed in a sparse population of HCC cells, and can also be expressed from non-malignant hepatocytes in chronic hepatitis and cirrhosis (Ohguchi <i>et al.</i> , 1998)(Johnson, 2001).	(Zhang <i>et al.</i> , 2012)
Enzyme-activated viral protein	MMP-activated MVF protein	MMP substrate site is inserted into MVF.	Efficacy dependent on tumour MMP expression. Could have broader cancer specificity	(Muhlebach <i>et al.</i> , 2010)
miRNA mediated control of virus gene expression in normal liver cells	mir-122 regulated adenovirus	mir-122 binding sites inserted into the 3' untranslated region of an adenovirus type 5 E1A-luciferase transcription cassette.	mir-122 expression is preserved in HCV-induced HCC, potentially rendering mir-122 regulated adenovirus ineffective in this subset of patients (Varnholt <i>et al.</i> , 2008).	(Cawood <i>et al.</i> , 2009)

AFP (alpha fetoprotein); MMP (membrane metalloproteinase); MVF (Measles virus fusion); miRNA (micro-RNA)

**Figure 1 – Therapeutic gene products expressed by engineered replication-competent adenoviruses and tested in pre-clinical models of HCC.** Infection of a malignant hepatocyte (illustrated by the large blue rectangular cell) by replication competent adenovirus results in the expression of engineered therapeutic genes. Suppressor of cytokine signalling (SOCS)-1 and SOCS3 inhibit JAK phosphorylation of STAT, thus attenuating cytokine signal transduction and suppressing tumour growth (Wei *et al.*, 2011) (Liu *et al.*, 2013). Tumour suppressor in lung cancer 1 (TSLC1) is a cell adhesion molecule whose overexpression inhibits cell growth and migration, and induces apoptosis (He *et al.*, 2012). Numerous engineered genes enhance apoptosis: Melanoma differentiation associated gene (mda)-7/IL-24 binding to its receptor triggers mitochondrial dysfunction and apoptosis, whilst receptor-independent tumour suppression is achieved via the induction of sustained ER stress (Xiao *et al.*, 2010). The hepatocellular carcinoma suppressor 1 (HCCS1) gene product activates the mitochondrial apoptotic pathway by inducing lysosomal protease efflux (Gan *et al.*, 2008) (Zhang *et al.*, 2008). SMAC (second mitochondria-derived activator of caspase) inhibits the activity of XIAP, a potent inhibitor of caspase activation that prevents apoptosis (Pan *et al.*, 2007) (Pei *et al.*, 2004). XIAP protein translation can also be knocked down using targeted short hairpin RNA (shRNA), sensitizing cells to pro-apoptotic signals such as tumour necrosis factor-related apoptosis inducing ligand (TRAIL) (Ye *et al.*, 2005) (Pan *et al.*, 2008). Other OV-encoded therapeutic proteins that act directly on malignant cells are the pro-apoptotic apoptin and the sodium-iodide symporter (NIS), a transmembrane glycoprotein, which transports out two sodium cations in return for one iodide anion. NIS proteins allow the intracellular concentration of radioactive iodide, inducing apoptosis (Zhang *et al.*, 2012) (Grunwald *et al.*, 2013). Several OV-encoded therapeutic proteins are secreted for paracrine effects on other cells within the tumour microenvironment; IL-12 drives the activation/differentiation of NK and T-cells, whilst the C-C chemokine ligand 5 (CCL5) induces NK cell activation and T-cell chemotaxis (Yang *et al.*, 2012) (Li *et al.*, 2013). Secreted endostatin acts on endothelial cells to inhibit migration and proliferation, and to induce apoptosis (Li *et al.*, 2005a).

**Table 2 – Engineering OV to enhance delivery and survival in pre-clinical HCC models**

<b>Mechanism</b>	<b>Description</b>	<b>Potential advantages</b>	<b>Reference</b>
Viral surface modification using polymers	Arginine-grafted bioreducible polymer or high molecular weight polyethylene glycol chemically conjugated to oncolytic adenovirus surface	Reduced hepatocyte infection and liver toxicity  Reduced neutralisation by antibodies	(Kim <i>et al.</i> , 2011) (Doronin <i>et al.</i> , 2009)
Virus-mediated inhibition of NK and NKT cells	VSV expressing a protein from human cytomegalovirus known to downregulate CD155.	Reduced NK and NKT cell recruitment to the site of viral infection, reducing virus inactivation	(Altomonte <i>et al.</i> , 2009)
Virus-mediated expression of chemokine-binding proteins	Recombinant VSV expressing high affinity chemokine-binding proteins; M3, from murine gammaherpesvirus-68, or equine herpes virus-1 glycoprotein G	Reduced neutrophil, NK and NKT cell recruitment to the site of viral infection, enhancing virus titres.	(Wu <i>et al.</i> , 2008) (Altomonte <i>et al.</i> , 2008)

**Table 3 – OV Tested in Pre-clinical Models of HCC**

Virus species	Name	Modifications	Assessed in Clinical Trials?	Pre-clinical HCC Model	Reference
Parvovirus H-1	H-1PV	Wild-type	Yes, glioma	Cell lines	(Moehler <i>et al.</i> , 2001)
HSV-1	G207	Deletion of both ICP34.5 neurovirulence genes & inactivation of ICP6 (ribonucleotide reductase) by insertion of the E.coli lacZ gene.	Yes; glioma	Cell lines and subcutaneous murine xenografts	(Song <i>et al.</i> , 2006) (Xue <i>et al.</i> , 2005)
	Cgal-Luc	Derived by repair of ICP4 (positive and negative regulation of virus genome) from CgalΔ3 virus, insertion of the <i>LacZ</i> gene into IGR54 and luciferase gene into IGR20.	No	Subcutaneous murine xenografts	(Argnani <i>et al.</i> , 2011)
	H6-Luc	Derived from the H6 mutant; syncytium forming (Syn <sup>+</sup> ), benzhydrazone (glycosylation inhibitor) resistant. Luciferase cassette inserted into IGR20.	The closely related HF10 mutant has been tested in multiple solid tumours		
	G92A	ICP4 regulated by the albumin enhancer/promoter, mutated US3 gene (inhibitor of virus-induced apoptosis), disrupted thymidine kinase gene and insertion of the E.coli <i>lacZ</i> gene.	No	Orthotopic murine xenografts	(Chung <i>et al.</i> , 2006)
	hrR3	ICP6 <i>LacZ</i> insertion mutant.	No		
Blue tongue virus	BTV-10	Wild-type, cell-culture adapted	No	Hep3B cell line	(Hu <i>et al.</i> , 2008)
	BTV-HC <sub>3</sub>	Wild-type, cell-culture adapted	No	Cell lines	(Chen <i>et al.</i> , 2007)
Measles virus (Edmonston)	MV-CEA	Expresses extracellular domain of the human carcinoembryonic antigen (CEA)	Yes, glioma and ovarian cancer	Cell lines and subcutaneous murine xenografts	(Blechacz <i>et al.</i> , 2006)
	MV-NIS	Expresses the human sodium iodide symporter (hNIS)	Yes, myeloma and multiple solid tumours		
	MV-GFP	Expresses green fluorescence protein. Human bone marrow-derived mesenchymal stem cells were infected with MV-GFP and systemically delivered in passively-immunised mice.	No	Orthotopic patient-derived HCC tissue xenografts	(Ong <i>et al.</i> , 2013)
Newcastle Disease Virus	NDFLtag-EGFP	Derived from the wild-type LaSota vaccine strain. Carrying enhanced green fluorescence protein.	No	Human and murine hepatic stellate cells	(Li <i>et al.</i> , 2009)
	NDV-Italien	Wild-type	No		
	rNDV/F3a a(L289A)	L289A mutation within the F (fusion) glycoprotein	No	Immunocompetent orthotopic murine model	(Altomonte <i>et al.</i> , 2010)
	NDV/Anh-EGFP	Derived from the wild-type Anhinga strain. Carrying enhanced green fluorescence protein.	No	Cell lines and subcutaneous immunocompetent murine model	(Wu <i>et al.</i> , 2014)
Vaccinia	GLV-1h68	Derived from the Lister strain and carries three gene cassettes: a <i>Renilla</i> luciferase-GFP (RUC-GFP) fusion cassette at the F14.5L locus, a reverse inserted human transferrin receptor and β-galactosidase cassette at the J2R locus (encodes thymidine kinase), and a β-glucuronidase cassette at the A56R locus (encoding hemagglutinin).	Yes, multiple solid tumours	Cell lines and murine xenografts	(Gentshev <i>et al.</i> , 2011)
	JX963	Western reserve expressing GM-CSF, with double deleted thymidine kinase and vaccinia growth factor genes.	The closely related vvDD-CDSR expressing cytosine deaminase and somatostatin receptor is being tested in solid tumours	Sorafenib-resistant cell lines	(Ady <i>et al.</i> , 2014)
				Orthotopic immunocompetent rabbit model	(Lee <i>et al.</i> , 2009)

**Table 4 – Completed HCC-directed clinical trials using oncolytic viruses.** Searches were performed on ClinicalTrials.gov, Current Controlled Trials, EU Clinical Trials Register and medline.

Virus	Phase	No of patients	Route	Delivered dose	Study Design	Anti-Cancer Effect	Grade III or IV Adverse Events	Reference
JX-594	2	25	IV followed by ITu	1x10 <sup>9</sup> PFU	Single treatment group. IV day 1, ITu days 8 and 22, sorafenib day 25	mRECIST disease control rate 62% for JX-594 and 59% after initiation of sorafenib	Not available	(Heo <i>et al.</i> , 2013b)
JX-594	2	30	ITu	1x10 <sup>8</sup> or 1x10 <sup>9</sup> PFU	Randomised comparison between low and high dose JX-594	OS 14.1 months in high dose group Vs 6.7 months in low dose group ( <i>P</i> =0.020)	Lymphopaenia, pyrexia hyperbilirubinaemia	(Heo <i>et al.</i> , 2013a)
JX-594	2*	120	IV followed by ITu	1x10 <sup>9</sup> PFU	JX-594 plus BSC or BSC only. IV day 1 followed by five ITu treatments	No significant overall survival advantage	Not available	(Transgene, 2013b)
Ad5 dl1520	2	10	IV followed by ITu	3x10 <sup>11</sup> PFU	Randomised comparison between PEI and Ad5 dl1520	1 patient had PR by RECIST and 4 had PD	None	(Habib <i>et al.</i> , 2002)

PEI (percutaneous ethanol injection); BSC (best supportive care); IV (intravenous); ITu (intratumoural); OS (overall survival); PFU (plaque forming units); PR (partial response); PD (progressive disease); RECIST(Response Evaluation Criteria in Solid Tumours); mRECIST (modified RECIST); \* TRAVERSE trial

**Table 5 – Ongoing HCC-directed clinical trials using oncolytic viruses.** Searches were performed on ClinicalTrials.gov, Current Controlled Trials and EU Clinical Trials Register.

<b>Virus</b>	<b>Phase</b>	<b>No of patients</b>	<b>Route</b>	<b>Study Design</b>	<b>Primary objective(s)</b>	<b>Progress</b>	<b>Trial identifier</b>
JX-594	2	21	IV	Single treatment group 5 x weekly infusions	Tumour response	Enrolment completed	NCT01636284
H101 recombinant human adenovirus type 5	3	120	HAI	Randomisation to adenovirus and TACE or TACE only	Overall survival	Recruiting	NCT01869088
VSV-hIFN- $\beta$	1	48	ITu	Modified "3+3" Fibonacci dose escalation	Maximum tolerated dose	Recruiting	NCT01628640

HAI (hepatic artery injection); TACE (trans-arterial chemo-embolization);

Figure  
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