Review

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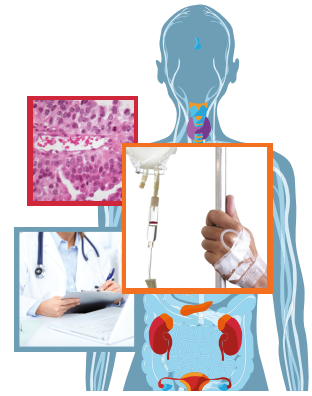

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Oncolytic virotherapy for anaplastic and poorly differentiated thyroid cancer: a promise or a clinical reality?

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Oncolytic viruses (OVs) selectively infect and lyse cancer cells. A direct lytic effect of OVs has been theorized in the initial studies; however, the antineoplastic effect of OVs is also due to the induction of an immune response against cancer cells. Anaplastic thyroid cancer is one of the most aggressive human malignancies with a short survival time of about 6–12 months from the diagnosis. The lack of effective therapies has prompted to investigate the efficacy of OVs in anaplastic thyroid carcinoma. Different OVs have been tested in preclinical studies, either as single agents or in combinatorial treatments. In this review, the results of these studies are summarized and future perspective discussed.

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Oncolytic viruses (OVs) are used to treat cancer due to their ability to selectively infect and lyse cancer cells, without harming normal cells or tissues [1].

Several viruses, either naturally occurring or developed through genetic engineering, are currently under investigation as oncolytic agents in clinical studies [2,3].

Currently, three OVs have been approved for cancer treatment. Rigvir, a wildtype nonpathogenic enteric cytopathic human orphan type 7 (ECHO-7) has been granted approval by the Latvian regulatory agency for the treatment of melanoma in 2004 [4]. However, this virus has not met the required regulation standard for European or US approval for clinical use.

In 2005, the oncolytic adenovirus H101 has been approved by PR China's State Food and Drug Administration for the treatment of head and neck cancer, and, so far, a large number of patients has been treated [5].

In 2015, a herpes simplex virus (HSV) 1 expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) named talimogene laherparepvec (Amgen, Inc., CA, USA) was approved by the EMA (European Medicines Agency) and US FDA for the treatment of advanced melanoma [6], finally establishing OVs as a new class of cancer therapeutic agents in the US and Europe.

Thyroid malignant neoplasm is a common endocrine malignancy, most thyroid carcinomas are well-differentiated carcinomas, belonging to the papillary or follicular histotype. Papillary thyroid carcinoma comprises 80–85% of all thyroid neoplasms, follicular thyroid carcinoma accounts for approximately 10–15% of cases. Papillary thyroid carcinoma and follicular thyroid carcinoma belong to the low-risk group, have a low recurrence rate (5%), a good prognosis and have an excellent survival rate (98%). Poorly differentiated thyroid carcinoma (PDTC) accounts for approximately 0.5–7% of all thyroid cancers. The least common histotype is the anaplastic thyroid carcinoma (ATC), accounting for approximately 2% of all thyroid malignancies. PDTC exhibits intermediate aggressive clinical characteristics between well differentiated thyroid carcinoma and ATC, with a 5-year disease-free survival and overall survival in 43 months. PDTC are often positive for thyroglobulin. ATC has a very poor prognosis, with a mean survival time of 2–6 months for ATC. ATC occurrence is characterized by a rapid tumor growth, trachea obstruction with respiratory distress and frequent distant metastases. ATCs are negative at immunohistochemical

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analysis for thyroid transcription factor-1, and thyroglobulin. Currently, treatments for both PDTC and ATC are still challenging. Multimodal treatment, surgery, chemotherapy and radiation obtained a very limited success [7–10]. The need for novel, effective therapies of ATC has prompted the investigation of OVs efficacy in ATC experimental models since 2001 [11]. The data obtained by different groups sustain the potential clinical efficacy of this treatment. Emerging evidences of the immune-stimulatory property of OVs against tumor cells and the immunosuppressive nature of ATC microenvironment further support the clinical use of OVs for ATC treatment.

In this review we will discuss the results of studies evaluating the efficacy of OVs in ATC models and the experimental approaches aimed to increase the efficacy of OVs against ATC cells.

Viruses & cancer treatment: a 100-year-old story

In early 1900s, studies reported cancer remissions after naturally acquired viral illnesses and the use of viruses for cancer treatment was hypothesized [12–14]. However, the lack of adequate technologies and knowledge in viral life cycle and pathogenic activity have been a hurdle for the clinical use of viruses.

Early clinical trials performed between 1950 and 1970 confirmed that viruses possessed an innate ability to kill cancer cells; however, dreadful side effects, such as hepatitis or neuroencephalitis, were also observed [12–14].

The interest in OVs was renewed in 1990s when the technological advancement together with a better understanding of the molecular alterations in cancer cells allowed manipulation of the viral genome [15] and the development of viruses selectively targeting tumor tissue with a reduced toxicity. In 1991, a landmark study reported that a modified HSV-1, with a mutation in the *TK* gene (TK-mutated) and with reduced neurotoxicity, replicated selectively in cancer cells and was active against glioma in mice brains [16].

In same years, another OV has been engineered: *dl*1520 (ONYX-015). *dl*1520 is an oncolytic adenovirus that carries the deletion of the *E1B55K* gene. The lack of E1B55K product makes the virus defective in p53 binding/degradation and nuclear export of viral mRNA, functions that are essential for viral propagation in normal cells [17,18]. In the great majority of cancer cells, the *E1B55K*-deletion is complemented by functional inactivation of p53 and alterations in mRNA export mechanisms. *E1B55K* is required for other important functions, and it has been observed that its deletion greatly impairs viral replication explaining the modest activity as single agent reported in clinical trials [18].

*dl*1520 was the first virus to enter Phase I clinical trials. Eighteen Phase I and II clinical trials were performed with *dl*1520 and safety was demonstrated with all routes of administration [19]. No Phase III clinical trial has ever been completed, due to legal issues , but in 2005 H101 an adenovirus with the same deletion of *dl*1520 was licensed in PR China for the treatment of head and neck cancers [5]. The approval was based on the results of a Phase III trial, with a 79% response rate in patients receiving H101 by intratumor administration in combination with cisplatin and 5-fluorouracil, while a 40% response rate was reported in control studies without virus treatment [5,20].

Overall, the clinical results obtained with *dl*1520 and H101 have been pivotal in demonstrating the safety profile of OVs, and paving the way for the development of more effective OVs.

Novel and more effective OVs were developed, employing different strategies and viral strains. In 2015, an HSV-1 expressing GM-CSF called talimogene laherparepvec has been approved by EMA and FDA for the treatment of advanced melanoma [6]. Other OVs (https://clinicaltrials.gov/ClinicalTrials.gov Identifiers: NCT03206073, NCT03294486, NCT03225989) are currently under clinical evaluation and the number of OVs approved for cancer treatment is expected to increase in the near future.

Recently, the interest toward OVs has been increased by observations showing that OVs exert potent immunostimulatory effects against tumor cells, and nowadays OVs are considered as immunotherapeutic agents [21]. The rational combination of OVs with immunotherapeutic checkpoint inhibitors is another relevant pro for their clinical use [22].

Tumor selectivity of OVs

OVs can be divided into two broad groups: OVs with a natural tropism toward cancer cells and OVs genetically engineered to selectively replicate in cancer cells. For both groups the deficiency in antiviral response in cancer cells is a crucial mechanism of tumor selectivity. In normal cells viral infection elicits the release of IFNs, produced following the detection of viral RNA, DNA or proteins by intracellular pathogen recognition receptors. This signaling pathway results in the expression of hundreds of effector genes known as IFN-stimulated genes. These genes are the basis for an elaborate effector mechanism aiming to the clearance of the viral infection [23,24]. Moreover, TLR activation due to the recognition of viral elements activate downstream pathways, leading to PKR

activation [23]. Phosphorylated PKR blocks protein synthesis preventing viral replication. Neoplastic cells have been shown to have an abnormal IFN pathways, and/or abnormal PKR activity, making them more susceptible to viral infection [24,25].

Selective OVs in thyroid cancer models

OVs with natural selectivity

Some viruses have a natural selectivity toward cancer cells (natural OVs), exploiting the aberrant signaling pathways that sustain cancer growth. For instance, constitutively active AKT pathway signaling serves as a sustained growth and survival signal in many different types of cancer [26]. Wang and colleagues demonstrated that the natural tropism of myxoma virus in cancer cells capitalizes on endogenous AKT activity via complex formation between AKT and M-T5, a myxoma viral protein [26]. A myxoma virus MYXV has been tested in a preclinical study for the treatment of ATC, although with a limited success in cell killing and in productive infection of ATC cells [27].

Vaccinia virus (VACV) shows a natural selectivity for tumors. The abnormal vascular structure of tumors likely represents the major determinant of the tropism [28]. Further selectivity for VACV can be achieved through the deletion of the *TK* gene, involved in nucleotide synthesis, limiting viral replication to nucleotide rich cancer cells [29,30]. Among VACVs, the most effective OV is JX-963. This virus has been engineered to target cancer cells presenting activation of the transcription factor E2F and the EGFR pathway, by deletion of the TK and vaccinia growth factor genes. It also expresses the human GM-CSF.

JX-963 has already been tested in a wide range of human cancer cell lines and in clinical trials [31].

GLV-1h68 is a replication-competent VACV targeting tumor cells. It bears mutations in two loci: *F14.5L, J2R* (encoding thymidine kinase) and *A56R* (encoding hemagglutinin). This virus also carries marker genes (*Renilla luciferase*, *GFP* and *β-gal*) that allow imaging of tumor-specific entry and replication [32]. GLV-1h68 has been tested in ATC cells and tumor xenografts, showing a significant inhibition of tumor growth after a single administration [33,34].

A variant of GLV-1h68 carrying *hNIS* symporter gene was developed showing re-expression of hNIS in ATC cells [35]. hNIS expression could be useful both as a marker gene for the tracking of the virus and as a potential sensitizer to radiotherapy using Iodine-131. A recombinant variant of tanapoxvirus has been used as OV. Tanapoxvirus is an attractive candidate for virotherapy, infected patients experience a mild and self-limiting febrile illness, no transmission from person to person has been observed [36]. Also for tanapoxvirus, ablation of the viral thymidine kinase has been used as a strategy to increase cancer cell selectivity. A tanapoxvirus has been already tested in studies involving thyroid cancer cells [27].

Other natural OVs include oncolytic attenuated measles virus (MV) [37], Mumps virus [38] and Newcastle Disease Virus [39]. The natural selectivity of these viruses might be related to the overexpression of surface molecules acting as viral receptors on cancer cells. Wild type and attenuated MVs have different receptors; attenuated MVs have a specific tropism for CD46 receptor. CD46 receptor protects cells from complement attack blocking the complement cascade and serving as co-factor for inactivation of C3b and C4b, thereby protecting neoplastic cells from complement mediated lysis [40]. Overexpression of CD46 has been observed in most human cancers including thyroid carcinomas [41].

In cells showing a high expression of CD46 receptor, common feature of cancer cells, infection with attenuated MV leads to an extensive intercellular fusion (cytopathic effect) resulting in the killing of cancer cells. Interestingly, levels of CD46 in cancer cells can predict the efficacy of oncolytic treatment with MV [42].

An attenuated MV, vaccine strain MVEdm, has been used in studies with thyroid carcinoma models. This virus is active against a broad range of human cancer cells and has been modified to include the *NIS* gene (MV-NIS), to allow infected cells to transport iodide ions into the cell. The inclusion of the *NIS* gene in the viral construct will render radioiodine-resistant ATC susceptible to radioiodine therapy.

In vitro and *in vivo* experiments demonstrated the oncolytic efficacy of MV-NIS in ATC-derived cell lines. Radioactive iodine uptake along with single-photon emission computed tomography imaging of xenografts confirmed NIS expression both *in vitro* and *in vivo*. Administration of radioiodine, to MV-NIS-treated KTC-3 tumors, showed an increased tumor cell killing [43].

Oncolytic parvovirus selective activity against human cancer cells relies on the intrinsic preference of this virus for proliferating and metabolically altered cancer cells. The oncoselectivity of parvovirus is a complex phenomenon based in part on multiple tumor cell-specific determinants. Molecular pathways involved in H-1 parvovirus (H-1PV) selective tumor targeting are described elsewhere [44,45]. The oncolytic H-1PV have not been tested yet in

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thyroid models. However, studies using H-1PV could be useful not only to test its efficacy against thyroid cancer cells, but also to evaluate the interactions between immune response and viral treatment in thyroid carcinomas. Indeed, the lack of immune competent animal models has prevented a better understanding of these interactions. The natural host of H-1PV is the rat and the virus is effective against rat tumor cells. Rat thyroid cell lines transformed with oncogenes involved in thyroid carcinogenesis and tumorigenic in syngeneic animals are available together with rat models of poorly differentiated and metastatic thyroid carcinoma [46–48]. These experimental models could be useful to analyze the immune stimulatory functions of H-1PV.

Tumor selectivity by viral genome modifications

Genome modification has been an important strategy to obtain a selective viral infection/replication in tumor cells. Two major strategies were exploited: re-targeting the selectivity of the viruses, and controlling the selectivity of replication *via* viral gene inactivation, transcriptional targeting, microRNA targeting sequences [1,49,50].

Here, we will discuss only the strategies regarding viruses used in studies focused on thyroid carcinomas.

The oncolytic adenovirus *dl*1520, bearing a *E1B55K* gene deletion, was the first OV used in thyroid carcinoma cell lines and ATC xenografts [11]. *dl*1520 showed to be active as single agent; however, its efficacy was increased when used in combination with doxorubicin, paclitaxel, ionizing radiation and lovastatin [51,52]. The results obtained in these preclinical studies have demonstrated the potential efficacy of *dl*1520 in combination with antineoplastic agents for the treatment of ATC.

The experience acquired with *dl*1520 led to the development of oncolytic adenoviruses with small specific gene deletions to retain both viral potency and tumor selectivity. By deleting the *E1ACR2* domain (24 amino acids), viral replication can only proceed in cells with deregulated growth control, with altered G1/S checkpoint and pRb/Ras signaling [53], found in the majority of cancer cells [54]. *dl*922–947, an adenoviral mutant with the *E1ACR2* deletion, showed a significantly higher efficacy than *E1B55K*-deleted variants.

*dl*922–947 has been extensively studied in thyroid carcinoma models alone or in combination with other agents, in other words, anti VEGF humanized antibody bevacizumab, ionizing radiation and other agents [55–58].

A conditionally replicative adenovirus (named 'HILMI'), replicating specifically in cells with an active Wnt/β-catenin pathway, was also developed.

HILMI replicates in cells by virtue of TCF response elements driving the expression of adenoviral genes *E1A* and *E1B*. A fraction of undifferentiated or poorly differentiated thyroid cancers contain mutations in *β-catenin* gene. Thyroid cancer cell lines, derived from undifferentiated or anaplastic carcinomas and with an active Wnt/β-catenin pathway, were efficiently killed by HILMI; indeed, prolonged survival of mice with ATC tumors was observed following HILMI administration [59].

ONYX-411 is a conditionally replicative oncolytic adenovirus tested in thyroid cancer experimental models. In particular, the expression of the *E1A* and *E4* genes, are under the control of the human E2F promoter, as E2F levels are high in tumor cells, this promotes viral replication. To prevent adenoviral-induced cell cycle progression in normal cells, the *E1A* gene of ONYX-411 was modified by deleting CR2 domain impairing pRB binding. ONYX-411 was shown to target and destroy ATC cell lines and reduced the growth of xenograft tumors *in vivo*.

Adenoviral mutant *dl*309 lacking several *E3* region genes *dl*309 was tested in human K1 cells derived spontaneously from a papillary thyroid carcinoma. E3 region proteins have a prominent role in immune evasion and prevent innate immunity. *dl*309 has enhanced cytopathogenicity compared with Ad5wt in range of human and mouse cells. To better establish viruses in inducing cell death, tumor-derived cell lines that have not undergone extensive passage were used. The K1 cell line are non-tumorigenic, contact-inhibited and retain DNA damage checkpoints [60]. Authors observed high levels of apoptosis in K1 infected cells likely correlated to the expression of viral gene *E1A*. However, *dl*309 virus has not been tested against tumorigenic ATC cell lines nor was tested its efficacy to stimulate an innate or acquired immune response against cancer cells.

Different OVs other than adenoviruses, were tested in thyroid carcinoma models. G207 is a conditionally-replicative HSV-1 with *ICP34.5* deletion and *UL39* disruption [61–63]. ICP34.5 is a neurovirulent factor that induces the dephosphorylation of the translation initiation factor eIF-2A, ultimately counteracting the host shutoff of protein synthesis. The *UL39* gene encodes for the large subunit of the ribonucleotide reductase (ICP6) required for viral DNA synthesis [64]. The selectivity is due to higher levels in cancer cells of the ribonucleotide reductase (an ICP6 cellular homolog that could provide the missing function) [65].

G207, combined with paclitaxel, showed synergistic cytotoxicity against ATC cells [66].

A variant of G207 expressing *Escherichia coli* LacZ (NV1023) has been also tested in thyroid models [67].

Table 1. Oncolytic viruses used in thyroid carcinoma models. Oncolytic viruses (adenoviruses, poxviruses and herpes viruses) have been tested in experimental models of thyroid carcinoma with interesting results. Data should be confirmed in orthotopic thyroid carcinoma models in order to better assess viral diffusion within the tumors.

Virus family	Virus type	ATC cell line	Xenograft study	Ref.
Poxvirus	Vaccinia virus Myxoma virus Tanapox virus	SW1736, U-HTh7, C643, THJ-11T, THJ-16T, THJ-21T, and THJ-29T, 8505C, ASH3, KMH2, BHT-101, Cal 62	No	[36]
	GLV-1h68	8505C, 8305C, KAT4C, KAT4, KAT18, DRO90-1	No	[42]
	GLV-1h68	8505C,DRO90-1	Yes	[43]
	GLV-1h153	8505C, 8305C, FRO	Yes	[44]
Adenovirus	ONYX-015	ARO, KAT4, FRO	Yes in combination with radiotherapy	[14,69]
	ONYX-015	ARO, KAT4, FRO, Cal62	Yes in combination with lovastatin	[60]
	*dl*922-947	ARO, FRO, KAT4	Yes in combination with bevacizumab	[70]
	dl922-947	BHT-101-5, FRO, Cal62	Yes in combination with ionizing radiation	[71]
	dl922-947	BHT-101-5, FRO, Cal62. 8505C	Yes in combination with AZD_{1152}	[72]
	dl922-947	BHT-101-5, FRO, Cal62	Yes in combination with olaparib	[73]
Herpes virus	NV1023	DRO90-1, ARO, KAT-4C, KAT-18	Yes	[74]
	G47Δ	ARO, FRO	Yes	[75]
	G207	DRO90-1, KAT-4	Yes in combination with paclitaxel	[76]

ATC: Anaplastic thyroid carcinoma.

G47Δ is another modified herpes OV with a deletion in the *γ34.5* gene encoding ICP34.5 protein and with the insertion of the *Escherichia coli LacZ* gene inactivating the *ICP6* gene. G47Δ demonstrated a greater replication capability and a higher antitumor efficacy than G207. G47Δ exhibited a significant efficacy against ATC xenografts [68].

In Table 1 are summarized the studies performed with OVs in anaplastic or poorly differentiated thyroid carcinoma models.

Mechanisms of action of OVs

Activation of cell death pathways by OVs & immune response

The mechanisms of action of OVs are not completely understood. A direct lytic effect has been theorized in the initial studies. More recent studies have proved indirect effects of OVs. These include the induction of innate and adaptive immune response against cancer cells and the re-shaping of tumor microenvironment (TME), (Figure 1) such as by inhibition of tumor angiogenesis [70,71]. All these mechanisms effectively contribute to the antitumoral effects [1].

The neoplastic cells in response to the infection of OVs activate different cell death pathways depending on the virus type, the cell type or a combination of both [72]. Cell death pathways include apoptosis, pyroptosis (caspase-1-dependent cell death), autophagic cell death, immunogenic cell death (ICD) and necrosis. The cell death pathway(s) activated by the infection with OVs, excluding apoptosis, are highly immunogenic [73,77].

ICD is characterized by the release of cytokines, tumor-associated antigens and other danger signals, including damage-associated molecular pattern molecules and pathogen-associated molecular pattern molecules, stimulating an immune response against infected and noninfected cancer cells [73,77]. Dying cells release a variety of cytokines into the local environment, such as IFNs, TNF-α and interleukins that further promote immune response [78–80].

Due to the cell-type dependent effects of the OVs on the activation of cell death pathways it is necessary to assess the effects of a specific virus in the context of the neoplastic cell type. Several studies have evaluated the effect of virotherapy in ATC cells. In particular, *dl*922–947 induced the activation of a programmed cell death that shares some features with apoptosis [56–58]. However, the treatment with caspase inhibitor zVAD-fmk was not able to modify cell survival, suggesting the involvement of a non-apoptotic pathways in *dl*922–947-induced death of ATC cells [58]. Authors have also excluded the activation of autophagy or the death by mitotic catastrophe [57]. The activation of a PARP-mediated cell death (parthanatos) in ATC cells infected with *dl*922–947 has been also

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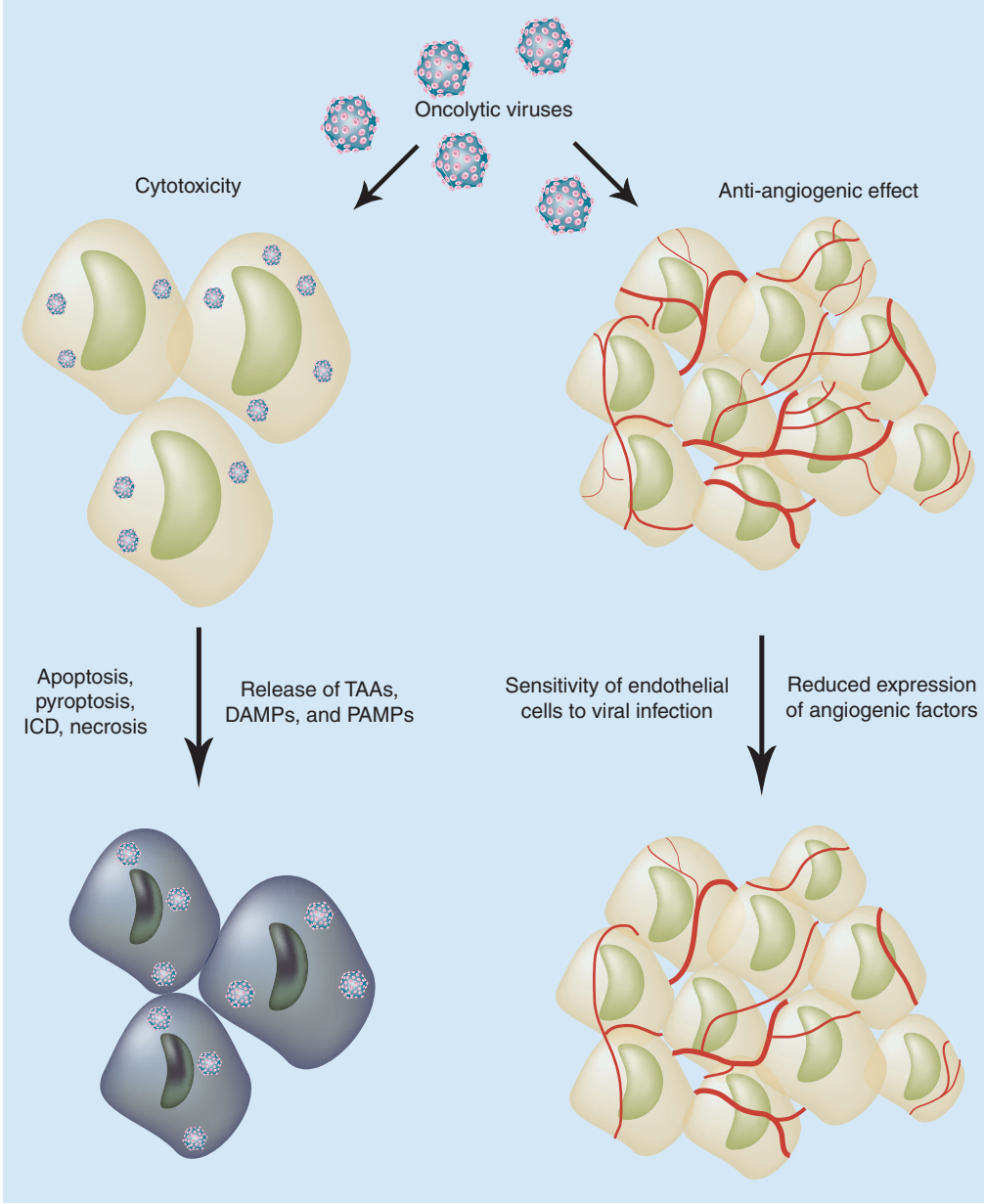

Figure 1. Effects of oncolytic viruses on tumor microenvironment in ATC.

analysed. AIF is a key mediator of parthanatos: upon PARP-1 activation, AIF translocates from the mitochondria to the nucleus. ATC cells infected with *dl*922–947 did not show any AIF translocation, leading to the exclusion of parthanatos activation [58].

So far, the involvement of pyroptosis or ICDs has not been evaluated in ATC cells infected with *dl*922–947, although the assessment of the cell death pathway activated by *dl*922–947 in ATC cells would be helpful to develop rationally-based combinatorial treatments.

The involvement of immune response in the antitumoral activity of OVs is now accepted. However, most of the preclinical studies on OVs have been performed using immunodeficient mice and human xenograft. The use of these models limits the study of the immune response, especially the adaptive response. Moreover, the lack of immune competent animal experimental models (lack of availability of mouse syngeneic cancer cells to engraft), or the natural tropism of the virus for human cells, but not mouse cells, represent a hurdle to these studies.

Nevertheless, experiments to address the immune response in ATC experimental tumors have been performed. In ATC xenografts (lacking the T cell-mediated immunity), *dl*922–947 adenovirus reduces the production of the monocyte attracting chemokine CCL2 and tumor-associated macrophage (TAM) density *in vivo*. TAM depletion in *dl*922–947-treated tumors is associated with increased expression of *Nos2*, a marker of pro-inflammatory M1 macrophages [81]. Moreover, an increased expression of *Ifng* mRNA encoding the protein IFNγ was observed. IFNγ may induce a switch toward a pro-inflammatory macrophage M1 phenotype [64]. However, this model is inadequate to fully recapitulate the mediated anti-tumor response elicited by virotherapy. A confirmation in an alternative immune competent model is probably required, although the preclinical studies performed with this virus are already sufficient for a clinical trial that could contribute to clarify this issue.

Modulation of TME by OVs

An effect on TME has been described for several OVs, including VACVs, parvovirus and adenoviruses in preclinical models and in clinical studies [81–83]. It involves the targeting of tumor-associated endothelial cells leading to a rapid destruction of the tumor vasculature and loss of perfusion within the tumor. The mechanisms underlying this process are poorly understood. Studies have shown that despite apparent profound and rapid loss of tumor perfusion, complete responses remain elusive and tumor regrowth occurs, suggesting a revascularization and neo-angiogenesis of treated tumors [82,83].

Oncolytic vaccinia virus possesses an anti-angiogenic potential and this effect can be exploited for synergistic combination with different antitumoral agents [83].

Proliferating human umbilical vein endothelial cells (HUVEC) and immortalized Kaposi sarcoma-derived human endothelial cells (KS-IMM) are sensitive to OV H-1PV. Although the cells undergo an abortive infection which does not lead to viral progeny production and spread, the infection of highly angiogenic KS-IMM cells significantly reduces tumor-forming ability after sub-cutaneous implantation and the infection inhibits the expression of the key angiogenesis factor, VEGF [74].

A significant reduction in microvessel density and a decrease in *CD31* mRNA (marker of endothelial cells) expression was observed in ATC xenograft tumors, confirming an innate anti-angiogenic effect of *dl*922–947 [58]. Another study not only confirmed this effect in ATC xenografts but further showed that *dl*922–947 treatment of ATC cells impairs the secretion of the pro-angiogenic chemokine IL-8 [81]. These findings were substantiated by *in vivo* results, showing that *dl*922–947 treatment of tumor xenografts established in athymic mice reduces *IL8* and *Cd31* mRNA level and overall the tumor microvascular density.

Combination therapies with OVs

OVs & chemotherapeutic drugs

Despite the efficacy of OVs against cancer cells, it is unlikely that tumor eradication could be obtained using OVs as single agents. In the initial studies, the combination of OVs with standard chemotherapeutics has been tested as a strategy to increase their efficacy [75]. The development of novel and more effective OVs, together with a better understanding of the interactions between OVs and tumor cells, has led to combinatorial treatments based on rational approaches [84].

The oncolytic adenovirus ONYX-015 has been tested in association with doxorubicin and paclitaxel against ATC cells. The combined treatment activated cell death more efficiently with respect to single treatments, likely because *E1A* gene products act on cell death pathways activation [11]. Lovastatin, a cholesterol-lowering drug also acting as an inhibitor of p21/ras activity, was used in conjunction with *dl*1520 to improve viral cytotoxicity. This combination increased the death of ATC cells and viral replication [52].

An obstacle to an efficient infection of tumor cells by OVs is represented by the abnormal tumor vascular structure and by the increased interstitial tumor pressure [1]. To improve viral distribution and diffusion, *dl*922–947 has been used in combination with bevacizumab, a humanized anti-VEGF antibody. The treatment of ATC xenografts with bevacizumab, prior to virus administration, normalized the vascular structure, therefore reducing interstitial pressure and allowing for a more efficient distribution of the virus within the tumor [55].

A typical feature of ATC cells infected by *dl*922–947 is represented by the increase of DNA content (>4N) and the accumulation of the cells in the G2/M phase of the cell cycle [77]. This effect is probably due to the abrogation of multiple cell cycle checkpoints by viral proteins. Indeed, *E1B* gene products prevent cell death and in combination with *E4*-gene products block the activation of the cell cycle checkpoints. Libertini *et al.* hypothesised that the block of the mitotic checkpoints could enhance the efficacy of *dl*922–947 [58]. The block of mitotic kinase Aurora B,

that is overexpressed in ATC, reduces the tumor growth [85]. A specific Aurora B kinase inhibitor (AZD1152) has been used in combination with *dl*922–947 showing additive/synergistic killing effects and a significant inhibition of tumor growth [57].

DNA damage response (DDR) represents a cellular defence against viruses. To hinder viral infection, the viral DNA is processed by the host cell as a damaged DNA. Viruses have evolved strategies to counteract DDR activity, which include degradation or mislocalization of key players involved in DDR [86–88]. In ATC cells, *dl*922–947 triggers an inefficient DDR, with an accumulation of γH2AX, a marker of DNA damage [71]. A specific-inhibitor of ATM (KU55933) increased the replication and the oncolytic activity of *dl*922–947, confirming that the DDR acts as a cellular defense and its inhibition could enhance the effects of OVs [56].

Other DNA repair pathways protect the integrity of the genome. Poly (ADP-ribose) polymerase 1 and 2 (PARP-1 and PARP-2) are key mediators in base excision repair (BER) pathway. PARP-1 is also involved in other DNA damage repair processes, including the repair of DNA double strand breaks [89]. During viral infections, cells are pushed toward S phase inducing the accumulation of single strand breaks (SSBs) as unscheduled DNA synthesis-associated DNA lesions [90]. Therefore, Passaro *et al.* have hypothesized that *dl*922–947 infection could induce PARP-1 activation. A robust protein PARylation in ATC cells infected with *dl*922–947 was observed, confirming their hypothesis. Cells defective in homologous recombination are highly sensitive to PARP-1 inhibition [91], due to an increasing genomic instability and cell death (synthetic lethality). Passaro *et al.* further demonstrated that *dl*922–947 impaired DDR and the combined treatment of *dl*922–947 with a specific PARP inhibitor (Olaparib) was effective against ATC cells. Indeed, this treatment, mimicking synthetic lethality, greatly increased DNA damage accumulation and cell death in ATC cells and tumor xenografts [58].

Ionizing radiation (IR) induces DNA damage and a subsequent block of the cell cycle with accumulation of cells either in G1 or G2 phase of the cell cycle, depending on the integrity of cell cycle checkpoints. Due to capability of oncolytic adenoviruses to greatly interfere with DDR, the association with IR has been tested in ATC models.

In an early study, the combination of ONYX-015 and radiotherapy was tested showing an increased cell killing and a delayed growth of ATC xenograft [51]. However, this initial study was only descriptive and the mechanisms leading to the enhanced cell killing were not investigated. In another study, Passaro *et al.* have shown that the IR combination enhances the effect of *dl*922–947 against ATC cells *in vitro*. In particular, a synergistic cell killing was observed only when irradiation was administered prior to viral infection and the efficacy of the combined treatment was confirmed in ATC xenografts [56].

The preclinical evaluation of OVs in combination with other agents for ATC treatment has been performed with oncolytic HSVs.

Lin and colleagues have tested oncolytic herpes viruses G207 and NV1023 in combination with adriamycin or paclitaxel in ATC cell lines [92]. The combination of G207 with paclitaxel showed synergistic effects in both cell lines. Although G207 viral entry and replication were not enhanced by paclitaxel, G207 in combination with paclitaxel significantly increased microtubule acetylation, mitotic arrest, aberrant chromatid separation, inhibition of cell cycle progression and apoptosis. The treatment of athymic mice bearing ATC xenografts confirmed the efficacy of this approach [92]. After the publication of this study it has been discovered that the ATC cell lines (KAT4 and DRO90–1) used by the authors to assess the efficacy of G207 in combination with paclitaxel, do not originate from thyroid carcinoma, and therefore it would be useful to confirm in cells of proved thyroid origin [93].

In Table 1 are summarized the combination studies performed with OVs.

Although the data obtained using OVs in combination with antineoplastic agents are encouraging, it is not possible to exclude that other OVs might display a superior cell killing in combinatorial treatments. Moreover, additional data might be useful for future clinical studies, such as the use of orthotopic models of ATC and *in vivo* imaging experiments to gain more data on viral administration, viral diffusion and spread to normal tissues.

Overall, these studies have confirmed that combinatorial approaches hold a potential for the treatment of ATC, suggesting a clinical evaluation. Currently used chemotherapeutic agents, such as doxorubicin and paclitaxel, have been tested in combination only with ONYX-015 and G207 and the results obtained in these studies need a confirmation with more potent OVs [11].

Other specific inhibitors have not been tested or are not used for the treatment of solid tumors [56–58], making the clinical evaluation of these combinations less likely.

PARP inhibitors are already used for the treatment of ovarian cancer patients with BRCA2- gene mutations and a clinical evaluation of the combined treatment with *dl*922–947 could be envisaged [58]. Finally, multimodality treatment of ATC already includes IR, therefore clinical evaluation of this rationally based combination is feasible [58].

Future perspective

Cancer immunotherapy is a clinically validated treatment for many solid tumors. Immunotherapy includes cancer vaccines, adoptive transfer of activated T or NK cells, the use of CAR T cells, OVs and antibodies blocking the so called immune checkpoint inhibitors, such as anti-CTLA4 and anti-PD-1/PD-L1 antibodies [94,95].

Immune checkpoint molecules are used by the immune system to maintain homeostasis, for instance, for the prevention of pathologic autoimmunity. Conversely, in tumors these signals are upregulated, allowing tumors to evade protective immune responses [95]. Immune checkpoint antagonists have been approved for the clinical use against different lesions [96]. However, it is accepted that this approach works better in neoplastic lesions with an existing antitumor immune response, whereas tumors with a low expression of PD-1/PDL-1 and minimal immune cells infiltration are less responsive. Moreover, even in neoplastic lesions such as melanoma, where checkpoint inhibitors have proved to be effective as single agents, only 20–25% of patients exhibit a durable response [96]. However, up to 50% tumor regressions have been observed using anti CTLA-4 in combination with anti PD1 [97], indicating that immune checkpoint inhibitors should not be used as single agents and suggesting their use in combination with other agents capable to stimulate a robust immune response against neoplastic cells.

Taking into account the capability of OVs to induce an immunogenic cell death and the subsequent activation of innate and/or adaptive immune response [77,78], OVs and immune checkpoint blockade could synergise in stimulating the immune system against the tumor [98]. Indeed, OVs treatment could stimulate the recruitment of effector T cells into the TME, whereas immune checkpoint blockade may sustain the potency of tumor infiltrating lymphocytes, TAM with an inflammatory M1 phenotype and NKs via the removal of inhibitory signals [95].

The efficacy of this strategy has been confirmed in several studies in different types of tumors [99].

ATC tissue is composed of about 60% of TAMs mixed with cancer cells and the increased number of TAMs in poorly differentiated carcinomas is associated with a decreased survival [100], probably reflecting an immunosuppression and trophic activity (M2 type) in response to Th2 cytokines (i.e., IL-10, TGF-β) [64]. Overall, in aggressive thyroid tumors a protumoral role of the infiltrating immune cells has been already established. Moreover, ATC cells express high levels of the enzymeindoleamine 2,3-dioxygenase (IDO1) that catalyzes the conversion of the amino acid tryptophan to the immunosuppressive molecule kynureine, thus causing halted growth of T cells [101].

PD-L1 expression is a potential biomarker to predict the response to anti-PD-1 or anti-PD-L1 agents [102]. In thyroid carcinomas a significant increase in membrane PD-L1 positivity was correlated with high risk of aggressive disease, distant metastasis or death. The combined cytoplasmic and membrane PD-L1 positivity was also associated with poor prognosis [103]. In another study PD-L1 has been shown to be highly expressed in a subset of patients with ATC [104].

Overall, these findings suggest a role for immunotherapeutic agents in patients with refractory thyroid cancer [105].

A potential immunostimulatory activity of *dl*922–947 has been shown in ATC models, thus making this virus an attractive candidate for combination treatments with anti-CTLA-4 and anti-PD-1/PD-L1 antibodies.

It is worth to note that the efficacy of *dl*922–947 has been assessed in immunocompromised animal models, obtaining only data suggestive of an NK cell activation [81]. Indeed, the use of immunocompromised models precludes to study the role of the immune adaptive response in adenoviral virotherapy, therefore data obtained in immune compromised models need to be confirmed in immune competent animals.

The studies in an immune competent setting require the availability of the specific murine cancer cell lines. Unfortunately, regarding the ATC only a limited number of cell lines are available [106,107]. Moreover, the use of murine cells encounters another problem when using viruses with human tropism. In fact, human-tropism viruses (i.e., adenoviruses, HSVs, etc) poorly replicate in mouse cell line, leading to an underestimation of the oncolytic effect of the virus. The use of more sophisticated immune competent models such as the humanized model is desirable. In humanized setting, the mice can be engrafted both with human cancer cells and human immune cells. However, this system is both challenging and expensive.

Alternatively, *in vitro* experiments may allow to investigate the effects of *dl*922–947 and immune checkpoint inhibitors on the stimulation of human NK cells, monocyte and monocyte-derived macrophages. Although these studies do not entirely recapitulate the complex interaction of the tumor microenvironment, data could be sufficient to understand the potential therapeutic efficacy of the combination.

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Conclusion

The lack of effective therapies for ATC emphasizes the need for novel treatments and the use of OVs was hypothesized since initial studies with ONYX-015 [11,51,52], and OVs could be helpful in improving disease control and survival of ATC patients.

Several issues remain to be addressed, such as which OV will be the more effective for ATC treatment and/or which administration route (intratumoral or I.V.) should be used. The effects of the OV-based treatments on distant metastases and the efficacy of OVs in combination also require assessement.

Clinical trials could be helpful to address the open questions, however, ATC is a rare lesion, found in only 2% of thyroid tumors, and most of the patients have a short survival time, making a clinical study difficult [10]. A compassionate treatment of ATC patients with *dl*922–947 could help in gaining clinical and efficacy data.

Although no clinical trials based on OVs for the treatment of ATC are in progress, viruses have already been used in patients with thyroid carcinoma. Indeed, in clinical trials aiming for the evaluation of OVs against solid tumors, thyroid carcinoma patients have been treated. These trials were not designed for the treatment of ATC and the virus was not previously tested in ATC models. In a Phase II clinical trial investigating, in patients with advanced cancers, the effects of intratumoral administration of a reovirus (REOLYSIN®) in combination with low-dose radiation, a single patient with thyroid carcinoma was enrolled and a partial response was observed [76].

Viruses with different mechanisms of action with respect of OVs have been also tested in clinical trial for thyroid carcinoma. VB-111 is a nonreplicating adenovirus [108], expressing a Fas-chimera transgene under the control of murine preproendothelin promoter that is active only against tumor vasculature. VB-111 is currently under evaluation in a Phase II clinical trials (ClinicalTrials. gov Identifier: NCT01229865, VB-111) in patients with advanced differentiated thyroid cancer. In this study, 44% of patients experienced tumor regression. Despite the lack of replication of VB-111 the results of this study could be useful for the future clinical use of replicating OVs, although the different mechanism of action of VB-111 does not allow to obtain conclusive indication on the therapeutic potential of OVs in ATC patients. Overall, these data obtained treating thyroid carcinoma patients with viruses are important for future studies.

Several OVs have been evaluated in preclinical studies, among these the adenoviral mutant *dl*922–947 is the most studied in ATC models, and its efficacy has been demonstrated in combination with different agents, including IR, making this virus a promising candidate for clinical studies [56–58,81].

In conclusion, the concept of OV-based cancer therapy is now shifted from the selective killing of cancer cells to immunostimulatory agents, opening a novel operational scenario and to an efficient coupling with immune checkpoint inhibitors. The efficacy of OVs has been clearly confirmed in the ATC preclinical scenario, strongly supporting its clinical use for the treatment of ATC.

Executive summary

- Oncolytic viruses (OVs) selectively infect and lyse cancer cells.
- Three OVs are used for cancer treatment in the world and other OVs are currently under investigation in clinical studies.
- T-VEC (talimogene laherparepvec) has been approved in Europe and the USA.
- OVs can be considered as immunostimulatory agents and their use in combination with inhibitors of immune checkpoints is under evaluation with promising results.
- Anaplastic thyroid carcinomas are resistant to currently available therapies and require more effective treatments.
- Preclinical studies have demonstrated the potential benefits of OVs against ATC *in vitro* and *in vivo*, both as single agents or in combinatorial treatments.

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