Virus Research 190 (2014) 53–59

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

# The control of anaplastic thyroid carcinoma cell lines by oncolytic poxviruses

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ARTICLE INFO

*Article history:*
Received 28 March 2014
Received in revised form 7 July 2014
Accepted 7 July 2014
Available online 16 July 2014

*Keywords:*
Anaplastic thyroid cancer
Oncolytic
Vaccinia virus
Myxoma virus
Tanapox virus

ABSTRACT

*Background and significance:* Anaplastic thyroid cancer (ATC) is rare, but its clinical presentation is often dramatic and aggressive and is nearly uniformly fatal. Oncolytic viral therapy is a potential strategy to improve outcomes for patients suffering with this disease.
*Methods:* Seven established ATC cell lines were infected with a panel of poxviruses to identify which virus had the most potential as an oncolytic agent. These included myxoma, vaccinia, and tanapox viruses, all modified to express green fluorescence protein (GFP). Viral proliferation was assessed by fluorescence and viral amplification. The effect on cell line growth was assessed by the Presto Blue metabolic assay and a live-dead assay. A replication assay was performed to assess the production of infectious progeny. An additional five ATC cell lines were tested using the assays described above for susceptibility to vaccinia virus.
*Results:* ATC cell lines were differentially susceptible to each virus. Vaccinia virus was superior to myxoma and tanapox viruses for the control of anaplastic thyroid cancer *in vitro*. Although subsequent investigation using an expanded panel of cell lines revealed differential susceptibility to vaccinia virus, effective control of cell proliferation was still achieved using higher titers.
*Conclusions:* Vaccinia virus was the most potent of the tested poxviruses and was highly effective in controlling proliferation and inducing cell death in ATC cell lines. The efficacy of these viruses offers hope for improving outcomes for patients suffering with ATC.



## 1. Introduction

The majority of thyroid malignancies are well-differentiated (papillary, follicular, medullary) and have an excellent prognosis (Hundahl et al., 1998). In contrast, anaplastic thyroid cancer (ATC) is perhaps the most aggressive human malignancy, with survival times that are typically counted in months and sometimes weeks (Taccaliti et al., 2012). Although ATC accounts for approximately 2% of thyroid malignancies, it causes 14–49% of thyroid cancer deaths (Hundahl et al., 1998; Kitamura et al., 1999). Its infrequent occurrence is often punctuated by dramatic disease presentation including airway distress, esophageal obstruction, rapid tumor growth and frequent distant metastases. In most cases, this is followed by rapid demise in as little as weeks to months. Surgery, chemotherapy and radiation have been tried alone or in combination with very limited success (Akaishi et al., 2011; Kebebew et al., 2005). Consequently, there is a dire need for novel, effective therapies for these patients.

Oncolytic poxviruses, particularly vaccinia virus (VACV), have demonstrated impressive efficacy *in vitro* and in xenograft models. They also induce partial and complete clinical responses in early phase clinical studies in a multitude of cancer (Russell et al., 2012). Multiple oncolytic viruses including vaccinia virus have been explored as a potential therapeutics for ATC in a preclinical setting

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http://dx.doi.org/10.1016/j.virusres.2014.07.009
0168-1702/

(Gholami et al., 2011; Huang et al., 2007; Reddi et al., 2012). The VACV studies from the same group initially employed VACV that expressed luciferase (Lin et al., 2008). However, clinical trials have not yet been started with these viruses in ATC. Early phase studies with poxviruses from other groups, investigating other cancers have demonstrated impressive partial and complete responses in a subset of patients, however this is often limited to less than 30% of the entire patient cohort (Russell et al., 2012). Given these low response rates, a combination of oncolytic viruses, delivered either simultaneously or sequentially, must be tested to optimize outcomes. We endeavored to study a panel of genotyped ATC cell lines with three different poxviruses (VACV, myxoma virus (MYXV) and Tanapox virus (TANV)) to determine the spectrum of efficacy of each virus. The most effective virus was then studied using an expanded panel of ATC cell lines. We hypothesized that poxviruses will demonstrate oncolytic activity in ATC cell lines and that cell lines less susceptible to one poxvirus would be more sensitive to infection by a different poxvirus.

## 2. Methods

### 2.1. Cell culture conditions

ATC cell lines SW1736, U-HTh7, and C643 were the kind gift of Dr. Nils Erik Heldin (Univ. of Uppsala, Sweden). The cell lines THJ-11T, -16T, -21T, and -29T were obtained from the Mayo Clinic. ATC cell lines 8505C, ASH3 and KMH2 were purchased from the Japanese Collection of Research of Bioresources Cell Bank (JCRB). Cell lines BHT-101 and Cal 62 were purchased from the DSMZ Cell Bank. Vero and owl monkey kidney (OMK) cells were obtained from the American Type Culture Collection and acted as positive controls for infection for VACV (Vero), MYXV (Vero) and TANV (OMK). ATC cells lines SW1736, U-HTh7, C643 and 8505C were grown in EMEM supplemented with 10% FBS (GIBCO) and penicillin (100 IU/mL) and streptomycin (100 μg/mL) (Invitrogen). Cell lines THJ-11T, -16T, -21T, and -29T were grown in RPMI 1640 supplemented with 10% FBS (GIBCO), 1× non-essential amino acids (Wisent), 1 mM sodium pyruvate (Wisent), 10 mM HEPES, penicillin (100 IU/mL) and streptomycin (100 μg/mL) (Invitrogen). BHT101 was grown in DMEM supplemented with 10% FBS (GIBCO), penicillin (100 IU/mL) and streptomycin (100 μg/mL) (Invitrogen) and human serum (Wisent). ATC cell lines Cal 62 and ASH3 were grown in DMEM supplemented with 10% FBS (GIBCO), penicillin (100 IU/mL) and streptomycin (100 μg/mL) (Invitrogen). The cell line KMH2 was grown in a 1:1 mixture of DMEM and RPMI 1640 supplemented with 10% heat-inactivated FBS (GIBCO), penicillin (100 IU/mL) and streptomycin (100 μg/mL) (Invitrogen). Vero and OMK were grown in DMEM/F12 supplemented with 10% FBS (GIBCO) and 400 ng/mL hydrocortisone (Wisent), penicillin (100 IU/mL) and streptomycin (100 μg/mL) (Invitrogen).

### 2.2. DNA extraction from cells and short tandem repeat (STR) profiling

DNA was extracted from cultured cells using the AllPrep DNA/RNA/Protein kit (Qiagen) following instructions provided by the manufacturer. For each cell line, 100 ng of DNA was analyzed by short tandem repeat (STR) profiling at The Center for Advanced Genomics (TCAG, Toronto, Canada). The lines were genotyped with a panel of 16 selected markers including the 8 Combined DNA Index System (CODIS) core STR loci (Lins et al., 1998) employed by the American Type Culture Collection (ATCC). All STR profiling results for the twelve cell lines were compared to the existing literature (Zhao et al., 2011).

### 2.3. Poxviruses

An enhanced green fluorescent protein (EGFP) cassette under the control of a synthetic poxvirus early/late promoter (Chakrabarti et al., 1997) was cloned into each of the following viruses: vaccinia virus (VACV; strain Copenhagen), tanapox virus (TANV; strain Kenya 1950) and myxoma virus (MYXV; strain Lausanne). All EGFP-expressing viruses were obtained from Dr. Grant McFadden, University of Florida, Gainesville, Florida. The construction and characteristics of these viral vectors have been described previously (Barrett et al., 2007; Campbell et al., 2010; Johnston et al., 2003; Nazarian et al., 2007).

### 2.4. Evaluation of infectivity

Cells were seeded ($4 \times 10^4$) into each well of a 48 well dish. Twenty-four hours later the cells had reached a confluence of 85–90% and virus was added at a multiplicity of infection (MOI) of 5, 0.5, 0.05, 0.005 plaque forming unit (pfu)/cell or left uninfected. Virus was allowed to adsorb for 1 h at either 4 °C or 37 °C and then the inoculum was removed. Cells were washed three times and complete medium was added. Cells were evaluated at 24, 48, 72 and 96 hours post infection (hpi). The presence of EGFP fluorescence was visualized by fluorescence microscopy and used as an indicator of viral entry, uncoating and gene expression. Plaque formation and morphology indicated viral replication. This protocol was repeated with VACV on the full panel of twelve ATC cell lines and the cells were evaluated at 48 hpi.

### 2.5. Metabolic assay

Cells were seeded ($4 \times 10^3$) into each well of a 96 well dish. After 24 h when the cells had reached 85–90% confluence, cells were left uninfected or virus was added and allowed to adsorb for 1 h at 37 °C at an MOI of 0.005, 0.05, 0.5 or 5. At 24, 48, 72 and 96 h, 10 μL of Presto Blue reagent (Invitrogen) was added to triplicate wells at each MOI. Fluorescence was quantified with a fluorimeter following a 60-min incubation. Metabolic assays were performed on the twelve-cell line panel with VACV and evaluated at 48, 72 and 96 hpi. UV-inactivated VACV (UV-VACV) served as a negative control.

### 2.6. Live dead assay

To measure the amount of cell death following treatment with virus, we performed live dead assays using trypan blue (Sigma). Trypan blue is a vital dye taken up by cells with damaged or leaky cell membranes. In contrast, healthy cells are able to exclude the dye and therefore appear uncolored or white. ATC cells from the SW1736, U-HTh7 and C643 lines were left untreated or infected with each experimental virus individually. At 24, 48, 72 and 96 hpi, the cells from untreated and infected samples were collected. An aliquot of collected cells (10 μl) was mixed with an equal volume of trypan blue (10 μl) and then the numbers of blue (dead) and white (live) cells were enumerated. These counts were done in triplicate. The combination of blue and white cells allowed us to determine the number of total cells at each time point and calculate the proportion of dead *versus* live populations. This assay was then repeated on the expanded panel of ATC cell lines infected with VACV.

### 2.7. Replication assay

In order to examine the replicative capacity of our experimental poxviruses in ATC cell lines, cells were seeded ($3 \times 10^5$/per well) into two wells of a 6 well dish. Twenty-four hours later the cells were 85–90% confluent and were infected with VACV, MYXV or