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All reovirus subtypes show oncolytic potential in primary cells of human high-grade glioma

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Abstract. Reoviridae are non-human pathogenic viruses. The family of reoviridae consists of 4 different subtypes. Many studies have proven that the Dearing subtype 3 has oncolytic potential. This potential is related to the RAS protein expression in tumour cells. The aim of this study, was to investigate whether all reovirus subtypes have oncolytic potential and whether there are differences in their efficacy, in particular for high-grade glioma. To evaluate the oncolytic potential, we performed an *in vitro* head-to-head study for all reovirus subtypes in 5 primary cell cultures of high-grade gliomas. The oncolytic activity was determined using end-point titration with observation of the cytopathogenic effect. For measurement of RAS activity, we performed an immunofluorescent detection stain on all cell cultures. For quantification of the virus, an RT-PCR measurement for all subtypes was performed. All reovirus subtypes showed oncolytic activity in the observed glioma biopsies. These observations correlated with RAS overexpression in the observed cells. All glioma biopsies overexpressed the RAS protein. The quantitative oncolytic potential differed in relation to the single observed cell culture and in relation to the chosen reovirus subtype. To our knowledge, this is the first study showing oncolytic activity for all reovirus subtypes. We show the relationship and correlation between RAS protein overexpression and vulnerability of cells to reovirus. Efficacy of the different subtypes is interindividually different and cannot be forecast.

Introduction

High-grade gliomas belong to a group of malignancies with a dismal prognosis. So far the median survival rate is approximately 14 months after diagnosis, despite treatment regimens including chemotherapy, radiotherapy and surgery.

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Gliomas are characterized by multiple cytogenetic and moleculargenetic disorders. Most important are mutations of suppressor gene p53 (>30%) and p16 (>50%), deletion of 10 (>60%) and 19q (25%) as well as amplification of the EGF receptor (>30%) and RAS-p21 (>75%). The RAS protein is essential for the intracellular pathway of extracellular signals for regulation of cell growth, survival and differentiation. In malignant diseases, RAS is often deregulated leading to an increased invasiveness and metastases rate combined with decreased apoptotic ability. Biochemical analyses of tumours have shown that the RAS protein cascade is affected in high-grade glioma. Unfortunately, RAS detection rates in glioma differ widely in literature (0-83%) (1-7). However, RAS could be an interesting target for possible treatment approaches in malignant gliomas. Certain phase I and II trials have identified RAS as a possible target for the Dearing strain (8).

Reoviruses are double-stranded RNA viruses. Most humans become infected by reoviruses during childhood. However, infection is generally (respiratory symptoms, diarrhea) mild or asymptomatic (9). Four different subtypes of reovirus are known, including Lang subtype 1, Jones subtype 2, and the two subtype 3 strains, Dearing and Abney. Differences between these strains are characterized by the cell attachment protein and its hemagglutinin activities, including the σ-1 protein sequences. Reoviruses have the ability to replicate in RAS-positive cells, resulting in the cell lysis of RAS-positive tumour cells (10). In the RAS overexpressing cell line NIH3T3, the replication rate was found to be significantly higher compared to the cell line NIH3T3 without overexpression of RAS or for the Dearing reovirus subtype 3 (ranging from factor 10 to 50). Loss of the cell defense mechanism against the reovirus was observed in these RAS protein overexpressing cells (11). However, data comparing the oncolytic ability of all reovirus subtypes do not yet exist (12).

The aim of this study was to comparatively evaluate the oncolytic potential of all reovirus subtypes using a head-to-head design on different primary human glioma cell cultures.

Materials and methods

We performed an *in vitro* head-to-head study for all reovirus subtypes in 5 cell cultures of high-grade gliomas established directly from 5 different patients.

Table I. Characterization of high-grade glioma biopsies by age, gender, tumour manifestation, histology, location and cytogenetics.

Cells	Age, gender, manifestation	Histology and location	Karyotype
T3868	49 years, f, primary	Partially astroglial differentiated anaplastic glioma of high malignancy, right temporal lobe	71-72,XXX,+1,+1,-4,+5,+5,-6, -6,+7,+7,-10,-10,+19,+22,+22
T3564	54 years, f, primary	Pons tumour, glioblastoma multiforme	47,XX,+7
T6217	70 years, f, relapse	Diffusely manifested astrocytary differentiated glioma with giant cells component, right temporal lobe	(A) 41-44,XY,+7,-10,+12,-13,-15, -18,-19,+20,+mar; (B) 81-102,XXYY, -4,+5,+6,+7,+7,+9,-10,-10,del(10)(q22), -13,-13,-14,-14,-15,-15,-18,-18,-19,+20, +1-4mar
T6208	53 years, m, primary	Diffuse high-grade glioma, negative for p21	77-82,XXYY,-6,-6,+7,-8,-8,-10,-10,-11, -13,-14,-14,-15,-15,-17,-18,-20,-22,-22, +1-3mar,dmin
T3095	52 years, m, primary	Glioblastoma multiforme, left occipital lobe	55-83,XY,+1,+3,+3,+4,+5,+6,+7,+7,+8, -10,+16,+17,+i(17q),+19,+21,+22,+5mar, +dmin

f, female; m, male.

Viruses. The reovirus subtypes, Lang, Jones, Dearing and Abney, were obtained from the ATCC (VR-230, VR-231 and VR-824, VR-232). They were propagated in suspension cultures of Vero cells (13) using the protocol of the ATCC for splitting and culturing.

Glioma cell culture. Tumour probes were extracted during neurosurgery and were then cultivated and cytogenetically analysed (Table I). All cell lines were grown in Eagle's minimal essential medium with L-glutamine and $NaHCO_3$ containing 10% heat-inactivated fetal bovine serum, non-essential amino acids and antibiotics (streptomycin and penicillin).

Immunofluorescent detection of RAS. The ImmunoCruz® Staining System (Mouse IgG) (Santa Cruz, CA, USA) was used according to the manufacturer's instructions. However the probes were further prepared for fluorescence by adding rhodamine red IgG antibody (Invitrogen®). Cells were then mounted in mounting medium and counterstained with DAPI diluted 1:10 in phosphate-buffered saline (Vectashield®).

Assay for oncolytic activity. The oncolytic activity and the replication potential was determined using end-point titration with observation of the cytopathogenic effect (CPE). A relevant CPE was defined as general degeneration of the cell lawn, which results in located detachment. A CPE of at least grade II was considered as positive. CPE was analysed every 24 h. $TCID_{50}$ was calculated by the method of Reed and Muench. Two different reference systems were generated to normalize the results: Titration of the input virus in Vero cells and titration by quantitative PCR (Table II).

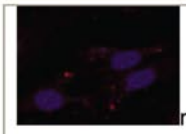
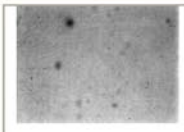
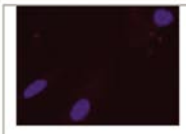
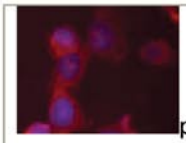
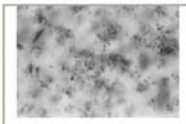
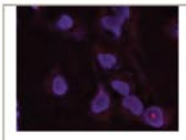
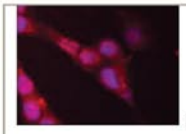
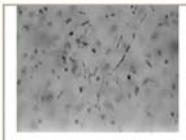
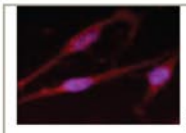
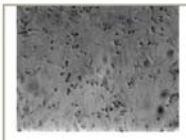
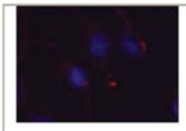
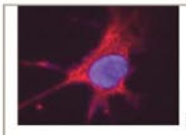
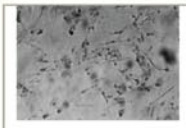
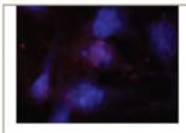
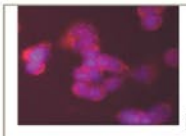
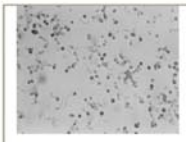
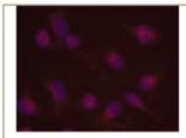
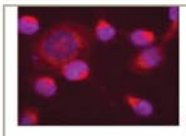
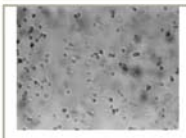
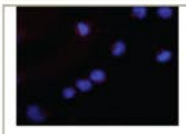
For titration of the virus using quantitative PCR as a reference, reovirus RNA was extracted according to the manufacturer's instructions using the Roche™ MagNA Pure LC Nucleic Acid Isolation Kit®. PCR was performed employing the LightCycler® Amplification Kit (LightCycler® RNA Amplification Kit HybProbe) using the following primer sequences: Reo-fw, 5'-gAATgCAgAACATgATTCAgCAT and Reo-rev, 5'-TAgCAgTATgCTCAgTAgAggTgg. Hybridisation probes used were: Reo-FL, 5'-gATTCTTTT TCTCCAggTAACgATTTTACTCATAT - FL and Reo-LC, 5'-LC Red640-ACTACCACTTTCCCgTCAggTTCAAC - PH (TIB Molbiol).

Table II. PCR cycle threshold to viral detection.

Virus	Cycle threshold value
Lang probe 1	27.09
Lang probe 2	26.19
Lang probe 3	25.92
Mean value Lang	26.40
Jones probe 1	32.01
Jones probe 2	31.84
Jones probe 3	32.41
Mean value Jones	32.09
Dearing probe 1	27.20
Dearing probe 2	25.53
Dearing probe 3	25.39
Mean value Dearing	26.04
Abney probe 1	30.41
Abney probe 2	28.71
Abney probe 3	27.63
Mean value Abney	28.92

The cycling conditions (45 cycles) were as follows: Reverse transcription of 30 min at 55˚C followed by an initial denatur-

Table III. Immunohistochemical proof of RAS expression in investigated cell cultures in comparison to light microscopical observation 72 h after infection with the Dearing subtype.

	Immunohistochemistry: H-RAS detection	t = 72 h after infection with strain Dearing	Immunohistochemistry: Negative control
Fibroblasts	 negative		
Vero cells	 positive		
T3095	 positive		
T6217	 positive		
T6208	 positive		
T3868	 positive		
T3564	 positive		

ation of 60 sec at 95˚C and cycles of denaturation of 5 sec each at 95˚C, annealing for 10 sec at 60˚C, and extension for 20 sec at 72˚C. Analyses were performed by a second derivative method. All experiments were run in triplicate on three different days.

Statistical analysis. Viral titers were normalised using two methods: i) Titration of the input virus in Vero cells as a reference, and ii) quantitative reovirus RNA PCR as a reference. To make both reference systems comparable, the titers were mathematically adjusted. Statistical analysis using JMP®, version 7.0 was performed using the Tukey-Kramer test. The statistical significance level used was $p<0.05$.

Results

All the reovirus subtypes showed oncolytic activity in the investigated glioma biopsies. This efficacy was related to the RAS expression in the examined cells. In the RAS-positive cells, oncolytic activity was observed. In the RAS-negative fibroblasts, all reovirus subtypes did not show relevant oncolysis (Table III).

Using Vero cells as a reference, the Lang strain showed the highest efficacy in 4 of the 5 cell cultures, compared to the Abney strain (1 of 5) (Fig. 1). The lowest replication was seen with the Jones subtype in 4 of the 5 cultures and the Dearing subtype in 1 of the 5 cultures.

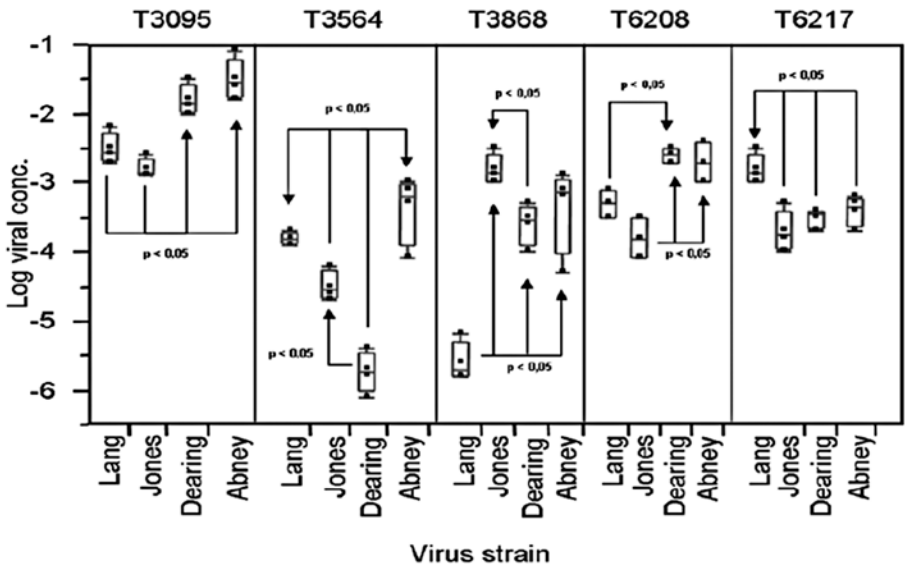


Figure 1. Mean logarithmic viral concentration in cell lines with PCR as a reference. Arrows indicate statistical significance ($p<0.05$). In T3095, the Jones strain replicated more efficiently (compared to: lg 1.2 for Abney, lg 0.9 for Dearing and lg 0.2 for Lang). In the cell line T3564, Dearing replicated more efficiently (compared to: lg 2.4 below Abney, lg 1,95 below Lang and lg 1.25 below Jones). In the cell line T3868, Lang replicated more efficiently (compared to: lg 2.9 below Jones, lg 2.6 below Abney and lg 2.1 below Dearing). In the cell line T6208, Jones replicated more efficiently (compared to: lg 1.2 below Dearing, lg 1.1 below Abney and lg 0.5 below Lang). In the cell line T6217, Jones replicated more efficiently (compared to: lg 0.9 below Lang, lg 0.3 below Abney and lg 0.2 below Dearing).

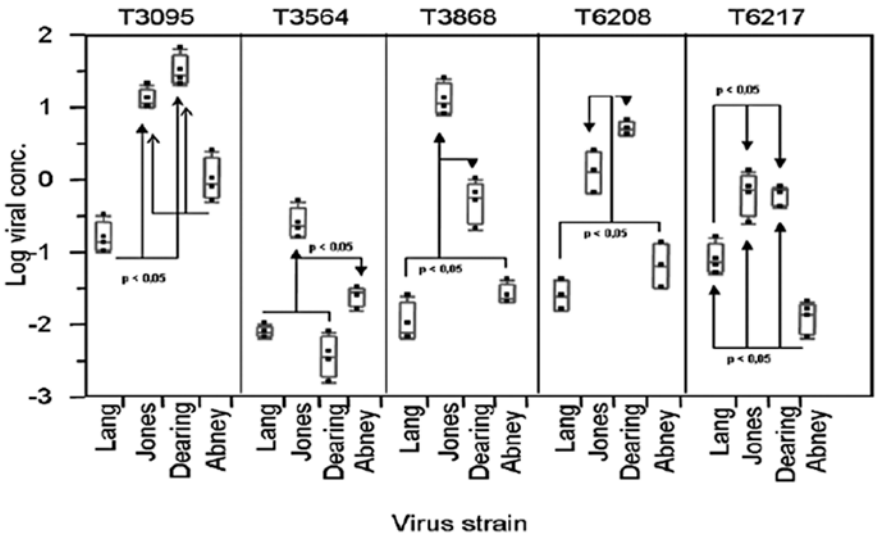


Figure 2. Mean logarithmic viral concentration in cell lines with Vero cells as a reference. Arrows indicate statistical significance ($p<0.05$). In T3095, the Lang strain replicated more efficienty (compared to: lg 2.3 for Dearing, lg 1.9 for Jones and lg 0.8 for Abney). In the cell line T3564, Dearing replicated more efficiently (compared to: lg 1.85 below Jones, lg 0.85 below Abney and lg 0.35 below Lang). In the cell line T3868, Lang replicated more efficiently (compared to: lg 3.1 below Jones, lg 1.7 below Dearing and lg 0.4 below Abney). In the cell line T6208, Lang replicated more efficiently (compared to: lg 2.1 below Dearing, lg 1.7 below Jones and lg 0.4 below Abney). In the cell line T6217, Abney replicated more efficiently (compared to: lg 1.7 below Jones and Dearing, and lg 0.8 below Lang).

In comparison to the results using Vero cells as a reference, the Jones strain showed the highest efficacy in 3 of the 5 cell lines, when titers were normalized to the viral load measured by PCR (Fig. 2). The Dearing and Lang subtypes replicated best in 1 of 5 cultures. Abney showed the lowest efficacy in 2 of the 5 glioma cell cultures, and all the other strains (Lang, Jones and Dearing) showed the lowest replication in 1 of the 5 cultures.

As indicated in the figures, the results varied considerably between the different glioma cell lines and between the two reference methods. The maximum difference in $TCID_{50}$ was lg 2.9 (Lang vs. Jones in T3868) with Vero cells as a reference. Using PCR as a reference, the maximal viral replication rate was lg 3.1 (Lang vs. Jones in T3868).

In general, the commercially available Dearing strain did not show the highest efficacy in all gliomas. Dearing was the

least effective strain in 2 of the 5 glioma cell lines. When comparing Dearing to the other subtypes, the $TCID_{50}$ titer of the other subtypes was up to 250 times higher than Dearing.

Discussion

The goal of this study was to characterize the oncolytic potential of all reovirus subtypes. This study proves that all reovirus strains have oncolytic activity in RAS-positive high-grade glioma. However, in RAS-negative fibroblasts, all reovirus subtypes did not show oncolytic activity. These observations are in accordance with previous studies (5,10,11,14-16).

To the best of our knowledge, only Dearing reovirus type 3 has been used in studies on oncolytic activity in cell culture, animal models and in humans. These studies resulted in a commercially available reovirus strain for human evaluation (14,17).

We used two different reference systems to normalize the input virus titer. Unfortunately, there is no gold standard to quantify the amount of infectious virus used as an input virus titer in the experiments. Generally, cell culture systems are used for this purpose. Vero cells are a well established cell culture system allowing the replication of many viruses. Using a cell culture system as a reference however, could lead to biased results, as Vero cells are not necessarily identical in their susceptibility to all virus subtypes. For this purpose, we chose to use an additional reference system, quantification by PCR. In this case, the susceptibility of a cell is no longer relevant. However, as RT-PCR detects not only RNA from infectious virus but RNA of non-infectious particles as well, quantification by PCR can also lead to biased results. Electron microscopy could be another alternative. However, it has the same disadvantages as RNA detection, as it is not clear whether a visible viral particle is still infectious. At present there is no reference system covering all these aspects.

Our results varied significantly depending on the reference system. Using PCR as a reference, the Jones subtype showed the highest efficacy in 3 of the 5 glioma cell cultures, whereas the opposite occured when using Vero cells as a reference (lowest efficacy in 4 of the 5 tumour cell cultures). This could be explained by the relatively low replication rate of the Jones strain in the Vero cells. Of note, the Dearing strain, which has already been established as tumourlytic agent in the treatment of malignancies, was not the most effective strain in all the 5 glioma cell cultures.

However, the quantitative differences between the strains in a given tumour cell culture were often less than factor 100 (Figs. 1 and 2). Nonetheless, whether these differences are clinically relevant in animal models or in humans needs to be evaluated.

The replication activity of a given virus differed significantly between the 5 cell cultures established from glioma patients. This is most likely due to an extended heterogeneity of the glioma tumour cells.

All reovirus strains replicated in all investigated glioma cell cultures, although each to a different extent. We could not see any systematic effects that might predict the replication activity in cell culture. It can be concluded that not only Dearing reovirus type 3 but all other reovirus subtypes could be used as oncolytic agents. The next step would be to prove this using animal models. Perhaps, if more than one virus proves to be beneficial, then this would lead to the testing of an individual glioma cell line with different strains, prior to the application of the virus as a tumourlytic agent in the future.

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