Immunohistochemical Characterization and Sensitivity to Human Adenovirus Serotypes 3, 5, and 11p of New Cell Lines Derived from Human Diffuse Grade II to IV Gliomas

Abstract

BACKGROUND: Oncolytic adenoviruses show promise in targeting gliomas because they do not replicate in normal brain cells. However, clinical responses occur only in a subset of patients. One explanation could be the heterogenic expression level of virus receptors. Another contributing factor could be variable activity of tumor antiviral defenses in different glioma subtypes.

METHODS: We established a collection of primary low-passage cell lines from different glioma subtypes (3 glioblastomas, 3 oligoastrocytomas, and 2 oligodendrogliomas) and assessed them for receptor expression and sensitivity to human adenovirus (HAd) serotypes 3, 5, and 11p. To gauge the impact of antiviral defenses, we also compared the infectivity of the oncolytic adenoviruses in interferon (IFN)-pretreated cells with IFN-sensitive Semliki Forest virus (SFV).

RESULTS: Immunostaining revealed generally low expression of HAd5 receptor CAR in both primary tumors and derived cell lines. HAd11p receptor CD46 levels were maintained at moderate levels in both primary tumor samples and derived cell lines. HAd3 receptor DSG-2 was reduced in the cell lines compared to the tumors. Yet, at equal multiplicities of infection, the oncolytic potency of HAd5 in vitro in tumor-derived cells was comparable to HAd11p, whereas HAd3 lysed fewer cells than either of the other two HAd serotypes in 72 hours. IFN blocked replication of SFV, while HAds were rather unaffected.

CONCLUSIONS: Adenovirus receptor levels on glioma-derived cell lines did not correlate with infection efficacy and may not be a relevant indicator of clinical oncolytic potency. Adenovirus receptor analysis should be preferentially performed on biopsies obtained perioperatively.

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Introduction

Diffusely infiltrating gliomas consist of WHO grade II to III astrocytic tumors; grade II to III oligodendrogliomas; and the most common form, grade IV glioblastomas [1], which feature a median life expectancy of 15 months with combined chemo- and radiotherapy [2]. Advanced gliomas typically feature inactivating
mutations in tumor suppressors RB1, p53, and p16 and activating mutations in tyrosine kinases, such as epidermal growth factor receptor (EGFR) [3]. Mutations in isocitrate dehydrogenase (IDH) 1 and 2, and O-methylguanine-DNA-methyltransferase (MGMT) promoter methylation guide treatment options and prognosis of particularly low-grade gliomas [1,4].

Oncolytic viruses offer an appealing option to conventional treatments because they can be selected to rely on oncogenic alterations for replication, averting toxicity to normal cells [5]. Human adenovirus serotype 5 (HAd5) has shown excellent safety in glioma patients, but tumor infection and regression only occur in a subset of patients [6,7]. HAd5 uses the cellular tight junction protein coxsackie-adenovirus receptor (CAR) for entry, whose expression in tumors may generally be low and could account for some of the observed patient response heterogeneity [8,9]. To improve the therapeutic efficacy of HAd5, its tropism has been increased by switching its receptor-binding domain, the so-called fiber knob, to that of other human adenovirus serotypes, such as HAd3 and HAd11p, which utilize more ubiquitous and cancer-overexpressed receptors for infection, desmoglein-2 (DSG-2) and cluster of differentiation 46 (CD46), respectively [10–12]. However, the expression patterns of also these receptors in tumors may be heterogenic, and as we and others have shown, the replication of some oncolytic viruses is also regulated intracellularly, particularly by components of the interferon (IFN)-inducible antiviral defense [13–15]. To complicate matters, most analyses of virus tropism and oncolytic efficacy to date have been made in established cell lines, which are prone to significant transcriptional and phenotypic deviation from the parental tumors they were derived from [16–18].

To consolidate the tumor grade-and-passage–dependent, virus entry-mediated, and IFN-regulated determinants of glioma permissiveness to oncolytic viruses, we established a collection of primary tumor cell lines from both low- and high-grade gliomas and compared the expression levels of CAR, DSG-2, and CD46 in the cell lines to the parental tumor tissue. We sought to correlate the different human adenovirus receptor levels to infection efficacy with the respective viruses, HAd5, HAd3, and HAd11p. To account for receptor-independent barriers to virus replication, we assessed whether IFN responsiveness of the primary tumor cell lines correlated with infection efficacy of these adenoviruses. Our results show that, within the glioma subgroups, individual tumors displayed highly variable levels of HAd receptors and that did not correlate with infection efficacy with any of the three HAd serotypes. In our limited sample set, there was also no association between virus serotype and glioma subtype. All tested glioma cells mounted effective antiviral defense, as observed by the complete inhibition of an IFN-sensitive reporter alphavirus [Semliki Forest virus (SFV)-VA7]. However, type I IFN-mediated antiviral signaling did not hinder replication of the tested adenoviruses. Our results support in general the continued development of oncolytic adenoviruses for glioma targeting and call for further investigation into the interconnection of virus tropism and therapeutic efficacy.

### Materials and Methods

#### Patients and Tumor Sample Collection

Samples were obtained from 17 adult glioma patients (range 27-76 years) operated on the NeuroCenter of Kuopio University Hospital during May 2012 to March 2014 (Table 1). For clarity, the cases are indicated as follows: AO, oligoastrocytoma; AOD, anaplastic oligodendroglioma; GB, glioblastoma; and OD, oligodendroglioma. The locations of gliomas are illustrated on representative magnetic resonance images (Supplementary Figure S1). Original diagnoses were confirmed by a neuropathologist (T.R.). Procedures of this study, which were in accordance with the ethical standards of the Helsinki Declaration, were approved by Research Ethics Committee of the Hospital District of Northern Savo. The written consent was obtained from all patients. Samples collected peroperatively were placed in ice-cold Opti-Mem Reduced Serum medium (31985, Gibco) when aimed for cell culture. Samples for histology were placed in 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4) overnight at +4°C.

#### Culturing of Cells

Obtained tumor was cut into smaller pieces with scalpel and McLhwan Tissue chopper (thickness 500 μm). Pieces were passed through a 21G needle followed by trituration and centrifugation. A portion of samples was treated enzymatically for 45 minutes at 37°C with Accutase (A6964, Sigma Aldrich) or 5 mg/ml collagenase (C0130, Sigma Aldrich) and 400 U/ml DNase I (D4527, Sigma Aldrich). Red blood cells were lysed with ammonium chloride (07800, Stem Cell Technologies). After two sequential centrifugations and washings, cells were suspended into culturing medium, and live cells were quantified by trypan blue exclusion. Cells were seeded in uncoated flasks (25 cm², Greiner CellStar) and cultivated under serum-free and serum-containing conditions at 37°C in 5% CO₂. Serum-free medium consisted of Dulbecco’s modified Eagle’s medium/F12 medium (21331, Gibco) containing 1% B27 supplement (12587 or 17504, Gibco), 1% N2 supplement (17502, Gibco), 1% Glutamax-I (Gibco 35050), 10 mM HEPES (H8878, Sigma), 20 ng/ml EGF (AF100-15, Peprotech), 10 ng/ml FGF (100-18B, Peprotech), 100 U/ml penicillin–0.1 mg/ml streptomycin (P0781, Sigma), and 1.25 μg/ml fungizone (15290, Gibco). Serum-containing medium included Dulbecco’s modified Eagle’s medium/F12 medium containing 1% Glutamax-I, 100 U/ml penicillin–0.1 mg/ml streptomycin, 1.25 μg/ml fungizone, and 10% FBS. 

#### Table 1. Characteristics of Glioma Patients

| Code-Subtype | Sex | Age | WHO Grade | Location of Tumor | Survival (Months) |
|--------------|-----|-----|-----------|------------------|------------------|
| Glioblastomas |     |     |           |                  |                  |
| 003-GB       | M   | 67  | IV        | Frontal          | 49               |
| 022-GB       | M   | 75  | IV        | Temporal         | 11               |
| 027-GB       | M   | 67  | IV        | Frontal          | 35               |
| 038-GB       | F   | 68  | IV        | Occipital        | 23               |
| Oligodendrogliomas |       |     |           |                  |                  |
| 004-OD       | M   | 44  | II        | Frontal          | A (57)           |
| 008-OD       | F   | 40  | II-III    | Insula           | A (56)           |
| 020-OD       | M   | 69  | II-III    | Frontal          | A (48)           |
| 014-AOD†     | M   | 39  | III-IV    | Temporal         | 9                |
| Oligoastrocytomas |       |     |           |                  |                  |
| 036-OA       | M   | 70  | II        | Temporal         | 7                |
| 015-OA       | M   | 35  | II-III    | Temporal         | A (51)           |
| 025-OA       | F   | 31  | II-III    | Frontal          | A (44)           |
| 039-OA       | M   | 51  | II-III    | Frontal          | A (37)           |
| 002-OA       | M   | 42  | III       | Parietal         | 45               |
| 016-OA       | M   | 27  | III       | Temporal + insula| A (51)           |
| 026-OA       | F   | 63  | III       | Frontal          | 23               |
| 034-OA       | M   | 39  | III       | Frontal          | A (39)           |
| Ependymomas  |     |     |           |                  |                  |
| 035-E ‡      | M   | 76  | II        | 4th ventricle    | 4                |

A, alive (in parenthesis months since operation); E, ependymoma; F, female; M, male.

† Anaplastic oligodendroglioma 4 years earlier.
‡ Ependymoma 8 years earlier.
§ IDHR132 mutation.
heat-inactivated fetal bovine serum (Bioclear). Cells were fed twice a week by replacing with 50% of fresh medium, with first feeding performed 1 day after plating. Serum-free conditions were switched to adherent cultures after day 4. Both in serum-free and in serum-containing cultures, plates coated with ECM gel (1:20 dilution) (E6909, Sigma) gave the most reliable growth. For cell culture splitting, cells were detached with Accutase and split 1:2 up to 1:4. Procedures for additional cell lines and preparation of rat primary cortical cells are described in Supplementary Methods.

**Immunohistochemistry of Paraffin-Embedded Sections and Immunocytochemistry of Cultivated Cells**

Paraffin-embedded-fixed samples were included in paraffin, cut into 7-μm sections, and stained with hematoxylin/eosin (H&E). For immunohistochemistry, antigen retrieval was done in citrate buffer (pH 6.0, 20 minutes), and endogenous peroxidase was blocked with 0.3% H2O2–methanol (20 minutes). For NeuN and CAR stainings, 0.1% Triton X-100 was included after peroxidase blocking. Sections were blocked with 1% BSA in PBS (A9647, Sigma Aldrich) and 1% normal goat/horse serum and stained using Vectastain ABC kits (PK-4001 or PK-6102, Vector Laboratories). Primary antibodies for cell type characterization were against astrocytic-like cells [polyclonal rabbit anti–glial fibrillary acidic protein (GFAP), Z0334, DAKO, 1:10,000], oligodendrocyte-like cells [monoclonal mouse anti-2′, 3′-cyclic nucleotide 3′-phosphodiesterase (CNPase), MAB326R, Millipore, 1:500], neurons [monoclonal mouse anti-NeuN, Millipore MAB377, 1:10,000], and proliferating cells [monoclonal mouse anti-human Ki-67 antigen, M7240, DAKO, 1:2000). Primary antibodies for virus receptor studies were against coxsackievirus receptor (monoclonal mouse anti-CAR, 05-644, Millipore, 1:50), antibodies for virus receptor studies were against coxsackievirus receptor (monoclonal mouse anti-CAR, 05-644, Millipore, 1:50), desmoglein-2 (rabbit polyclonal anti-DSG-2, H-145, sc-20,115, Santa Cruz Biotechnology Inc., 1:300), and cluster of differentiation 46 (CD46) (MAB384, Sigma Aldrich, 1:200), and proliferating cells (monoclonal mouse anti-NeuN, Millipore MAB377, 1:10,000), and endogenous peroxidase was blocked with 0.3% H2O2

**Viruses and Infections In Vitro**

Cells were infected with the replication-competent Ad of serotype 3 recombinant rAd3ΔE3GFP [19], referred here as HAd3; the conditionally replicating adeno-virus serotype 5 construct Ad5-CRAD [20], referred to as HAd5; or the replication competent serotype 11p virus RCAd11pE1GFP [21], referred to as HAd11p. These viruses were amplified in A549 human lung carcinoma cells and functional titered obtained by TCID50 assay.

Patient glioma-derived cells (12,000 or 15,000/well) and commercial cell lines (A549, 293, A172, U87-MG; 25,000/well) were plated on 48-well plates and monitored using Zeiss Axios Observer Z1 microscope with 10× objective. For study of virus IFN sensitivity, cells were pretreated for 4 hours with human IFN β (500 U/ml or 2000 U/ml; PPT-262, Biosite). SFV VA7-EGFP produced in BHK-21 cells was used as a reference for an IFN-sensitive virus [22,23].

**Cell Viability**

Cell viability was assayed with MITT Cell Proliferation Kit I (Roche) reading absorbances by VICTOR plate reader (PerkinElmer-Wallac, Turku, Finland). Results are expressed as means of absorbance difference (A595nm−A630nm).

**Statistical Analyses**

Cell viability data were analyzed with unpaired t test or one-way analysis of variance (followed by Bonferroni test) (Origin and GraphPad software).

**Results and Discussion**

**Establishment of Primary Human Glioma Cell Lines**

Primary surgical samples were obtained from 17 patients featuring diffusely infiltrating gliomas from grade II to IV (Table 1, Supplementary Figure S1). Superior cell viability was achieved when cells from the peripherally collected glioma samples were extracted purely mechanically omitting proteolytic enzymes (not shown). We were able to establish cell lines from 14 of the 17 patient samples in serum containing medium and in two thirds of cases in serum-free conditions using serum-free neurosphere and monolayer techniques [24,25]. Because of favorable growth properties, we chose to continue work with eight cell lines representing each of the glioma subtypes (three glioblastomas, two oligodendrogliomas, three oligoastrocytomas). Although cells grown in serum-free conditions may more faithfully retain glioma phenotypic
features than cells grown in serum-containing medium [26,27], they grow much more slowly and were prone to detach. Therefore, to ensure sufficient cell numbers for functional experiments, cell lines kept in serum-containing medium were used, with infections performed on cells in passages 6 to 14. To provide basal biological information about the eight chosen cell lines, we assessed by Western blotting the expression of common glioma hallmarks, such as select genes commonly deleted, mutated, amplified, or otherwise indicative of glioma growth, including TP53, IDH1, MGMT, and EGFR. Overall, none of these markers were prominently expressed in the established cell lines (Supplementary Figure S2).

**Cell Type and Virus Receptor Expression in Patient Tumors and Derived Cell Lines**

We next compared expression of cell-specific differentiation markers and virus receptors in the primary glioma tissue samples and the derived cell lines by immunohisto-/fluorescence labeling (summarized in Table 2). Astrocytic marker GFAP was strongly expressed in both the parental tumors and the derived cell lines in all cases (Supplementary Figure S3), in agreement with other studies and as expected for gliomas [17,18,27–29]. The oligodendrocytic marker CNPase was found in sporadic cells in tumor samples but was only detected in a punctate pattern in cultured cells originating from one oligoastrocytoma case, 025-OA, and from both the oligodendro-glioma cases, 004-OD and 020-OD. As expected, the neuronal marker NeuN was found only sporadically in the original tumors and was absent from all the derived cell lines, in line with the ontogeny of gliomas [30,31].

Efficacy of adenoaviruses infection of cultured cells has been shown to depend on cognate virus receptor expression levels [8,9,32]. Correspondingly, it is possible that the unfavorable results in recent phase II and III clinical trials treating glioblastoma patients with human adenovirus serotype 5–derived constructs [6,7] may partly derive from insufficient expression of CAR on glioma cells [32]. In our samples, CAR was expressed at low level in sporadic cells in the primary tumors and was almost absent in the derived cell lines (Table 2, Figure 1). Although CAR has been detected in several commercial and low-passage primary glioma cell lines in earlier studies [9,32–37], the general expression level also in these studies has been low. Moreover, as CAR has inhibitory properties for cancer invasion, its expression is generally reduced in advanced gliomas compared to lower-grade tumors [9,38,39].

DSG-2 belongs to the cadherin family of transmembrane glycoproteins and serves as receptor for HAd3 entry, but little is known about DSG-2 expression in gliomas. In colon carcinoma xenografts, loss of DSG-2 led to reduced epithelial cell proliferation and tumor growth [40]. In the present study, DSG-2 subjectively appeared more abundant in the parental tumors than CAR, and its expression in the derived cell lines was more obvious than CAR, although still weak compared to the positive controls (Table 2, Figure 1). Adenovirus 11p receptor CD46 was maintained at moderate/strong levels in both primary tumors and derived cell lines (Table 2, Figure 1), consistent with previous investigations [32,36,37,41]. The results by immunofluorescence staining in fixed cells were largely mirrored by Western blotting of corresponding cell lysates (Supplementary Figure S4).

**Glioma Permissiveness to Oncolytic Adenoviruses In Vitro Maybe Not Primary Receptor Dependent**

Given the small sample size in the present study, it was no surprise that HAd serotype receptor expression did not correlate with any particular glioma subclass. Next, to determine whether the glioma subclass and/or virus receptor levels would predict infection efficacy with the corresponding oncolytic adenoviruses, established cell lines were infected at multiplicity of infection (MOI) 10 and followed under fluorescence microscope for 72 hours, at the end of which oncolytic potency was determined by viability assay. SFV VA7 served as a broad-tropism oncolytic control, which also potentially can reveal the presence of basal antiviral defenses. Our results showed comparable tumor killing by HAd5 and HAd11p in all tested cell lines (Figure 2). Expression of GFP was more intense in the HAd11p-infected cells than in the HAd5-infected cells (Supplementary Figure S5), which is likely due to differences in the respective virus designs (in HAd5 GFP expression is transcriptionally linked to E1B), as the cytopathic effects indicative of virus replication were very similar between the two serotypes. In contrast, HAd3 reduced cell viability by at most 40% and then only in the lower-grade glioma cell lines (particularly from oligoastrocytomas), with the glioblastoma cell lines showing only minor loss of viability (Figure 2). Based on GFP expression, in 72 hours, HAd3 had only infected a fraction of primary glioma cells compared to the other two serotypes (Supplementary Figure S5). SFV infection progressed similarly in all samples, except in 026-OA, where infection appeared less efficient than in the other cell lines, constituting an interesting case for further study with regard to cell-intrinsic resistance to oncolytic SFV replication (Figure 2).

Interestingly, while CAR was virtually absent in the established glioma cell lines, infection and oncolytic potency of HAd5 were at least as pronounced as for HAd11p, whose receptor CD46 was clearly expressed in all studied cell lines (Figures 1 and 2). Overall, in our limited sample set, the level of these adenovirus receptors did not

**Table 2. Expression Analysis of Parental Tumors and Glioma-Derived Cell Lines**

| Cell Type Markers | Virus Receptors |
|-------------------|-----------------|
| **NeuN** | **CAR** | **CD46** | **DSG-2** |
| Code | Parental Tumor | Cell Line | Parental Tumor | Cell Line | Parental Tumor | Cell Line | Parental Tumor | Cell Line |
| 003-GB | – | – | ++ | +++ | ++ | – | – | + |
| 027-GB | + | + | – | – | + | – | – | + |
| 036-GB | – | – | – | – | – | +/– | – | – |
| 004-OD | + | – | + | +++ | ++ | – | – | + |
| 020-OD | + | – | ++ | + | + | – | + | + |
| 015-OA | + | + | – | – | + | – | ++ | ++ |
| 025-OB | + | + | – | – | + | – | ++ | ++ |
| 026-OA | – | – | – | – | – | – | ++ | ++ |

– = no expression; +/+ = negligible expression; + = weak expression; ++ = moderate expression; +++ = strong expression.
correlate with oncolytic potency \textit{in vitro} (which serves as an indicator for infection efficiency). However, because numerous previous studies have demonstrated a functional role for the virus receptors in adenovirus oncolytic potency \cite{8,9,35,42,43}, it is preferable to measure virus receptor levels perioperatively rather than from derived cell lines due to potential loss of expression during passaging. Moreover, the therapeutic efficacy with oncolytic viruses in general is not solely determined by infection capacity but also by immunogenicity of virus-induced cell death \cite{44}, which complicates predictions of clinical outcome based on infection data \textit{in vitro}.

While relative comparisons between these adenovirus serotypes in our study is difficult due to fundamental differences in virus design, the HAd3 fiber knob attached onto an HAd5-based backbone, termed HAd5/3, did display greater infection capacity than the parental HAd5 virus \cite{45}, arguing for the possibility to optimize oncolytic adenoviruses for glioma targeting further by combining the strongest human adenovirus backbone with the best receptor-binding domain. Serotype 3 also has DSG-2-dependent tight-junction-opening properties which have not been described for other serotypes, making the virus still an appealing contender for glioma targeting \cite{46}.

\textbf{Figure 1.} Expression of adenovirus receptors (CAR, CD46, and DSG-2) in glioma tissues and cultured glioma cells. (A) Adenovirus receptor expression (brown color) was variable in paraffin sections of patient gliomas for which hematoxylin (blue color) was used as counterstain. Anaplastic oligodendroglioma 014-AOD was used as positive control in CAR and CD46 staining and rat kidney in DSG-2 staining. Among gliomas especially, 014-AOD had abundant expression of adenovirus receptors. Scale bar 100 \(\mu\)m. (B) Fluorescence images of immunocytochemically (red color) stained cultured cells. Nuclei were stained with DAPI (blue color). Especially CD46, receptor for serotype 11p, was well preserved among patient glioma-derived cells. A549 and 293 cells were used as positive controls. Scale bar 20 \(\mu\)m.
Gliomas Capable of Mounting Interferon-Dependent Antiviral Responses But These Not Hindering Human Adenovirus Oncolysis

We previously demonstrated that some human commercial glioma cell lines as well as murine gliomas can mount both intrinsic and IFN-mediated resistance against oncolytic SFV [23]. To test whether primary human glioma cell lines can mount IFN-triggered antiviral defenses and whether such resistance could limit the replication of the three different adenovirus serotypes used in the present study, we pretreated for 4 hours our eight primary cell lines, two commercial glioma cell lines (A172 and U87), and IFN-responsive A549 cells with recombinant human IFN beta (2000 U/ml) and infected them with the three human adenovirus serotypes. SFV was used as an indicator for productive antiviral defense. The experiments revealed that only SFV replication was blocked by the IFN pretreatment, demonstrating that the primary glioma cell lines are capable of responding to IFN (Figure 3). While it has been shown that serotype 5 oncolytic adenovirus replicates more slowly in several types of epithelial cancer cells featuring constitutive expression of select IFN-stimulated genes, such as MxA [13,14], it is possible that such resistance differs functionally from the response to brief IFN pretreatment. Alternatively, as SFV (control virus) was inhibited, we conclude that oncolytic adenoviruses can replicate even in IFN-exposed glioma cells, and thus lack of clinical efficacy may result from yet undiscovered factors.

Conclusions
We describe here establishment of new glioma cell lines derived not only from glioblastomas but also from lower-grade gliomas including oligoastrocytoma and oligodendrogliomas. Along with clinical data and original tumors, their immunophenotypic characterization is presented. We show that, compared to human adenoviruses (serotypes 3, 5, and 11p), the VA7-EGFP alphavirus vector based on SFV is superior in infection and killing of low-passage human glioma cells. Human adenoviruses might, however, be more suitable for therapeutic use, as they seem capable of replicating despite IFN response, although recently also more IFN-I resistant versions of SFV have been described by us [47]. To sum up, clinical efficacy may be influenced by factors not fully reflected under culture conditions.

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Conflict of Interest
Ya-Fang Mei states that adenovirus serotype 11p vector is under European patent process (application EP20100822318). Other authors declare no conflicts of interest.

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Figure 3. Effect of exogenous interferon on infection with adenovirus (HAd) and SFV. Viability of human cells is determined 3 days after infection and is given as percentage of control (ctrl, 100%; average ± S.E.M., HAd3, and HAd11p) and 1 for SFV. IFN-*** = at least 3 parallel wells/group) at the same time point.

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