Treatment of metastatic renal cancer with capsid-modified oncolytic adenoviruses

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Abstract
Renal cancer is a common and deadly disease that lacks curative treatments when metastatic. Here, we have used oncolytic adenoviruses, a promising developmental approach whose safety has recently been validated in clinical trials. Although preliminary clinical efficacy data exist for selected tumor types, potency has generally been less than impressive. One important reason may be that expression of the primary receptor, coxsackie-adenovirus receptor, is often low on many or most advanced tumors, although not evaluated in detail with renal cancer. Here, we tested if fluorescence-assisted cell sorting could be used to predict efficacy of a panel of infectivity-enhanced capsid-modified marker gene expressing adenoviruses in renal cancer cell lines, clinical specimens, and subcutaneous and orthotopic murine models of peritoneally metastatic renal cell cancer. The respective selectively oncolytic adenoviruses were tested for killing of tumor cells in these models, and biodistribution after locoregional delivery was evaluated. In vivo replication was analyzed with noninvasive imaging. Ad5/3-Δ24, Ad5-Δ24RGD, and Ad5.pK7-Δ24 significantly increased survival of mice compared with mock or wild-type virus and 50% of Ad5/3-Δ24 treated mice were alive at 320 days. Because renal tumors are often highly vascularized, we investigated if results could be further improved by adding bevacizumab, a humanized antivascularendothelial growth factor antibody. The combination was well tolerated but did not improve survival, suggesting that the agents may be best used in sequence instead of together. These results set the stage for clinical testing of oncolytic adenoviruses for treatment of metastatic renal cancer currently lacking other treatment options. [Mol Cancer Ther 2007;6(10):2728–36]

Introduction
Each year over 200,000 cases of renal cell carcinoma are detected (1); one third of patients have metastases at diagnosis, and many operated patients relapse (2). Unfortunately, radiotherapy, chemotherapy, or hormonal therapies have little effect in this disease, and therefore, it is the sixth leading cause of cancer deaths in developed nations (3). Immunomodulating agents, such as IFN-α and interleukin 2, have shown antitumor activity in 7% to 15% of patients according to a metaanalysis (4), but metastatic disease can be cured only very rarely (5). Antiangiogenic molecules, including bevacizumab, sorafenib, and sunitinib, have increased the time to progression in patients with metastatic disease, but a survival advantage has not yet been shown (6). Thus, the long-term prognosis for patients with advanced renal cell cancer remains poor, and novel and more effective therapies are needed.

A promising approach for treatment of tumors resistant to available therapies is utilization of oncolytic viruses, which achieve tumor cell killing by replication per se (7). Adeno-
Approaches for transductional targeting include 5/3 serotype chimerism (17) and incorporation of RGD-4C (18) or polylysine chains (pK) into the adenovirus fiber, which is responsible for tropism (19, 20). The receptors for serotype 3 adenoviruses are not fully known, but CD46 may play a role (21–23). RGD-4C binds to the αvβ3 class of integrins (24, 25), and polylysine chains give the virus tropism toward heparan sulfate proteoglycans (HSPG; ref. 26). We hypothesized that these capsid modifications would enhance transduction of renal cancer cells, which would subsequently lead to an increased oncolytic effect and antitumor efficacy with the respective oncolytic viruses in vitro and in vivo. However, receptor expression is likely to vary from tumor to tumor and, therefore, different viruses might be most useful for different patients. Thus, we hypothesized that fluorescence-activated cell sorting (FACS) would be useful as a predictive tool for personalized treatment.

A promising approach for rendering the replication of adenoviruses tumor specific is deletion of 24 bp (Δ24) from the constant region 2 of E1A. Consequently, the mutant E1A is unable to bind Rb for liberation of E2F, which is required for effective induction of virus replication in quiescent cells. However, because cancer cells, including renal cell cancers, are universally deficient in the p16-Rb pathway, abundant E2F is available and replication and cell killing can proceed, whereas normal cells are spared (27–29). We hypothesized that capsid-modified Δ24 oncolytic adenoviruses would be effective in killing renal cancer cells.

Although oncolytic viruses are effective against tumor agents, the complexity of advanced tumor masses may present challenges for curative treatment. Therefore, it is likely that the efficacy of early generation oncolytic viruses can be improved by combination with other modalities, which may also be useful because of nonoverlapping side effects and the potential for additive or synergistic efficacy. A defective von Hippel Lindau pathway may be a characteristic feature of most, if not all, renal cancers, which makes antiangiogenic approaches attractive (30). Therefore, we investigated whether the efficacy of oncolytic adenoviruses could be further improved with bevacizumab, one promising agent for the treatment of metastatic renal cell cancer.

### Materials and Methods

#### Cell Lines and Tissue

786-O, ACHN, Caki-2, and 769-P are human clear cell carcinoma kidney cancer cell lines and were obtained from American Type Culture Collection. All cell lines were maintained in recommended conditions. Kidney tumor samples were obtained with signed informed consent and ethical committee permission from patients undergoing surgery at Helsinki University Central Hospital.

#### Adenoviruses

Viruses were propagated as reported (31), and their main features are described in Table 1.

#### Flow Cytometric Analysis for Receptor Expression

Renal cancer cells were incubated with anti-CD46 (BD Biosciences), anti-integrin α3 β3 (Chemicon International), anti-integrin αv β3 (Chemicon International), anti-HSPG (Seikagaku), anti-CAR RmcB antibodies, or FACS buffer. Cells were then washed and incubated with phycoerythrin-labeled goat anti-mouse immunoglobulin polyclonal

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**Table 1. Adenoviruses used in this study**

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<th>Virus name</th>
<th>E1 reporter gene</th>
<th>Fiber</th>
<th>Main receptors</th>
<th>Ratio*</th>
<th>Reference</th>
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<td>Luciferase</td>
<td>Serotype 3 knob</td>
<td>CD46 and unknown (receptor X)</td>
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</tr>
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<td>RGD motif in HI loop</td>
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<td>7 lysine residues at COOH terminus</td>
<td>HSPGs and CAR</td>
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<td>GFP + luciferase</td>
<td>RGD motif in HI loop and 7 lysine residues at COOH terminus</td>
<td>αvβ integrins and HSPGs and CAR</td>
<td>38</td>
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<td>7 lysine residues at COOH terminus</td>
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<tr>
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<td>αvβ integrins and CAR</td>
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<td>—</td>
<td>Wild type</td>
<td>CAR</td>
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</table>

*Ratio of viral particles to plaque forming units, a quality control measure and indicator of viral packaging efficacy.

¹ A 24-bp deletion in constant region 2 of E1A region of adenovirus genome mediates selectivity to p16/Rb pathway mutant tumor cells.
antibody (BD Biosciences) before flow cytometry. For analysis of clinical samples, tumor pieces were minced, suspended in growth medium with 0.2 wünsch units/mL liberase blendzyme (Roche Diagnostics), and incubated at 37°C for 2 h for enzymatic dissociation before FACS. To analyze receptor expression of tumors grown in mice, ACHN cells were injected i.p. into severe combined immunodeficiency mice and tumors were harvested after 30 days. Tumors were cut into small pieces and enzymatically dissected, and FACS was done as above.

**Marker Gene Transfer Assays**

Cells were infected with replication-deficient, marker gene–expressing viruses for 30 min, and then washed and incubated with complete growth medium at 37°C. At 24 h later, luciferase (Luciferase Assay System, Promega) or β-gal (Galacto Light Plus, Tropix) assays were done according to the manufacturer’s manual. Clinical samples were minced and washed twice. Samples were resuspended in 2% RPMI and then infected with 5,000 virus particles per cell. Luciferase or β-gal assays were done as above.

**In vitro Cytotoxicity Assays**

Renal cancer cells (10⁴ on 96-well plates) were infected with indicated viruses. After 1 h, infection medium was replaced with medium containing 5% FCS, which was changed every other day. Eight to eleven days later, cell viability was analyzed with MTS assay (Cell Titer 96 AQoneous One Solution Cell Proliferation Assay, Promega).

**Animal Experiments**

All animal experiments were approved by Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland. Female nude or severe combined immunodeficiency mice were purchased from Taconic at the age of 4 to 5 weeks and quarantined for 2 weeks. Mice were frequently monitored for their health status and euthanized as soon as any sign of pain or distress was noticed.

For analysis of biodistribution, ACHN cells were infected i.p. into severe combined immunodeficiency mice, and 35 days later, 10⁵ virus particles of replication-deficient, marker gene–expressing viruses were given i.p. At 48 h later, mice were killed, and selected organs were collected and analyzed for marker gene expression as described earlier.

In the subcutaneous tumor model, 786-O tumors were grown in nude mice, and a mixture of 3 × 10⁶ virus particles of oncolytic virus and 3 × 10⁸ virus particles of Ad5/3luc1 was injected i.t. on days 1, 2, and 3. Tumor volume was calculated V = L × H² × 0.52. On days 4 and 10, mice were injected i.p. with 4.5 mg d-luciferin (Promega) and, after 10 min, imaged for bioluminescence with IVIS imaging system series 100 using Living Image v2.5 software (Xenogen).

For in vivo survival studies, ACHN cells were injected i.p. into severe combined immunodeficiency mice. At 7, 14 and 21 days later, 10⁸ virus particles of indicated viruses were injected i.p. Bevacizumab (90 µg; Avastin, Genentech) was given i.p. once weekly for 5 weeks starting at day 11.

**Statistical Analysis**

To compare differences between groups, two-tailed Student’s t test was used and a P value of <0.05 was considered significant. P values of the in vivo s.c. experiment were calculated by Mann-Whitney test (SPSS 13.0). Data of survival experiments were plotted as Kaplan-Meier graphs, and a log-rank t test (SPSS 13.0) was used for pairwise comparison of groups.

**Results**

**Predicting the Efficacy of Gene Delivery to Renal Cancer Cell Lines with FACS**

Expression of receptors proposed relevant for entry of the viruses used was determined with FACS (Supplementary Fig. S1). 786-O cells showed high expression of CD46 and HSPG, as well as αvβ3 and αvβ5 integrins, whereas CAR expression was low (Supplementary Fig. S1A). Fittingly, >300-fold improved transgene expression was obtained with Ad5.pK7 (GL) (target receptor: HSPG; Fig. 1A). Ad5/3luc1 (proposed receptors: “receptor X” and CD46) also increased gene delivery up to 120-fold versus the CAR binding virus.

CD46 was highly expressed on ACHN cells compared with CAR, and also high expression of αvβ3 and αvβ5 integrins was seen (Supplementary Fig. S1B). Ad5/3luc1 showed >400-fold increased transgene activity (Fig. 1B) with this cell line.

On Caki-2 cells, high CD46 and HSPG expression was seen (Supplementary Fig. S1C). Gene delivery with Ad5pK21-LacZ (target receptor: HSPG) was >100-fold and with Ad5/3luc1 68-fold higher than with the unmodified control virus (Fig. 1C).

Compared with CAR, higher HSPG, CD46, and αvβ3 and αvβ5 integrin expression was seen on 769-P cells (Supplementary Fig. S1D). With this cell line, transgene expression was up to 130-fold higher with Ad5.pK7 (GL) than with the unmodified control virus (Fig. 1D), and Ad5/3luc1 showed 65-fold increase.

For all cell lines, viruses with polylysine modifications or 5/3 chimerism showed significantly enhanced gene delivery compared with the respective wild-type capsid control viruses, and the degree of enhancement could be estimated by FACS.

**Using FACS for Prediction of Gene Delivery with Capsid-Modified Adenoviruses to Clinical Renal Cell Cancer Samples**

Because adaptation to growth in vitro may render cell lines distinct from clinical tumor specimens (33–35), we sought to corroborate cell line findings with freshly excised tumor samples from kidney cancer patients. Despite contaminating normal cells not present in cell lines (which would dilute the data), we found higher expression of HSPGs compared with CD46, αvβ3 and αvβ5 integrins, and

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*Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).*
CAR (Supplementary Fig. S2). Ad5pK21-LacZ yielded a 17-fold increase in gene delivery, whereas Ad5/3luc1 and Ad5.pK7 (GL) exhibited 4.5-fold and 2.5-fold increases, respectively (Fig. 2A).

In another clinical sample, Ad5.pK7 (GL) exhibited a 15-fold increase in gene delivery, whereas Ad5pK21-LacZ and Ad5.RGD.pK7 (GL) yielded 7.5-fold and 6.5-fold higher values than controls (Fig. 2B). In a third sample, Ad5/3luc1 resulted in 6.5-fold higher gene delivery compared with control, and Ad5pK21-LacZ and Ad5.pK7 (GL) showed increases of 4 and 3.8 (Fig. 2C). The sizes of the samples available did not allow FACS analysis to be done from all samples.

Increasing Oncolytic Potency with Capsid-Modified Conditionally Replicating Adenoviruses

In vitro

With all cell lines, Ad5/3-D24 exhibited the highest oncolytic effect (Fig. 3). However, also the oncolytic effect of Ad5.pK7-D24 was improved over or similar to positive controls in three of four cell lines (Fig. 3A–C). In general, oncolytic potency correlated well with FACS and luciferase expression (Figs. 1 and 2; Supplementary Figs. S1 and S2), but Ad5/3-D24 seemed to be somewhat more oncolytic than predicted by gene transfer.

Increasing Antitumor Efficacy in a Subcutaneous Renal Cancer Model with Capsid-Modified Oncolytic Adenoviruses

Untreated tumors grew rapidly, whereas Ad5.pK7-D24 and Ad5-Δ24RGD showed the highest antitumor effect compared with mock (both P < 0.001; Fig. 4A). In these groups, 50% of injected tumors disappeared completely. Treatment with Ad5/3-D24 also resulted in a significant antitumor effect (P < 0.001 compared with mock) but complete tumor eradication was not seen. Tumors injected with replication-deficient control virus Ad5LacZ did not become smaller, whereas replication-competent control viruses without capsid modifications (Ad5 wild type and Ad5-Δ24E3) resulted in significant antitumor efficacy compared with mock group (P < 0.001).

To show in vivo replication of the viruses and to assess amplification kinetics, subcutaneous tumors were coinfected with oncolytic virus and luciferase expressing E1-deleted (i.e., nonreplicating) virus. Presence of the two viruses in the same cell allows both viruses to replicate (36–39). Therefore, the degree of luciferase expression correlates with replication of the oncolytic adenoviruses. On day 4 (Fig. 4B), 1 day after the last virus injection, Ad5/3-Δ24–injected tumors showed the highest photon emission. On day 10 (Fig. 4C), all values were ~200 times smaller, but Ad5/3-Δ24–injected tumors continued to exhibit the strongest light emission. With the most effective viruses, few tumor cells remained on day 10 (Ad5.pK7-D24 and Ad5-Δ24RGD), and consecutively photon emission was low. Fittingly, the decrease in light emission between days 4 and 10 was smallest with the Ad5LacZ-injected tumors.

Receptor Expression In vivo and Biodistribution of Capsid-Modified Adenoviruses in an Orthotopic Mouse Model of Metastatic Renal Cancer

To develop an orthotopic model wherein the tumor environment would resemble the clinical situation as closely as possible, we grew human renal cell tumors i.p. in mice. Then we studied if gene delivery could be predicted with flow cytometry (Supplementary Fig. S3). We found higher expression of CD46 and HS PG than CAR in both samples, whereas αvβ3 and αvβ5 integrins were expressed at about the same levels.
Biodistribution of capsid-modified viruses in tumor-bearing mice was evaluated after i.p. delivery (Fig. 5A), which might be a relevant treatment approach in this patient population. Ad5/3luc1 and Ad5lucRGD delivered 5.6-fold ($P = 0.03$) and 4-fold, respectively, more luciferase to tumor tissues than the isogenic control virus Ad5luc1. Ad5.pK7 (GL) resulted in 2.1-fold higher luciferase expression compared with its isogenic control virus Ad5 (GL). Transduction of the liver, which is the most crucial organ with regard to side effects, was similar between the capsid-modified viruses and controls (no significant differences), and the same was true for heart and kidneys. However, in the lungs, Ad5/3luc1 ($P = 0.024$) and Ad5lucRGD ($P = 0.017$) delivered significantly more luciferase than Ad5luc1, and in the spleen, Ad5/3luc1 was significantly more effective than Ad5luc1 ($P = 0.023$). At 6-fold higher luciferase expression in tumor tissue versus liver, heart, kidneys, lungs, and spleen. These findings might predict toxicity data similar to adenovirus 5, which has been very safe in treatment of >4,000 published cancer patients (8–11).

Improving Survival of Mice Bearing Peritoneally Disseminated Renal Cell Cancer with Oncolytic Adenoviruses Alone or in Combination with Bevacizumab

Median survival for the mock group was 41 days, whereas mice treated with bevacizumab had a median survival of 54 days (Fig. 5B). Treatment with wild-type Ad300wt prolonged median survival (73 days) significantly ($P = 0.031$) compared with mock. Ad5.pK7-$\Delta 24$–treated mice had a median survival of 105 days, and treatment with Ad5.pK7-$\Delta 24$ in combination with bevacizumab resulted in a median survival of 67 days ($P$ values compared with mock, <0.001 and 0.008). Administration of Ad5-$\Delta 24$RGD alone and in combination with bevacizumab increased median survival (127 and 126 days) significantly when compared with mock (both $P$ values, <0.001) and when compared with Ad300wt ($P < 0.001$ and 0.004). Impressively, 50% of mice in the Ad5/3-$\Delta 24$ (without bevacizumab) group were alive without signs of illness after a follow-up of 320 days ($P$ values compared with mock and Ad300wt, both <0.001). In contrast, only 14% of mice treated with Ad5/3-$\Delta 24$ in combination with bevacizumab were still alive.

Discussion

Renal cell cancer is a common disease with poor prognosis, and patients with metastatic disease cannot be cured with current modalities. One promising experimental approach is adenoviral cancer gene therapy, whose safety and preliminary efficacy have recently been shown in clinical trials (8–11). However, few patients with advanced cancer have been cured, and therefore, room for improvement remains. We have developed a number of novel oncolytic agents that enter tumor cells through CAR-independent mechanisms, which might be useful for avoiding inefficient transduction due to variable or low CAR expression that has been reported common in many tumor types.

Because expression of the receptors relevant for entry of these viruses can vary between individual tumors, we...
sought to identify a method that could predict which virus would be most useful for each tumor. In FACS analysis of tumor cell lines, clinical tumor specimens and tumor xenografts, CD46, HSPGs, and integrins, were expressed to higher degrees than CAR. Consequently, transductional targeting with 5/3, pK7, pK21, and RGD capsid modifications resulted in markedly increased levels of gene delivery and antitumor efficacy. This suggests that it might be useful to analyze receptor expression before deciding which virus would be most useful for each patient, assuming a panel of treatment agents were available.

Although in general surface receptor expression predicted oncolytic potency quite accurately and increases in gene transfer translated into enhanced potency, Ad5/3-Δ24 seemed to perform slightly better than expected. Oncolytic potency may be affected by the speed of virion packaging and release, and as seen previously in many models, these attributes seem to be optimal in Ad5/3-Δ24 (24, 31, 34). This has also been studied formally (40). Whereas Ad5.pK7-Δ24 and Ad5-Δ24RGD were able to significantly prolong survival of mice bearing orthotopic metastatic kidney tumors, Ad5/3-Δ24 was most effective.

In a biodistribution experiment using locoregional delivery in an orthotopic renal cell cancer model, we found that capsid-modified adenoviruses had significantly higher in vivo tropism to tumor tissue than to organs relevant with

Figure 3. Increasing the potency of oncolytic adenoviruses with capsid modifications. Four renal cancer cell lines were infected with oncolytic adenoviruses featuring capsid modifications or wild-type capsids. After 8 to 10 d, cell viability was measured with MTS assay and plotted relative to samples not infected. Bars, SE; stars, significantly improved cell killing over wild-type virus Ad300wt (P < 0.05) at 10 virus particles per cell.

Figure 4. Increasing in vivo antitumor effect by capsid modification of oncolytic adenoviruses. Tumors were induced by subcutaneous inoculation of 786-O cells. When tumors reached the size of 5 × 5 mm², oncolytic adenoviruses and control viruses were injected i.t. on day 1, 2, and 3 (A). Tumor volume on day 1 was set as 100% in each group. Tumors treated with Ad5-Δ24RGD, Ad5.pK7-Δ24, and Ad5/3-Δ24 were significantly smaller than mock-treated tumors (all P values <0.001). To noninvasively assess in vivo replication of the oncolytic adenoviruses, coinfection with a luciferase-expressing virus was done. On day 4 (B) or day 10 (C), D-luciferin was injected i.p., mice were imaged, and photon emission was quantified as a surrogate for virus replication. Note the different scales of the x axis. Bars, SE.
Regarding toxicity, including liver and spleen. i.p. delivery is gaining in popularity for treatment of ovarian cancer, and therefore, the approach is clinically feasible for treatment of peritoneally disseminated cancer (41, 42). Although not widely used for treatment of renal cell cancer, it might be useful in the context of oncolytic viruses, because of high local concentration with reduced systemic exposure. Furthermore, nearly all renal cell cancer patients with metastatic disease feature also an intraperitoneal mass (43). Although the lung is the most common organ for distant metastases also intraperitoneal disease without metastases elsewhere is quite common (44).

Human tumors are more complex than the murine models used here. They are larger, slower growing, feature necrotic, hyperbaric, acidic, and hypoxic regions. Therefore, their treatment may be more difficult, and it is possible that combination with other antitumor approaches might be useful. For renal cell cancer, antiangiogenic approaches are appealing due to universal von Hippel Lindau pathway defects, which result in high expression of vascular endothelial growth factor and subsequent strong angiogenic signaling, as corroborated in recent human trials (45, 46). Despite obvious efficacy, complete responses have been rare, a survival benefit has not yet been shown, and few or no patients are cured. This suggests that antiangiogenic treatment alone may not be effective enough and that its greatest utility might be realized in combination regimens.

A combination of an antiangiogenic agent and oncolytic adenovirus could have additive or synergistic effects because antiangiogenic therapy can reduce intratumoral pressure and reorganize leaky blood vessels, which might facilitate dissemination of oncolytic viruses (47). Alternatively, if oncolytic viruses enter an area of the tumor through the vasculature, they might potentiate the antiangiogenic effect by killing tumoral areas near blood vessels. Furthermore, the two approaches might work on different cell populations.

Conversely, antagonistic action could also result, if intratumoral dissemination is compromised due to...
collapse of vasculature. Moreover, oncolytic viruses may exert part of their effect via release into the systemic circulation and subsequent reinfestation of tumors, but this might be compromised if vessels are not available anymore (47).

To evaluate these aspects, we tested the combination of oncolytic adenoviruses with bevacizumab, the most clinically established antiangiogenic agent. Bevacizumab in combination with Ad5:pK7-242 led to decreased median survival compared with virus alone. Ad5/3-Ad24 alone treatment led to 50% survival at the end of the experiment, whereas the combination with bevacizumab resulted in only 14% survival (all P values <0.001 compared with mock, Ad300wt, and bevacizumab alone). Overall, there was little, if any, evidence of additive or synergistic efficacy. Instead, there were more early deaths due to tumor progression in the combination arms. Therefore, our opinion is that these data do not support combining these viruses with bevacizumab. Instead, consecutive therapy could be more useful to gain the full therapeutic effect of each drug without the risk of antagonism.

In conclusion, our results suggest that capsid-modified oncolytic adenoviruses are effective in murine models of renal cell cancer and thus set the stage for clinical testing of the agents. Since all three capsid modified viruses show antitumor effect, one could alternate viruses within the treatment regimen to avoid virus neutralization by antibodies, which are conformation specific (48). It has been shown that slight changes in the capsid allow escape from preexisting neutralizing antibodies (32, 49). Sequential treatment of patients with antiangiogenic and oncolytic agents might be useful for increasing the therapeutic options available to patients with currently incurable disease.

References

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