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Abstract

Oncolytic adenoviruses have been safe in clinical trials but the efficacy has been mostly limited. All published trials have been performed with serotype 5 based viruses. The expression level of the Ad5 receptor CAR may be variable in advanced tumors. In contrast, the Ad3 receptor remains unclear, but is known to be abundantly expressed in most tumors. Therefore, we hypothesized that a fully serotype 3 oncolytic adenovirus might be useful for treating cancer. Patients exposed to adenoviruses develop high titers of serotype-specific neutralizing antibodies, which might compromise re-administration. Thus, having different serotype oncolytic viruses available might facilitate repeated dosing in humans. Ad3-hTERT-E1A is a fully serotype 3 oncolytic adenovirus controlled by the promoter of the catalytic domain of human telomerase. It was effective in vitro on cell lines representing seven major cancer types, although low toxicity was seen in non-malignant cells. In vivo, the virus had anti-tumor efficacy in three different animal models. Although in vitro oncolysis mediated by Ad3-hTERT-E1A and wild-type Ad3 occurred more slowly than with Ad5 or Ad5/3 (Ad3 fiber knob in Ad5) based viruses, in vivo the virus was at least as potent as controls. Anti-tumor efficacy was retained in presence of neutralizing anti-Ad5 antibodies whereas Ad5 based controls were blocked. In summary, we report generation of a non-Ad5 based oncolytic adenovirus, which might be useful for testing in cancer patients, especially in the context of high anti-Ad5 neutralizing antibodies.
Oncolytic adenovirus based on serotype 3

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Abstract

Oncolytic adenoviruses have been safe in clinical trials but efficacy has been mostly limited. All published trials have been performed with serotype 5 based viruses. The expression level of the Ad5 receptor CAR may be variable in advanced tumors. In contrast, the Ad3 receptor remains unclear but is known to be abundantly expressed in most tumors. Therefore, we hypothesized that a fully serotype 3 oncolytic adenovirus might be useful for treating cancer. Patients exposed to adenoviruses develop high titers of serotype specific neutralizing antibodies, which might compromise re-administration. Thus, having different serotype oncolytic viruses available might facilitate repeated dosing in humans. Ad3-hTERT-E1A is a fully serotype 3 oncolytic adenovirus controlled by the promoter of the catalytic domain of human telomerase. It was effective in vitro on cell lines representing seven major cancer types, while low toxicity was seen in non-malignant cells. In vivo, the virus had anti-tumor efficacy in three different animal models. Although in vitro oncolysis mediated by Ad3-hTERT-E1A and wild type Ad3 occurred more slowly than with Ad5 or Ad5/3 (Ad3 fiber knob in Ad5) based viruses, in vivo the virus was at least as potent as controls. Anti-tumor efficacy was retained in presence of neutralizing anti-Ad5 antibodies while Ad5 based controls were blocked. In summary, we report generation of a non-Ad5 based oncolytic adenovirus, which might be useful for testing in cancer patients, especially in the context of high anti-Ad5 neutralizing antibodies.
**Introduction**

Gene therapy and oncolytic viruses are promising ways of treating cancers incurable with current therapies. Treatment approaches take advantage of molecular differences between normal and tumor cells. Various strategies are currently in clinical development with adenoviruses as the most popular vehicle 1, 2. Adenoviruses are useful tools for both gene delivery and oncolytic cell killing. In the former approach, E1-deleted, “replication deficient” viruses are typically used, while the latter harnesses the cell killing potential of viral replication *per se*. Typically, viruses are modified to retain replication competence in tumor but not normal cells 3.

An optimal oncolytic adenovirus would infect and replicate only in cancer cells and would be potent enough to kill all cancer cells before the immunological response neutralizes the virus. Making certain deletions or adding tissue specific promoters can make the virus more selective while adding transgenes can make the approach more potent 4. Nevertheless, in the context of advanced tumor masses, it is unlikely that a single round of treatment would eradicate the tumor. Although in theory virus replication can proceed as long as there are tumor cells, in practice intratumoral barriers limit efficacy 5, 6. Therefore, re-administration of the virus is likely to be required for increasing efficacy. A key factor limiting systemic re-administration is the neutralizing antibody response induced by the virus 7, 8.

More than 50 serotypes of adenoviruses have been identified. Nevertheless, all cancer trials utilizing recombinant adenoviruses have featured subgroup C viruses, which in most cases has been Ad5. Furthermore, while biological selection has been used to identify a non-Ad5 based oncolytic virus 9, and an E1 containing but non-selective Ad11p based virus has been recently reported 10, most tumor selective viruses have been based on Ad5. According to previous publications, there are several advantages to using serotype chimeric capsids in the context of an otherwise serotype 5 virus 11-15. Even though serotype chimerism allows partial escape from pre-existing neutralizing antibodies against Ad5, and vice versa, the fact that most of the virus capsid is...
still from Ad5 renders the escape incomplete 8. Therefore, it would be advantageous to use a fully non-Ad5 virus, especially in the context of high titers of neutralizing antibodies against Ad5 16. This situation might arise as a result of natural infection or subsequent to treatment with an Ad5 based oncolytic virus.

In this study, we hypothesised that an oncolytic adenovirus based on serotype 3 could be constructed and would allow tumor cell killing. Some candidate receptors for adenovirus 3 have been proposed, but these findings have been disputed in other publications and therefore the cellular entry of Ad3 remains poorly understood 17-20. Nevertheless, it has been possible to show that the receptor(s) most relevant for Ad3 are highly expressed in many cancer cell types 15, 17, 21-25 in contrast to the Ad5 receptor CAR which may be frequently downregulated in advanced tumors 13, 23, 26-28.

Wild type Ad3 causes mainly respiratory infections and conjunctivitis in humans 29. The complete DNA sequence was reported in 2005 and it has only 62.75% identity with serotype 5. The genomic organisation is similar to other human Ads having early and delayed early transcription units, including late and major late units 18.

In recent decades research on telomeres and telomerases has progressed rapidly. Telomerase activation is a critical step in human carcinogenesis, and most human tumors feature activity of telomerase. This feature is closely linked with activity of catalytic subdomain hTERT promoter, which has been suggested to be active in most human tumors and may therefore be a useful tumor specific promoter 30-33.

In this study, we constructed the first fully serotype 3 based oncolytic adenovirus Ad3-hTERT-E1A. Efficacy analysis revealed oncolytic potency in all tumor cell lines tested, except a line known to lack the Ad3 receptor. The virus was attenuated in non-tumor cells. Efficacy was also seen in three animal models of human cancer and the virus was not blocked by anti-Ad5 antibodies.
Results

Construction and replication of Ad3-hTERT-E1A
Following standard cloning procedures, Ad3-hTERT-E1A was rescued and grown to large scale. PCR and sequencing was used to confirm the structure of the virus (Fig. 1). Methodology established for Ad5 based viruses had to be optimized to account for the slower replication of Ad3 wild type and Ad3-hTERT-E1A. To estimate functional titer, the usual 10-day cytopathic effect assay was not sufficient and therefore we developed a more dynamic progressive infectivity assay (Fig. 1c), where cytopathic effect was allowed to develop until the titer plateaued. We believe this represents the actual functional titer of the virus.

Oncolytic potency of Ad3-hTERT-E1A on cancer cell lines in vitro
The ability of Ad3-hTERT-E1A to kill cancer cells in vitro was analyzed by infecting monolayers representing seven different tumor types (Fig. 2 and Figure S1). In all malignant cell lines Ad3-hTERT-E1A showed complete oncolysis while the non-replicative Ad5/3luc1 virus showed no cell killing (p<0.05). However, as anticipated based on experience from growing and titering the virus, Ad3-hTERT-E1A was somewhat slower than the serotype 5 viruses.

Tumor selectivity of Ad3-hTERT-E1A
For analyzing tumor selectivity, we infected HUVEC (human umbilical vein endothelial cells) and FSH173WE (fibroblasts) with the same viruses and performed cell killing assays (Fig. 3). No cell killing was seen at low virus concentrations and at higher doses Ad3-hTERT-E1A was less toxic than the control viruses Ad3wt, Ad5wt, Ad5/3-hTERT-E1A, Ad5/3-Δ24 (0.1, 1 and 10 VP/cell P<0.05). From previous work with Ad5/3 chimeras, we knew that LNM-35/EGFP cannot be infected with Ad5/3 and thus may lack the Ad3 receptor 34. Fittingly, no cell killing was seen with Ad3-hTERT-E1A or Ad3 wild type.
**Oncolytic potency in vivo**

We tested the efficacy of Ad3-hTERT-E1A in mice bearing PC-3MM2 prostate cancer xenografts ([Fig. 4](#)). Ad3-hTERT-E1A was able to reduce tumor growth significantly in comparison to PBS injections ($P=0.0035$). Interestingly, even though the virus was slower than positive controls *in vitro*, there was no difference in efficacy *in vivo*. In mice with A549 xenografts, the Ad3-hTERT-E1A group had the smallest tumors from day 17 onwards. At day 17 a borderline difference ($P=0.051$) could be seen comparing Ad3-hTERT-E1A and the PBS. At day 30 Ad3-hTERT-E1A had reduced tumor growth significantly ($P=0.01$) more than Ad5/3-hTERT-E1A, which is known to be a highly potent oncolytic adenovirus 35, 36. Therefore, Ad3-hTERT-E1A proved highly potent *in vivo*.

Subcutaneous tumors may not be optimal surrogates of human cancers and therefore we proceeded to utilize an orthotopic model featuring intraperitoneally disseminated carcinomatosis induced with luciferase expressing SKOV3Luc cells ([Figs. 4c,d](#)). Ad3-hTERT-E1A significantly reduced the luciferase signal as compared to PBS treated mice ($P<0.0001$). There was no difference in efficacy between the different replication competent viruses. Ad3-hTERT-E1A extended the median survival of mice from 34 to 46 days (PBS *versus* Ad3-hTERT-E1A), which resulted in a significant difference in Kaplan-Meier survival analysis ($P=0.01$). One out of the 7 mice treated with Ad3-hTERT-E1A survived until the end of the experiment (day 120) and may have been cured as no evidence of tumor could be detected in luciferase imaging or autopsy and its behavior was normal.

**Neutralization of Ad3-hTERT-E1A by anti-Ad5 antibodies**

Many humans have circulating anti-Ad5-antibodies and they can also become induced as a result of treatment with oncolytic adenovirus. Since Ad3-hTERT-E1A is based fully on serotype 3, it might
be able to escape neutralization by anti-Ad5 antibodies. This was tested in mice with orthotopic SKOV3-Luc tumors. Anti-Ad5 antibodies blocked Ad5 wild type (P=0.003, day 21) and the 5/3 chimeric virus (P=0.03, day 14) (knob from Ad3 but otherwise Ad5) but not Ad3-hTERT-E1A (Fig. 5).
Discussion

Oncolytic adenoviruses are promising anti-tumor agents. However, achieving efficacy against advanced solid tumors may require multiple rounds of administration. Although efficacy of intratumoral injection does not seem to be compromised by neutralizing antibodies, metastatic disease might well benefit from systemic delivery or from the prolonged systemic dissemination which results from intratumoral injection. These, however, are attenuated by high neutralizing antibodies titers. Therefore, it would be advantageous if a non-Ad5 based oncolytic adenovirus would be available for use in patients with high anti-Ad5 neutralizing antibodies titers, or previously treated with Ad5 agents.

Currently, little has been published on serotype 3 adenoviruses in the context of gene therapy. However, a wild type Ad3 virus was studied under normoxic and hypoxic conditions. The same group recently published ColoAd1, a complex Ad3/Ad11p chimeric virus, which was made by pooling an array of serotypes and then passaging the pools under conditions that invite recombination between serotypes. These highly diverse viral pools were then placed under stringent directed selection to generate and identify potent agents. The authors reported that the Ad3/Ad11p chimeric virus was 2-3 orders of magnitude more potent and selective than the parent serotypes or the most clinically advanced oncolytic Ad, ONYX-015, in vitro. These results were further supported by in vivo and ex vivo studies. However, the safety and efficacy of this agent may be difficult to predict due to the complex chimerism involved. In the same publication a direct comparison of wild type Ad5 and Ad3 was reported on some normal and tumor cell lines with data well in accord to our findings.

We constructed Ad3-hTERT-E1A, which contains the hTERT promoter up-stream of the E1A transcription site for tumor specific replication. hTERT is universally expressed in tumors but not
normal tissues and may therefore be a useful pan-carcinoma promoter 42. In vitro, the virus seemed slower in reaching the maximum titer and the functional infectivity assay required 35 days to show maximum cytopathic effect. Although both fully Ad3 viruses eventually killed all cancer cells in a cytotoxicity assay, they seemed slower than Ad5 based oncolytic agents, including a 5/3 serotype chimera. In non-cancer cells, Ad3-hTERT-E1A was less toxic than the control viruses, including wild type Ad3. Ad3-hTERT-E1A was not able to kill cells that lack the Ad3 receptor. Despite being somewhat slower than controls in vitro, Ad3-hTERT-E1A was at least as oncolytic as Ad5 and Ad5/3 based controls in vivo. This finding has two interpretations. First, faster replication may not automatically mean better tumor control. Slower dissemination might result in fewer necrotic or hypoxic areas which can compromise viral penetration. Second, expression of viral receptors relevant for replication may not be expressed identically in vitro and in vivo. Ad3-hTERT-E1A binds to the currently unknown Ad3 receptor. However, this receptor has been proposed to be expressed to high degree in tumor cells 15, 17, 21-25, 43. Also, it does not seem to be downregulated during carcinogenesis, as proposed for the Ad5 receptor CAR 13, 23, 26-28. Identification of the receptor might help in understanding of the behaviour of Ad3-hTERT-E1A.

One potential concern with using the hTERT promoter is toxicity to telomerase positive normal tissue stem cells. However, since an hTERT regulated oncolytic adenovirus (based on serotype 5) has been safely used in phase 1 trials 44, the promoter may be feasible for human use. Nevertheless, Ad3-hTERT-E1A has different tropism than Ad5. In particular, Ad3 based viruses may be able to enter stem cell type cells better than Ad5 20, 35, 45, 46. In theory, this might be a safety concern in the context of normal tissue stem cells, some of which might be positive for the Ad3 receptor and hTERT. However, since most humans have sustained wild type adenovirus type 3 infections without detectable symptoms of normal tissue stem cell loss, these cells may have other ways of
protecting against adenovirus. Also since the mitotic rate of normal stem cells is low, the activity of hTERT might be lower compared to aggressive tumors.

In summary, we have constructed the first selectively oncolytic adenovirus fully based on serotype 3. This may also be the first non-Ad5 based oncolytic adenovirus controlled by a tumor specific promoter. Efficacy was seen in tumor cell lines and the virus was at least as potent as Ad5 or Ad5/3 based controls in several murine models of human cancer and the virus was not blocked by anti-Ad5 antibodies. Further studies are needed to study murine biodistribution and toxicity, prior to possible human testing. Eventually, Ad3-hTERT-E1A might be a useful agent for clinical testing in patients with anti-Ad5 neutralizing antibodies or previously treated with an Ad5 based agent.
**Materials and Methods**

**Cell culture**

293 cells were purchased from Microbix (Toronto, Canada). 293-2v6-11 cells contain an ponasteron-inducible E4orf6 region 47. 911-1c11 were kept in 1mg/ml G418 18. SKOV3.ip1 ovarian adenocarcinoma cell line was obtained from Dr. Price (M. D. Anderson Cancer Center, Houston, TX). Firefly luciferin-expressing ovarian adenocarcinoma cell line SKOV3-luc was kindly provided by Dr. Negrin (Stanford Medical School, Stanford, CA). PC-3MM2 highly metastatic hormone refractory subline of prostate carcinoma was a kind gift of Isaiah J. Fidler (MD Anderson Cancer Center, Houston, TX). LNM35/EGFP, a highly lymphogenous metastatic subline of a human large cell carcinoma of the lung, was provided by Takashi Takahashi (The Honda Research Institute, Japan). The Human Umbilical Vein Endothelial Cells (HUVEC) were bought from Lonza (Basel, Switzerland). The following cell lines were obtained from ATCC (Manassas, VA): ACHN kidney cell carcinoma, A549 lung adenocarcinoma, FHS173WE human fibroblast cell line, HCT116 colorectal carcinoma, CAMA-1 breast adenocarcinoma, PANC-1 pancreatic epithelioid carcinoma. All cell lines were cultured under recommended conditions.

**Construction of Ad3-hTERT-E1**

For cloning of Ad3-hTERT-E1 three different PCR were performed on Ad3-wt plasmid pKSB2Ad3wt 18, two of them to exclude the original TATA-box from Ad3wt sequence. First PCR product included left ITR with MluI digestion site until the TATA-box (forward primer: 5’-CAT GGT ACC CAA GTG TGT CGC TGT CGA GT-3’, reverse primer 5’-CAT GAT ATC AGC GAT CAG CTG ACA CCT AC-3’; adding KpnI site in front and EcoRV site at the end), second started directly after the TATA-box until first half of E1A region (forward primer: 5’-CAT GAT ATC GTG ACC CAA GTG TGT CGC TGT CGA GT-3’, reverse primer 5’-CAT GAT ATC GTG CCA GCG AGA AGA GTT TT-3’, reverse primer 5’-CAT TCT AGA GCG AGC AGC ACA ATA GTT CTT TCA-3’; adding EcoRV site at front and XbaI at the end). A third PCR amplified 1600
bp of the end of adenovirus type 3 including right ITR. The respectively digested first two PCR products around the TATA-box were cloned into KpnI/XbaI digested pUC19 cloning vector resulting in pGBAd3E1, including a TATA-box excluded start of Ad3 sequence. hTERT promoter derived from pBT255 was digested with KpnI/XhoI, blunted, and cloned in front of the E1 region by EcoRV digestion of pGBAd3E1 resulting in pGBAd-3hTERT-E1.

The third PCR product with the end of Ad3 sequence was now cloned into NotI/blunted pShuttle, followed by digestion with HindIII, and subsequently cloned into HindIII digested pGBAd3-hTERT-E1 resulting in pSGBAd3-hTERT-E1 as shuttle vector with end and modified start of adenovirus 3 genome. Finally pSGBAd3-hTERT-E1 was digested with XhoI/MscI, resulting in the plasmid being opened up in between the modified start and preserved end of adenovirus 3 sequence, and homologous recombined with MluI digested and thus backbone-free pKSB2Ad3wt, to reduce background due to same resistance gene and achieve a plasmid with Ad3-hTERT-E1 sequence (pKGB-Ad3-hTERT-E1).

The recombinant Ad3-hTERT-E1A virus was rescued by transfecting 911-1c11 cells with MluI-digested pKGB-Ad3-hTERT-E1. Subsequent amplification of a first large Ad3-hTERT-E1A virus stock was done in 293-2v6-11 cells, followed by 2nd amplification in A549 cells. All tests done in this paper are made with the 2nd virus stock. After amplification the viruses were purified on double cesium chloride gradients. The stocks were confirmed not to have serotype 3 wild type or serotype 5 contamination by PCR. Later the hTERT (295bs) insertion area was controlled to be correct by sequencing the purified DNA of the virus. The area was sequenced with ten primers and the expected sequence for 1244bps was seen (Fig. 1).

Other adenoviruses

Please see table 1 for adenoviruses used in this study.
**Progressive infectivity assay**

The cells were plated on 96-well plates, 10 000 cells/well and rows were infected the next day by lowering dilution from $10^{-5}$ to $10^{-12}$ of Ad3-hTERT-E1A, each dilution in ten duplicates. Infection was done in DMEM, 2% FBS. Plates were observed by microscope and cytopathic effect of the virus wells were compared to the mock wells. From the observations pfu/ml was calculated in the similar way than in TCID$_{50}$ (Adeasy manual, Agilent Technologies, Inc. 2008). The media (DMEM, 5% FBS) was changed every 4 to 7 days.

**In vitro cytotoxicity assays**

Cells were plated at 10 000 cells/well on 96 well plates. The next day, cells in triplicates or quadruplicates were infected with the viruses at 0.1 to 100 VP/cell. Infection was done in 2% fetal bovine serum (FBS). The plates were regularly observed and the growth media was changed every 3-5 days. Six to twenty days later, as optimal for each line, cell viability was analyzed with MTS assay (Cell Titer 96 AQueous One Solution Cell Proliferation Assay, Promega). MTS analyses were performed on the following days: PC-3MM2 on day 8, A549 on day 17, HCT116 on day 6, SKOV3.ip1 on day 18, HUVEC on day 11, FSH174WE on day 13, LNM-35/EGFP on day 10, CAMA-1 on day 11, PANC-1 on day 20 and ACHN on day 11.

**Animal experiments**

All animal experiments were approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland. Mice where frequently monitored for their health status and euthanized as soon as signs for pain or distress was noticed. For the subcutaneous model female NMRI nude mice were used (Charles River, Germany). They were ordered at the age of 3-4 weeks and quarantined for 2 weeks. For the intraperitoneal model female
fox chase SCID mice (Charles River, Germany) were used. They were ordered at the age of 6-7 weeks and quarantined for 4 months.

**Subcutaneous animal experiment**

Mice received subcutaneous injections of $2 \times 10^6$ PC-3MM2 or $5 \times 10^6$ A549 cells into both flanks. After 11 (A549) or 13 (PC-3MM2) days tumors reached injectable size and were treated with $10^8$VP in 50µl PBS. Controls received PBS only. The injection was repeated after one and two weeks. Tumors were measured frequently and the volume was calculated $V = L \times H^2 \times 0.52$. The measured volume before the first treatment was regarded as 100% and the tumor growth was compared to this. With the A549 group some of the tumors treated with PBS grew aggressively and the mice had to be euthanized after two and a half weeks.

**Intraperitoneal animal experiment**

An orthotopic model of peritoneally disseminated ovarian cancer was developed by injecting $5 \times 10^6$ SKOV3-luc cells intraperitoneally in 300µl of pure DMEM into SCID mice. After three days the mice ($n=7$) were imaged noninvasively and treated intraperitoneally by injecting PBS or $10^8$VP in PBS per mouse. The mice were imaged on day 3, 7, 14, 21, 28, 35 and 42 using IVIS 100 (Xenogen, Alameda, CA, USA) to estimate the number of tumor cells in the mice. For bioluminescence imaging, 150mg/kg D-luciferin (Promga, Madison, WI, USA) was injected intraperitoneal and captured 10min later with 10s exposure time, 1f/stop, medium binning and open filter. During imaging the mice were in isoflurane gas anesthesia. Images were overlaid with Living Image 2.50 (Xenogen). Total flux (photons/s) was measured by drawing regions of interest (ROI) around the peritoneal area of the mice. Background was subtracted.

**Neutralizing antibody experiment**
Anti-Ad5 neutralizing antibodies were produced as reported 8. Briefly, a non-replicative Ad5(GL) virus was injected subcutaneously on days 0, 3 and 6 into 16 immunocompetent NMRI mice. A month later blood was collected and allowed to clot. The serum (which contains the Ad5 neutralising antibodies) was then heated to 56°C for 40 minutes to inactivate complement and frozen.

In the second phase of the experiment 5x10^6 SKOV3-luc cells (human ovarian cancer) were injected intraperitoneally into SCID mice on day 0 (N=5/group). For treatment of the mice, 10^9 VP with and without the Ad5 neutralizing antibody serum was injected intraperitoneally on days 3, 7 and 14. For each mouse 50ul of serum was mixed with 50ul of PBS containing the virus. The number of tumor cells as a function of time was estimated with repeated luciferase imaging of live animals as in the intraperitoneal efficacy experiment above.

**Statistical analysis**

Nonparametric Mann-Whitney test (SPSS 15.0 for Windows) was used to compare two independent samples for all the *in vitro* and some of the *in vivo* data. The analysis of tumor size was performed using a repeated measures model with PROC MIXED (SAS Ver. 9.1). Models were run with the tumor size measurements in the natural metric and log transformed. The effects of treatment group, time in days and the interaction of treatment group and time were evaluated by F tests. Curvature in the models was tested for by a quadratic term for time. The a priori planned comparisons of specific differences in predicted treatment means averaged over time and at the last timepoint were computed by t-statistics. For the A549 experiment we compared the groups at 17 and 30 days and the PC-3MM2 at 30 days and time-averaged. Survival was evaluated using the Kaplan-Meier survival plot with log rank regression (SPSS 15.0 for Windows). For all analyses a two-sided P value of <0.05 was deemed statistically significant.
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**Supplementaty material**

**Figure S1**

Cell killing assays with CAMA-1 (breast cancer), PANC-1 (pancreas cancer) and ACHN (renal cancer) cells. Ad3-hTERT-E1A showed cell killing in all of these cancer types. In all experiments Ad3-hTERT-E1A was significantly more efficient than the replication deficient control Ad5/3luc1 in 100VP/cell (P<0.05). Bars indicate SE.

**Figure S2**

In vivo antitumor efficacy of Ad3-hTERT-E1A. A bioluminescence picture taken on day 28.

**Figure S3**

An electron microscope picture of the Ad3-hTERT-E1A.
References


Figure legends

Fig. 1
(a) Structure of Ad3-hTERT-E1A. The hTERT promoter (295bp) was inserted to replace the TATA-box in front of the E1A region of the adenovirus 3. Arrows indicate the sequenced area (1244bp). The sequencing confirms that the backbone is adenovirus serotype 3, that the hTERT promoter is in the right location and that the promoter is correct.
(b) Progressive infectivity assay with Ad3-hTERT-E1A. The cells were plated on 96-well plates and rows infected by lowering dilution from $10^{-5}$ to $10^{-12}$ of Ad3-hTERT-E1A, each dilution in ten duplicates. Plates were observed by microscope and virus wells were compared to the uninfected mock wells. From the observations pfu/ml was calculated in the similar way than in TCID$_{50}$. The media (DMEM, 5% FBS) was changed every 4 to 7 days. The pfu/ml titer plateaus after 30 days, suggesting slow replication kinetics for Ad3-hTERT-E1A in vitro. The experiment was performed on three cell lines, and twice on A549 cells.

Fig. 2
Cell killing assays with cancer cell lines. PC-3MM2 (prostate cancer), A549 (lung cancer), HCT116 (colon cancer) and SKOV3.ip1 (ovarian cancer) cells were used. Ad3-hTERT-E1A showed cell killing in all cell lines (P<0.05 versus Ad5/3luc1, the replication deficient control, at 10 VP/cell). Ad5wt, Ad5/3-hTERT-E1A and Ad5/3-Δ24 killed cells faster than Ad3-based viruses. Bars indicate SE.

Fig. 3
Cell killing assays with non-malignant and Ad3 receptor deficient cells. HUVEC (human umbilical vein endothelial cells) and FSH173WE (fibroblasts) were used to represent non-tumor cells. Ad3-hTERT-E1A did not differ from replication deficient Ad5/3luc1 at low doses and killed
significantly less cells than positive controls Ad3wt, Ad5wt, Ad5/3-Δ24 at 0.1, 1 and 10 VP/cell (P<0.05). From previous studies 34 it was known that LNM-35 (lung cancer) cells lack the Ad3 receptor. Ad3wt and Ad3-hTERT-E1A did not show any cytotoxicity on these cells. Bars indicate SE.

**Fig. 4**

*In vivo* efficacy of Ad3-hTERT-E1A

(a) PC-3MM2 (highly metastatic hormone refractory prostate carcinoma) tumors were grown in nude mice. 10⁹ VP was injected into each tumor weekly for a total of three injections and tumors were measured every 2-3 days. Tumors treated with Ad3-hTERT-E1A grew more slowly than PBS treated tumors (P=0.0035). Bars indicate SE.

(b) Similar data were seen in mice with A549 (lung cancer) tumors although the PBS group had to be terminated early due to large tumor size. On day 17 a difference bordering on significance (P=0.051) was seen comparing PBS and Ad3-hTERT-E1A. Also, on day 30 Ad3-hTERT-E1A was found significantly (P=0.01) better than Ad5/3-hTERT-E1A, a highly potent positive control. Bars indicate SE.

(c) Luciferase expressing SKOV3-luc ovarian cancer cells were grown intraperitoneally in SCID mice. A single intraperitoneal 10⁹ VP virus injection was performed and the number of tumor cells as a function of time was estimated with repeated luciferase imaging of live animals. A significant difference in the luciferase signal was seen between all virus treated groups and the PBS group (P<0.0001), and there were no differences between virus groups. In this experiment Ad5wt could be included as an additional positive control. Bars indicate SE.

(d) In survival analysis, all virus treated groups survived longer than the PBS group (P≤0.01). The only long term survivor from the experiment was one out of 7 mice from the Ad3-hTERT-E1A
group. It was healthy and tumor free in imaging and autopsy at 120 days, when the experiment was ended.

**Fig. 5**

*In vivo* Ad5 neutralising antibody experiment.

A non-replicative Ad5(GL) virus was injected subcutaneously on days 0, 3 and 6 into immunocompetent NMRI mice. A month later serum containing the Ad5 neutralising antibodies were collected, heated and frozen. In the second phase of the experiment $5 \times 10^6$ SKOV3-luc cells (human ovarian cancer) were injected intraperitoneally into SCID mice on day 0. $10^9$VP diluted in PBS, with or without the Ad5 neutralizing antibody serum, was then injected intraperitoneally on days 3, 7 and 14. The number of tumor cells as a function of time was estimated with repeated luciferase imaging of live animals. A significant difference in luciferase signal was seen between Ad5wt mixed with serum and Ad5wt without serum ($P=0.003$, day 21) and between Ad5/3-hTERT-E1A with and without serum ($P=0.03$, day 14). Ad3-hTERT-E1A was not blocked by the anti-Ad5 serum. Bars indicate SE.
Table 1. Adenoviruses used in this study

<table>
<thead>
<tr>
<th>Virus name</th>
<th>Backbone serotype</th>
<th>Fiber knob serotype</th>
<th>E1A</th>
<th>Ratio (VP/ pfu)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad5 wt</td>
<td>5</td>
<td>5</td>
<td>Wild type</td>
<td>19</td>
<td>ATCC (Ad300 wt strain)</td>
</tr>
<tr>
<td>Ad5/3-hTERT-E1A</td>
<td>5</td>
<td>3</td>
<td>Controlled by hTERT promoter (mediates selectivity to telomerase active cells)</td>
<td>10</td>
<td>35 (Named here Ad5/3-hTERT-Δgp)</td>
</tr>
<tr>
<td>Ad5/3-Δ24</td>
<td>5</td>
<td>3</td>
<td>24bp deletion (mediates selectivity to p16/Rb pathway mutant tumor cells)</td>
<td>22</td>
<td>48</td>
</tr>
<tr>
<td>Ad5/3luc1</td>
<td>5</td>
<td>3</td>
<td>Deleted (makes virus replication deficient)</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Ad3 wt</td>
<td>3</td>
<td>3</td>
<td>Wild type</td>
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<td>18</td>
</tr>
<tr>
<td>Ad3-hTERT-E1A</td>
<td>3</td>
<td>3</td>
<td>Controlled by hTERT promoter (mediates selectivity to telomerase active cells)</td>
<td>9</td>
<td>This article</td>
</tr>
</tbody>
</table>
Fig. 2 Hemminki et al
Fig. 3 Hemminki et al
Fig. 4 Hemminki et al
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