

ONCOLYTIC ADENOVIRAL VECTORS AND METHODS AND USES RELATED THERETO

Field of the invention

[0001] The present invention relates to the fields of life sciences and medicine. Specifically, the invention relates to cancer therapies. More specifically, the present invention relates to oncolytic adenoviral vectors and cells and pharmaceutical compositions comprising said vectors. The present invention also relates to a use of said vectors in the manufacture of a medicament for treating cancer in a subject and a method of treating cancer in a subject. Furthermore, the present invention relates to methods of producing GM-CSF in a cell and increasing tumor specific immune response in a subject, as well as uses of the oncolytic adenoviral vector of the invention for producing GM-CSF in a cell and increasing tumor specific immune response in a subject.

Background of the invention

[0002] Cancer can be treated with surgery, hormonal therapies, chemotherapies and/or radiotherapies but in many cases, cancers, which are often characterized by an advanced stage, cannot be cured with present therapeutics. Therefore, novel cancer cell targeted approaches such as gene therapies are needed.

[0003] During the last twenty years gene transfer technology has been under intensive examination. The aim of cancer gene therapies is to introduce a therapeutic gene into a tumor cell. These therapeutic genes introduced to a target cell may for example correct mutated genes, suppress active oncogenes or generate additional properties to the cell. Suitable exogenous therapeutic genes include but are not limited to immunotherapeutic, anti-angiogenic, chemoprotective and "suicide" genes, and they can be introduced to a cell by utilizing modified virus vectors or non-viral methods including electroporation, gene gun and lipid or polymer coatings.

[0004] Requirements of optimal viral vectors include an efficient capability to find specific target cells and express the viral genome in the target cells. Furthermore, optimal vectors have to stay active in the target tissues or cells. All these properties of viral vectors have been developed during the last decades and for example retroviral, adenoviral and adeno-associated viral vectors have been widely studied in biomedicine.

[0005] To further improve tumor penetration and local amplification of the anti-tumor effect, selectively oncolytic agents, e.g. conditionally replicat-

ing adenoviruses, have been constructed. Oncolytic adenoviruses are a promising tool for treatment of cancers. Tumor cells are killed by oncolytic adenoviruses due to a replication of the virus in a tumor cell, the last phase of the replication resulting in a release of thousands of virions into the surrounding tumor tissues for effective tumor penetration and vascular re-infection. Tumor cells allow replication of the virus while normal cells are spared due to engineered changes in the virus genome, which prevent replication in non-tumor cells.

[0006] In addition to replication mediated cell killing, oncolytic adenoviruses can also be armed with different therapeutic transgenes. This approach combines the advantages of conventional gene delivery with the potency of replication competent agents. One goal of arming viruses is induction of an immune reaction towards the cells that allow virus replication. Virus replication alone, although immunogenic, is normally not enough to induce effective anti-tumor immunity. To strengthen induction of therapeutic immunity, viruses can be armed with stimulatory proteins such as cytokines for facilitation of the introduction of tumor antigens to antigen presenting cells such as dendritic cells, and their stimulation and/or maturation. Introduction of immunotherapeutic genes into tumor cells and furthermore, translation of the proteins, leads to activation of the immune response and efficient destruction of tumor cells. The most relevant immune cells in this regard are natural killer cells (NK) and cytotoxic CD8+ T-cells.

[0007] Adenoviruses are medium-sized (90–100nm), nonenveloped icosahedral viruses, which have double stranded linear DNA of about 36 kilo base pairs in a protein capsid. The viral capsid has fiber structures, which participate in attachment of the virus to the target cell. First, the knob domain of the fiber protein binds to the receptor of the target cell (e.g. CD46 or coxsackievirus adenovirus receptor (CAR)), secondly, the virus interacts with an integrin molecule and thirdly, the virus is endocytosed into the target cell. Next, the viral genome is transported from endosomes into the nucleus and the replication machinery of the target cell is utilized also for viral purposes (Russell W.C. 2000, J General Virol 81, 2573-2604).

[0008] The adenoviral genome has early (E1-E4), intermediate (IX and IVa2) and late genes (L1-L5), which are transcribed in sequential order. Early gene products affect defense mechanisms, cell cycle and cellular metabolism of the host cell. Intermediate and late genes encode structural viral proteins for production of new virions (Wu and Nemerow, 2004, Trends

Microbiol 12: 162–168; Russell W.C. 2000, J General Virol 81, 2573-2604; Volpers C. and Kochanek S. 2004, J Gene Med 6 suppl 1, S164-71; Kootstra N.A. and Verma I.M. 2003, Annu Rev Pharmacol Toxicol 43, 413-439).

[0009] More than 50 different serotypes of adenoviruses have been found in humans. Serotypes are classified into six subgroups A-F and different serotypes are known to be associated with different conditions i.e. respiratory diseases, conjunctivitis and gastroenteritis. Adenovirus serotype 5 (Ad5) is known to cause respiratory diseases and it is the most common serotype studied in the field of gene therapy. In the first Ad5 vectors E1 and/or E3 regions were deleted enabling insertion of foreign DNA to the vectors (Danthinne and Imperiale 2000). Furthermore, deletions of other regions as well as further mutations have provided extra properties to viral vectors. Indeed, various modifications of adenoviruses have been suggested for achieving efficient anti-tumor effects.

[0010] For example, patent EP1377671 B1 (Cell Genesys, Inc.) and application US2003/0104625 A1 (Cheng C. et al.) describe an oncolytic adenoviral vector encoding an immunotherapeutic protein granulocyte-macrophage colony-stimulating factor (GM-CSF). Also, publication EP1767642 A1 (Chengdu Kanghong Biotechnologies Co., Ltd.) points out oncolytic adenoviral vectors having improved effects on human immune response.

[0011] Still, more efficient and accurate gene transfer as well as increased specificity and sufficient tumor killing ability of gene therapies are warranted. Safety records of therapeutic vectors must also be excellent. The present invention provides a cancer therapeutic tool with these aforementioned properties by utilizing both oncolytic and immunotherapeutic properties of adenoviruses in a novel and inventive way.

Summary

[0012] The object of the disclosure is to provide novel methods and means for achieving the above-mentioned properties of adenoviruses and thus, solving the problems of conventional cancer therapies. More specifically, the disclosure provides novel methods and means for gene therapy.

[0013] The present application describes construction of recombinant viral vectors, methods related to the vectors, and their use in tumor cells lines, animal models and cancer patients.

[0014] The present disclosure relates to an oncolytic adenoviral vector comprising an adenovirus serotype 5 (Ad5) nucleic acid backbone, a 24 bp

deletion (D24) in the Rb binding constant region 2 of adenoviral E1 and a nucleic acid sequence encoding a granulocyte-macrophage colony-stimulating factor (GM-CSF) in the place of the deleted gp19k/6.7K in the adenoviral E3 region.

[0014a] In a first aspect, there is provided an oncolytic adenoviral vector, characterized by comprising an adenovirus serotype 5 (Ad5) nucleic acid backbone, a 24 bp deletion (D24) in the Rb binding constant region 2 of E1 and a nucleic acid sequence encoding a human granulocyte-macrophage colony-stimulating factor (GM-CSF) in the place of the deleted gp19k/6.7K in the E3 region.

[0015] The present disclosure further relates to a cell comprising the adenoviral vector of the disclosure.

[0015a] In a second aspect, there is provided a cell, characterized by comprising the adenoviral vector as defined above.

[0016] The present disclosure also relates to a pharmaceutical composition comprising the adenoviral vector of the disclosure.

[0016a] In a third aspect, there is provided a pharmaceutical composition, characterized by comprising the adenoviral vector as defined above.

[0017] In a fourth aspect, there is provided a use of the adenoviral vector as defined above in the manufacture of a medicament for treating cancer in a subject.

[0018] The present disclosure also relates to a method of treating cancer in a subject, wherein the method comprises administration of the vector or pharmaceutical composition of the disclosure to a subject.

[0019] Furthermore, the present disclosure also relates to a method of producing GM-CSF in a cell, wherein the method comprises:

- a) carrying a vehicle comprising an oncolytic adenoviral vector of the disclosure to a cell, and
- b) expressing GM-CSF of said vector in the cell.

[0019a] In a fifth aspect, there is provided a method of producing GM-CSF in a cell, characterized in that the method comprises:

- a) carrying a vehicle comprising an oncolytic adenoviral vector as defined above to a cell, and
- b) expressing GM-CSF of said vector in the cell.

[0020] Furthermore, the present disclosure also relates to a method of increasing tumor specific immune response in a subject, wherein the method comprises:

- a) carrying a vehicle comprising an oncolytic adenoviral vector of the disclosure to a target cell or tissue,
- b) expressing GM-CSF of said vector in the cell, and
- c) increasing amount of cytotoxic T cells and/or natural killer cells in said target cell or tissue.

[0021] In a sixth aspect, there is provided a use of the oncolytic adenoviral vector as defined above for producing GM-CSF in a cell.

[0022] Still, the present disclosure relates to the oncolytic adenoviral vector of the disclosure for producing GM-CSF in a cell.

[0023] Still, the present disclosure also relates to a use of the oncolytic adenoviral vector of the disclosure for increasing tumor specific immune response in a subject.

[0024] Still, the present disclosure relates to the oncolytic adenoviral vector of the disclosure for increasing tumor specific immune response in a subject.

[0025] The present disclosure provides a tool for treatment of cancers, which are refractory to current approaches. Also, restrictions regarding tumor types suitable for treatment remain few compared to many other treatments. In fact all solid tumors may be treated with the present disclosure. Larger tumors by mass and more complex tumors can be cured by the present disclosure. The treatment can be given intratumorally, intracavitary, intravenously and in a combination of these. The approach can give systemic efficacy despite local injection. The approach can also eradicate cells proposed as tumor initiating ("cancer stem cells").

[0026] Besides enabling the transport of the vector to the site of interest the vector of the disclosure also assures the expression and persistence of the transgene. Furthermore, immune response against the vector as well as the transgene is minimized.

[0027] The present disclosure solves a problem related to therapeutic resistance of conventional treatments. Furthermore, the present disclosure provides tools and methods for selective treatments, without toxicity or damages in healthy tissues. Advantages of the present disclosure include also different and reduced side effects in comparison to other therapeutics. Importantly, the approach is synergistic with many other forms of therapy including chemotherapy and radiation therapy, and can therefore be used in combination regimens.

[0028] Induction of an immune reaction towards cells that allow replication of unarmed viruses is normally not strong enough to lead to development of therapeutic tumor immunity. In order to overcome this weakness, the present disclosure provides armed viruses with a potent inducer of anti-tumor immunity. The present disclosure achieves cancer therapy, wherein tumor cells are destroyed by virion caused oncolysis. In addition, various different mechanisms activating human immune response, including activation of natural killer cells (NK) and dendritic cells (DC) are influenced.

[0029] Compared to adenoviral tools of the prior art, the present disclosure provides a more simple, more effective, inexpensive, non-toxic and/or safer tool for cancer therapy. Furthermore, helper viruses are not needed.

[0030] The novel products of the disclosure enable further improvements in cancer therapy.

Brief description of the figures

[0031] Figure 1 shows a schematic of pAd5-D24-GMCSF. The virus bears a 24 base pair deletion in the constant region 2 of E1A. gp19k and 6.7K in E3 have been replaced with the cDNA of human GM-CSF. ADP refers to the adenovirus death protein.

[0032] Figure 2 shows a schematic of the first step of cloning to generate shuttle plasmid (pTHSN) bearing GM-CSF.

[0033] Figure 3 shows a schematic of the second step of cloning to generate the plasmid containing all the adenoviral genes with the 24 base pair deletion in E1 region (which mediates selective replication in cancer cells).

[0034] Figure 4 shows a schematic of the third step of cloning to generate the plasmid containing all the adenoviral genes except for gp19k and 6.7K which have been replaced by GM-CSF (pAd5D24.GM-CSF (SEQ ID NO 8)). Ad5-RGD-D24-GMCSF (SEQ ID NO 9), Ad5/3-D24-GMCSF (SEQ ID NO 7) and Ad5-pK7-D24-GMCSF (SEQ ID NO 10) were created similarly.

[0035] Figures 5a-d show that GMCSF expression does not impair virus replication and cell killing effect. Figure 5a represents results of MTS assay showing lung cancer derived (A549) cell killing efficiency of the new generated virus Ad5-D24-GMCSF. Figure 5b represents results of MTS assay showing killing of JIMT-1 cancer initiating cells ("cancer stem cells") with Ad5-D24-GMCSF. Figure 5c represents results of MTS assay showing breast cancer cells (MDA-MB-436) killing efficiency of the new generated virus Ad5-D24-GMCSF. Figure 5d represents results of MTS assay showing MDA-MB-436 killing efficiency of the new generated viruses Ad5-D24-GMCSF, Ad5-RGD-D24-GMCSF and Ad5/3-D24-GMCSF.

[0036] Figure 6a shows adenovirus-coupled expression of human GMCSF. A549 cell line was infected with Ad5D24 or Ad5D24-GMCSF, media was collected over time and analyzed for expression of GMCSF by FACSAR-RAY.

[0037] Figure 6b shows that adenovirus-expressed GMCSF retains its biological activity in human lymphocytes. TF1 cells, which require human GMCSF for staying alive, were cultured in the presence of human recombinant GMCSF (E. coli-produced, purchased from Sigma) or supernatant from Ad5-D24-GMCSF infected cells.

[0038] Figures 7a and 7b show *in vitro* analysis of transduction of a patient tumor to test the infectivity of an Ad5-based virus.

[0039] Figure 7c shows a prediction of tumor transduction with Ad5-D25-GMCSF by staining its receptor CAR (coxackie-adenovirus receptor) in archival tumor specimens.

[0040] Figure 8a shows *in vivo* efficiency of Ad5-D24-GMCSF in Syrian Hamsters (permissive for human adenovirus replication) bearing pancreatic cancer tumors. Both Ad5D24 and Ad5-D24-GMCSF eradicate the tumors within 16 days following the treatments. 1×10^9 VP of virus were administered on days 0, 2 and 4.

[0041] Figure 8b shows that intratumoral injection of Ad5-D24-GMCSF resulted in high levels of hGMCSF in serum of Syrian Hamsters. Animals treated with Ad5D24E3 or Ad5-D24-GMCSF were sampled on day 4 and the concentration of human GMCSF in serum was assessed by FACSARRAY.

[0042] Figure 8c shows that curing of HapT1 tumors with Ad5-D24-GMCSF (but not with Ad5D24) protected Syrian hamsters from subsequent re-challenge with HapT1. This demonstrates that Ad5-D24-GMCSF can induce a tumor specific immune response. Animals previously treated with Ad5D24 or Ad5D24-GMCSF (Figure 8a) were re-challenged with the same tumor and tumor growth was measured over time.

[0043] Figure 8d shows induction of a tumor specific immune response by Ad5-D24-GMCSF, curation of HapT1 tumors with Ad5-D24-GMCSF did not protect Syrian hamsters from Hak tumors. Animals with HapT1 tumors previously treated with Ad5D24, or Ad5D24-GMCSF (Figure 8a) were re-challenged with a different tumor and tumor growth was measured over time.

[0044] Figure 9a shows efficacy of Ad5-D24-GMCSF in combination with metronomic oral cyclophosphamide. 88% tumor reduction was observed by CT scan 71 days following the beginning of the treatment.

[0045] Figure 9b shows efficacy of the treatment with Ad5D24-GMCSF in an ovarian cancer patient treated with Ad5-D24-GMCSF. CT scan showed complete disappearance of all measurable tumors as indicated by the arrow.

[0046] Figures 10a-d show that Ad5-D24-GMCSF elicited a T cell-response against both tumor epitopes and adenovirus (present in tumor cells). T cells harvested from patients treated with Ad5-D24-GMCSF were analyzed

by IFN-gamma ELISPOT upon stimulation with a mix of peptide from Adenovirus 5 and a mix of peptide from the tumor antigen survivin.

[0047] Figure 11 shows induction of Adenovirus Hexon-specific T cells. Leukocytes harvested from patients treated with Ad5-D24-GMCSF were stained with a CD3, CD8 and hexon-specific tetramer antibodies and analyzed by flow cytometry before and after the treatment. Treatment increased hexon specific cytotoxic T-cells from 0.21 to 2.72%.

[0048] Figure 12 shows a reduction of circulating T regulatory cells in patient R73. PBMCs which are positive for CD4, negative for CD127 but high in Foxp3 are considered effective T regulatory cells.

[0049] Figures 13a-i show schematics of cloning to generate shuttle plasmid (pTHSN) bearing GM-CSF, to generate the plasmid containing all the adenoviral genes with the 24 base pair deletion in E1 region (which mediates selective replication in cancer cells) and to generate the Ad5/3-D24-GMCSF plasmid containing all the adenoviral genes except for gp19k and 6.7K which have been replaced by GM-CSF (pAd5D24.GM-CSF).

[0050] Figure 14 shows a nucleotide sequence of Ad5/3-D24-GMCSF. Bolded region indicates the D24 deleted E1A region (nucleotides 563-1694). Underlined region indicates GMCSF (nucleotides 28380-28814). Region in italics indicates the Ad3 knob region (nucleotides 31701-32272). The sequence of the figure corresponds to sequence SEQ ID NO 7.

[0051] Figure 15 shows survival plot for Ad5/3-D24-GMCSF treated patients.

Detailed description of the invention

Adenoviral vector

[0052] In Ad5, as well as in other adenoviruses, an icosahedral capsid consists of three major proteins: hexon (II), penton base (III), and a knobbed fiber (IV), along with minor proteins: VI, VIII, IX, IIIa, and IVa2 (Russell W.C. 2000, J General Virol 81, 2573-2604). Proteins VII, small peptide *mu*, and a terminal protein (TP) are associated with DNA. Protein V provides a structural link to the capsid via protein VI. Virus encoded protease is needed for processing some structural proteins.

[0053] The oncolytic adenoviral vector of the present invention is based on an adenovirus serotype 5 (Ad5) nucleic acid backbone, a 24 bp deletion (D24) in the Rb binding constant region 2 (CR2) of adenoviral E1 and a nucleic acid sequence encoding a granulocyte-macrophage colony-stimulating

factor (GM-CSF) in the place of the deleted gp19k/6.7K in the adenoviral E3 region (Figure 1). In a preferred embodiment of the invention, the adenoviral vector is based on a human adenovirus.

[0054] Ad5 genome contains early (E1-4), intermediate (IX and IVa2) and late (L1-5) genes flanked by left and right inverted terminal repeats (LITR and RITR, respectively), which contain the sequences required for the DNA replication. The genome contains also packaging signal (ψ) and major late promoter (MLP).

[0055] Transcription of the early gene E1A starts the replication cycle followed by expression of E1B, E2A, E2B, E3 and E4. E1 proteins modulate cellular metabolism in a way that makes a cell more susceptible to virus replication. For example they interfere with NF- κ B, p53, and pRb-proteins. E1A and E1B function together in inhibiting apoptosis. E2 (E2A and E2B) and E4 gene products mediate DNA replication and E4 products also effect virus RNA metabolism and prevent host protein synthesis. E3 gene products are responsible for defending against the host immune system, enhancing cell lysis, and releasing of virus progeny (Russell W.C. 2000, J General Virol 81, 2573-2604).

[0056] Intermediate genes IX and IVa2 encode minor proteins of the viral capsid. Expression of the late genes L1-5, which lead to production of the virus structural components, the encapsidation and maturation of virus particles in the nucleus, is influenced by MLP (Russell W.C. 2000, J General Virol 81, 2573-2604).

[0057] Compared to a wild type adenovirus genome, the adenoviral vector of the invention lacks 24 base pairs from CR2 in E1 region, specifically in E1A region, and gp19k and 6.7K in E3 region. In a preferred embodiment of the invention, in addition to partial regions E1 and E3, the oncolytic adenoviral vector of the invention further comprises one or more regions selected from a group consisting of E2, E4, and late regions. In a preferred embodiment of the invention, the oncolytic adenoviral vector comprises the following regions: a left ITR, partial E1, pIX, pIVa2, E2, VA1, VA2, L1, L2, L3, L4, partial E3, L5, E4, and a right ITR. The regions may be in any order in the vector, but in a preferred embodiment of the invention, the regions are in a sequential order in the 5' to 3' direction. Open reading frames (ORFs) may be in the same DNA strand or in different DNA strands. In a preferred embodiment of the invention, the E1 region comprises a viral packaging signal.

[0058] As used herein, expression "adenovirus serotype 5 (Ad5) nucleic acid backbone" refers to the genome or partial genome of Ad5, which comprises one or several regions selected from a group consisting of partial E1, pIX, pIVa2, E2, VA1, VA2, L1, L2, L3, L4, partial E3, L5 and E4 of Ad5 origin. One preferred vector of the invention comprises nucleic acid backbone of Ad5. In another preferred vector, the adenoviral nucleic acid backbone is mostly derived from Ad5 and combined with a portion (e.g. a part of the capsid structure) of Ad3.

[0059] As used herein, expression "partial" region refers to a region, which lacks any part compared to a corresponding wild type region. "Partial E1" refers to E1 region with D24 and "partial E3" refers to E3 region lacking gp19k/6.7K.

[0060] As used herein, expressions "VA1" and "VA2" refer to virus associated RNAs 1 and 2, which are transcribed by the adenovirus but are not translated. VA1 and VA2 have a role in combating cellular defence mechanisms.

[0061] As used herein, expression "a viral packaging signal" refers to a part of virus DNA, which consists of a series of AT-rich sequences and governs the encapsidation process.

[0062] 24 base pair deletion (D24) of E1 affects CR2 domain, which is responsible for binding the Rb tumor suppressor/cell cycle regulator protein and thus, allows the induction of the synthesis (S) phase i.e. DNA synthesis or replication phase. pRb and E1A interaction requires eight amino acids 121 to 127 of the E1A protein conserved region (Heise C. et al. 2000, Nature Med 6, 1134-1139), which are deleted in the present invention. The vector of the present invention comprises a deletion of nucleotides corresponding to amino acids 122-129 of the vector according to Heise C. et al. (2000, Nature Med 6, 1134-1139). Viruses with the D24 are known to have a reduced ability to overcome the G1-S checkpoint and replicate efficiently only in cells where this interaction is not necessary, e.g. in tumor cells defective in the Rb-p16 pathway (Heise C. et al. 2000, Nature Med 6, 1134-1139; Fueyo J et al. 2000, Oncogene 19, 2-12).

[0063] The E3 region is nonessential for viral replication *in vitro*, but the E3 proteins have an important role in the regulation of host immune response i.e. in the inhibition of both innate and specific immune responses. The gp19k/6.7K deletion in E3 refers to a deletion of 965 base pairs from the ade-

noviral E3A region. In a resulting adenoviral construct, both gp19k and 6.7K genes are deleted (Kanerva A et al. 2005, Gene Therapy 12, 87-94). The gp19k gene product is known to bind and sequester major histocompatibility complex I (MHC1) molecules in the endoplasmic reticulum, and to prevent the recognition of infected cells by cytotoxic T-lymphocytes. Since many tumors are deficient in MHC1, deletion of gp19k increases tumor selectivity of viruses (virus is cleared faster than wild type virus from normal cells but there is no difference in tumor cells). 6.7K proteins are expressed on cellular surfaces and they take part in downregulating TNF-related apoptosis inducing ligand (TRAIL) receptor 2.

[0064] In the present invention, the GM-CSF transgene is placed into a gp19k/6.7k deleted E3 region, under the E3 promoter. This restricts transgene expression to tumor cells that allow replication of the virus and subsequent activation of the E3 promoter. E3 promoter may be any exogenous or endogenous promoter known in the art, preferably endogenous promoter. In a preferred embodiment of the invention, a nucleic acid sequence encoding GM-CSF is under the control of the viral E3 promoter.

[0065] GM-CSF participates in immune response by acting through various mechanisms including recruitment of natural killer (NK) cell and stimulation of antigen presenting cells (APC). APC can then recruit, activate and target T-cells towards the tumor. The nucleotide sequence encoding GM-CSF may be from any animal such as a human, ape, rat, mouse, hamster, dog or cat, but preferably GM-CSF is encoded by a human sequence. The nucleotide sequence encoding GM-CSF may be modified in order to improve the effects of GM-CSF, or unmodified i.e. of a wild type. In a preferred embodiment of the invention, a nucleic acid sequence encoding GM-CSF is of a wild type.

[0066] The vector of the invention may also comprise other modifications than partial deletions of CR2 and E3 and insertion of GM-CSF sequence as mentioned above. In a preferred embodiment of the invention, all the other regions of the Ad5 vector are of a wild type. In another preferred embodiment of the invention, the E4 region is of a wild type. In a preferred embodiment of the invention, a wild type region is located upstream of the E1 region. "Upstream" refers to immediately before the E1 region in the direction of expression. E1B region may also be modified in the vector of the invention.

[0067] Insertion of exogenous elements may enhance effects of vectors in target cells. The use of exogenous tissue or tumor-specific promot-

ers is common in recombinant adenoviral vectors and they can also be utilized in the present invention. For example, viral replication can be restricted to target cells for example by promoters, which include but are not limited to CEA, SLP, Cox-2, Midkine, hTERT, variants of hTERT, E2F, variants of E2F, CXCR4, SCCA2 and TTS. They are usually added to control E1A region, but in addition to or alternatively, other genes such as E1B or E4 can also be regulated. Exogenous insulators i.e. blocking elements against unspecific enhancers, the left ITR, the native E1A promoter or chromatin proteins may also be included in recombinant adenoviral vectors. Any additional components or modifications may optionally be used but are not obligatory in the vectors of the present invention.

[0068] Most adults have been exposed to the most widely used adenovirus serotype Ad5 and therefore, the immune system can rapidly produce neutralizing antibodies (NAb) against them. In fact, the prevalence of anti-Ad5 NAb may be up to 50%. It has been shown that NAb can be induced against most of the multiple immunogenic proteins of the adenoviral capsid, and on the other hand it has been shown that even small changes in the Ad5 fiber knob can allow escape from capsid-specific NAb. Modification of the knob is therefore important for retaining or increasing gene delivery in the context of adenoviral use in humans.

[0069] Furthermore, Ad5 is known to bind to the receptor called CAR via the knob portion of the fiber, and modifications of this knob portion or fiber may improve the entry to the target cell and cause enhanced oncolysis in some cancers (Ranki T. et al. 2007, Int J Cancer 121, 165-174). Indeed, capsid-modified adenoviruses are advantageous tools for improved gene delivery to cancer cells.

[0070] In one embodiment of the invention, the oncolytic adenoviral vector comprises a capsid modification. As used herein "capsid" refers to the protein shell of the virus, which includes hexon, fiber and penton base proteins. Any capsid modification i.e. modification of hexon, fibre and/or penton base proteins known in the art, which improves delivery of the virus to the tumor cell, may be utilized in the present invention. Modifications may be genetic and/or physical modifications and include but are not limited to modifications for incorporating ligands, which recognize specific cellular receptors and/or block native receptor binding, for replacing the fiber or knob domain of an adenoviral vector with a knob of other adenovirus (chimerism) and for adding specific

molecules (e.g. FGF2) to adenoviruses. Therefore, capsid modifications include but are not limited to incorporation of small peptide motif(s), peptide(s), chimerism(s) or mutation(s) into the fiber (e.g. into the knob, tail or shaft part), hexon and/or penton base. In a preferred embodiment of the invention, the capsid modification is Ad5/3 chimerism, insertion of an integrin binding (RGD) region and/or heparin sulphate binding polylysine modification into the fiber. In a specific embodiment of the invention, the capsid modification is Ad5/3 chimerism.

[0071] As used herein, "Ad5/3 chimerism" of the capsid refers to a chimerism, wherein the knob part of the fiber is from Ad serotype 3, and the rest of the fiber is from Ad serotype 5.

[0072] As used herein, "RGD region" refers to the arginine-glycine-aspartic acid (RGD) motif, which is exposed on the penton base and interacts with cellular av integrins supporting adenovirus internalization. In a preferred embodiment of the invention, the capsid modification is a RGD-4C modification. "RGD-4C modification" refers to an insertion of an integrin binding RGD-4C motif in the HI loop of the fiber knob domain. 4C refers to the four cysteins, which form sulphur bridges in RGD-4C. Construction of recombinant Ad5 fiber gene encoding the fiber with the RGD-4C peptide is described in detail for example in the article of Dmitriev I. et al. (1998, Journal of Virology, 72, 9706-9713).

[0073] As used herein, "heparan sulphate binding polylysine modification" refers to addition of a stretch of seven lysines to the fiber knob c-terminus.

[0074] Expression cassettes are used for expressing transgenes in a target, such as a cell, by utilizing vectors. As used herein, the expression "expression cassette" refers to a DNA vector or a part thereof comprising nucleotide sequences, which encode cDNAs or genes, and nucleotide sequences, which control and/or regulate the expression of said cDNAs or genes. Similar or different expression cassettes may be inserted to one vector or to several different vectors. Ad5 vectors of the present invention may comprise either several or one expression cassettes. However, only one expression cassette is adequate. In a preferred embodiment of the invention, the oncolytic adenoviral vector comprises at least one expression cassette. In a preferred embodiment of the invention, the oncolytic adenoviral vector comprises only one expression cassette.

[0075] A cell comprising the adenoviral vector of the invention may be any cell such as a eukaryotic cell, bacterial cell, animal cell, human cell, mouse cell etc. A cell may be an *in vitro*, *ex vivo* or *in vivo* cell. For example, the cell may be used for producing the adenoviral vector *in vitro*, *ex vivo* or *in vivo*, or the cell may be a target, such as a tumor cell, which has been infected with the adenoviral vector.

[0076] In a method of producing GM-CSF in a cell, a vehicle comprising the vector of the invention is carried into a cell and furthermore, GM-CSF gene is expressed and the protein is translated and secreted in a paracrine manner. "A vehicle" may be any viral vector, plasmid or other tool, such as a particle, which is able to deliver the vector of the invention to a target cell. Any conventional method known in the art can be used for delivering the vector to the cell.

[0077] Tumor specific immune response may be increased in a subject by the present invention. Cytotoxic T cells and/or natural killer cells are stimulated, produced and targeted as a consequence of GM-CSF expression. In a preferred embodiment of the invention, amount of natural killer and/or cytotoxic T cells is increased in a target cell or tissue. In order to follow or study the effects of the invention, various markers of immune response (e.g. inflammatory markers) may be determined. The most common markers include but are not limited to increase in pro-inflammatory cytokines, tumor or adenovirus specific cytotoxic T-cells, recruitment and activation of antigen presenting cells or increase in size of local lymph nodes. The levels of these markers may be studied according to any conventional methods known in the art, including but not limited to those utilizing antibodies, probes, primers etc. such as ELISPOT assay, tetramer analysis, pentamer analysis and analysis of different cell types in blood or in tumors.

Cancer

[0078] The recombinant Ad5 vectors of the invention have been constructed for replication competence in cells, which have defects in the Rb-pathway, specifically Rb-p16 pathway. These defective cells include all tumor cells in animals and humans (Sherr C.J. 1996, Science 274, 1672-1677). In a preferred embodiment of the invention, the vector is capable of selectively replicating in cells having defects in the Rb-pathway. As used herein "defects in the Rb-pathway" refers to mutations and/or epigenetic changes in any genes or proteins of the pathway. Due to these defects, tumor cells overexpress E2F

and thus, binding of Rb by E1A CR2, that is normally needed for effective replication, is unnecessary.

[0079] Any cancers or tumors, including both malignant and benign tumors as well as primary tumors and metastasis may be targets of gene therapies. In a specific embodiment of the invention the cancer is any solid tumor. In a preferred embodiment of the invention, the cancer is selected from a group consisting of nasopharyngeal cancer, synovial cancer, hepatocellular cancer, renal cancer, cancer of connective tissues, melanoma, lung cancer, bowel cancer, colon cancer, rectal cancer, colorectal cancer, brain cancer, throat cancer, oral cancer, liver cancer, bone cancer, pancreatic cancer, choriocarcinoma, gastrinoma, pheochromocytoma, prolactinoma, T-cell leukemia/lymphoma, neuroma, von Hippel-Lindau disease, Zollinger-Ellison syndrome, adrenal cancer, anal cancer, bile duct cancer, bladder cancer, ureter cancer, brain cancer, oligodendroglioma, neuroblastoma, meningioma, spinal cord tumor, bone cancer, osteochondroma, chondrosarcoma, Ewing's sarcoma, cancer of unknown primary site, carcinoid, carcinoid of gastrointestinal tract, fibrosarcoma, breast cancer, Paget's disease, cervical cancer, colorectal cancer, rectal cancer, esophagus cancer, gall bladder cancer, head cancer, eye cancer, neck cancer, kidney cancer, Wilms' tumor, liver cancer, Kaposi's sarcoma, prostate cancer, lung cancer, testicular cancer, Hodgkin's disease, non-Hodgkin's lymphoma, oral cancer, skin cancer, mesothelioma, multiple myeloma, ovarian cancer, endocrine pancreatic cancer, glucagonoma, pancreatic cancer, parathyroid cancer, penis cancer, pituitary cancer, soft tissue sarcoma, retinoblastoma, small intestine cancer, stomach cancer, thymus cancer, thyroid cancer, trophoblastic cancer, hydatidiform mole, uterine cancer, endometrial cancer, vagina cancer, vulva cancer, acoustic neuroma, mycosis fungoides, insulinoma, carcinoid syndrome, somatostatinoma, gum cancer, heart cancer, lip cancer, meninges cancer, mouth cancer, nerve cancer, palate cancer, parotid gland cancer, peritoneum cancer, pharynx cancer, pleural cancer, salivary gland cancer, tongue cancer, tonsil cancer.

Pharmaceutical composition

[0080] A pharmaceutical composition of the invention comprises at least one type of the vectors of the invention. Furthermore, the composition may comprise at least two, three or four different vectors of the invention. In addition to the vector of the invention, a pharmaceutical composition may also comprise any other vectors, such as other adenoviral vectors, other therapeu-

tically effective agents, any other agents such as pharmaceutically acceptable carriers, buffers, excipients, adjuvants, antiseptics, filling, stabilising or thickening agents, and/or any components normally found in corresponding products.

[0081] The pharmaceutical composition may be in any form, such as solid, semisolid or liquid form, suitable for administration. A formulation can be selected from a group consisting of, but not limited to, solutions, emulsions, suspensions, tablets, pellets and capsules.

[0082] In a preferred embodiment of the invention, the oncolytic adenoviral vector or pharmaceutical composition acts as an *in situ* cancer vaccine. As used herein "*in situ* cancer vaccine" refers to a cancer vaccine, which both kills tumor cells and also increases the immune response against tumor cells. Virus replication is a strong danger signal to the immune system (=needed for a TH1 type response), and thus acts as a powerful costimulatory phenomenon to GM-CSF mediated maturation and activation of APCs, and recruitment of NK cells. Tumor cell lysis also helps to present tumor fragments and epitopes to APCs and furthermore, costimulation is produced by inflammation. Thus, an epitope independent (i.e. not HLA restricted) response is produced in the context of each tumor and therefore takes place *in situ*. Tumor specific immune response is activated in the target cell as well as the surrounding cells, e.g. in the target tissue.

[0083] The effective dose of vectors depends on at least the subject in need of the treatment, tumor type, location of the tumor and stage of the tumor. The dose may vary for example from about 10^8 viral particles (VP) to about 10^{14} VP, preferably from about 5×10^9 VP to about 10^{13} VP and more preferably from about 8×10^9 VP to about 10^{12} VP. In one specific embodiment of the invention the dose is in the range of about 5×10^{10} - 5×10^{11} VP.

[0084] The pharmaceutical compositions may be produced by any conventional processes known in the art, for example by utilizing any one of the following: batch, fed-batch and perfusion culture modes, column-chromatography purification, CsCl gradient purification and perfusion modes with low-shear cell retention devices.

Administration

[0085] The vector or pharmaceutical composition of the invention may be administered to any eukaryotic subject selected from a group consisting of plants, animals and human beings. In a preferred embodiment of the in-

vention, the subject is a human or an animal. An animal may be selected from a group consisting of pets, domestic animals and production animals.

[0086] Any conventional method may be used for administration of the vector or composition to a subject. The route of administration depends on the formulation or form of the composition, the disease, location of tumors, the patient, comorbidities and other factors. In a preferred embodiment of the invention, the administration is conducted through an intratumoral, intramuscular, intra-arterial, intravenous, intrapleural, intravesicular, intracavitary or peritoneal injection, or an oral administration.

[0087] Only one administration of oncolytic adenoviral vectors of the invention may have therapeutic effects. However, in a preferred embodiment of the invention, oncolytic adenoviral vectors or pharmaceutical compositions are administered several times during the treatment period. Oncolytic adenoviral vectors or pharmaceutical compositions may be administered for example from 1 to 10 times in the first 2 weeks, 4 weeks, monthly or during the treatment period. In one embodiment of the invention, administration is done three to seven times in the first 2 weeks, then at 4 weeks and then monthly. In a specific embodiment of the invention, administration is done four times in the first 2 weeks, then at 4 weeks and then monthly. The length of the treatment period may vary, and for example may last from two to 12 months or more.

[0088] In order to avoid neutralizing antibodies in a subject, the vectors of the invention may vary between treatments. In a preferred embodiment of the invention, the oncolytic adenoviral vector having a different fiber knob of the capsid compared to the vector of the earlier treatment is administered to a subject. As used herein "fiber knob of the capsid" refers to the knob part of the fiber protein (Figure 1).

[0089] The gene therapy of the invention is effective alone, but combination of adenoviral gene therapy with any other therapies, such as traditional therapy, may be more effective than either one alone. For example, each agent of the combination therapy may work independently in the tumor tissue, the adenoviral vectors may sensitize cells to chemotherapy or radiotherapy and/or chemotherapeutic agents may enhance the level of virus replication or effect the receptor status of the target cells. The agents of combination therapy may be administered simultaneously or sequentially.

[0090] In a preferred embodiment of the invention, the method or use further comprises administration of concurrent radiotherapy to a subject. In

another preferred embodiment of the invention, the method or use further comprises administration of concurrent chemotherapy to a subject. As used herein "concurrent" refers to a therapy, which has been administered before, after or simultaneously with the gene therapy of the invention. The period for a concurrent therapy may vary from minutes to several weeks. Preferably the concurrent therapy lasts for some hours.

[0091] Agents suitable for combination therapy include but are not limited to All-trans retinoic acid, Azacitidine, Azathioprine, Bleomycin, Carboplatin, Capecitabine, Cisplatin, Chlorambucil, Cyclophosphamide, Cytarabine, Daunorubicin, Docetaxel, Doxifluridine, Doxorubicin, Epirubicin, Epothilone, Etoposide, Fluorouracil, Gemcitabine, Hydroxyurea, Idarubicin, Imatinib, Mechlorethamine, Mercaptopurine, Methotrexate, Mitoxantrone, Oxaliplatin, Paclitaxel, Pemetrexed, Temozolomide, Teniposide, Tioguanine, Valrubicin, Vinblastine, Vincristine, Vindesine and Vinorelbine.

[0092] In a preferred embodiment of the invention, the method or use further comprises administration of verapamil or another calcium channel blocker to a subject. "Calcium channel blocker" refers to a class of drugs and natural substances which disrupt the conduction of calcium channels, and it may be selected from a group consisting of verapamil, dihydropyridines, gallopamil, diltiazem, mibefradil, bepridil, fluspirilene and fendiline.

[0093] In a preferred embodiment of the invention, the method or use further comprises administration of autophagy inducing agents to a subject. Autophagy refers to a catabolic process involving the degradation of a cell's own components through the lysosomal machinery. "Autophagy inducing agents" refer to agents capable of inducing autophagy and may be selected from a group consisting of, but not limited to, mTOR inhibitors, PI3K inhibitors, lithium, tamoxifen, chloroquine, bafilomycin, temsirolimus, sirolimus and temozolomide. In a specific embodiment of the invention, the method further comprises administration of temozolomide to a subject. Temozolomide may be either oral or intravenous temozolomide.

[0094] In one embodiment of the invention, the method or use further comprises administration of chemotherapy or anti-CD20 therapy or other approaches for blocking of neutralizing antibodies. "Anti-CD20 therapy" refers to agents capable of killing CD20 positive cells, and may be selected from a group consisting of rituximab and other anti-CD20 monoclonal antibodies. "Approaches for blocking of neutralizing antibodies" refers to agents capable of in-

hibiting the generation of anti-viral antibodies that normally result from infection and may be selected from a group consisting of different chemotherapeutics, immunomodulatory substances, corticoids and other drugs. These substances may be selected from a group consisting of, but not limited to, cyclophosphamide, cyclosporin, azathioprine, methylprednisolone, etoposide, CD40L, CTLA4lg4, FK506 (tacrolimus), IL-12, IFN-gamma, interleukin 10, anti-CD8, anti-CD4 antibodies, myeloablation and oral adenoviral proteins.

[0095] The oncolytic adenoviral vector of the invention induces virion mediated oncolysis of tumor cells and activates human immune response against tumor cells. In a preferred embodiment of the invention, the method or use further comprises administration of substances capable to downregulating regulatory T-cells in a subject. "Substances capable to downregulating regulatory T-cells" refers to agents that reduce the amount of cells identified as T-suppressor or Regulatory T-cells. These cells have been identified as consisting one or many of the following immunophenotypic markers: CD4+, CD25+, FoxP3+, CD127- and GITR+. Such agents reducing T-suppressor or Regulatory T-cells may be selected from a group consisting of anti-CD25 antibodies or chemotherapeutics.

[0096] In a preferred embodiment of the invention, the method or use further comprises administration of cyclophosphamide to a subject. Cyclophosphamide is a common chemotherapeutic agent, which has also been used in some autoimmune disorders. In the present invention, cyclophosphamide can be used to enhance viral replication and the effects of GM-CSF induced stimulation of NK and cytotoxic T-cells for enhanced immune response against the tumor. It can be used as intravenous bolus doses or low-dose oral metronomic administration.

[0097] Any method or use of the invention may be either *in vivo*, *ex vivo* or *in vitro* method or use.

[0098] The present invention is illustrated by the following examples, which are not intended to be limiting in any way.

Examples

Example 1. Cloning of three D24-GM-CSF type viruses

- PCR out hGM-CSF,
- create SunI/MunI sites => 445 bp (pORF-GM-CSF as a template)
- SunI/MunI digestion of PCR product and pTHSN
- Sticky-end ligation =>

- *PmeI* linearized pShuttle-D24 + pTG3602 => pAd5-D24
- Ad5-D24-GM-CSF (SEQ ID NO 8: E1A region with D24 deletion in nucleotide positions 563-1524 and a fiber region in nucleotide positions 30490-32236)
 Homol recomb: *SrfI* linearized pAd5-D24 + *FspI* linearized
 pTHSN-GM-CSF => pAd5-D24-GM-CSF
 PacI linearization & transfection => Ad5-D24-GM-CSF

[0099] All phases of the cloning were confirmed with PCR and multiple restriction digestions. The shuttle plasmid pTHSN-GMCSF was sequenced. Absence of wild type E1 was confirmed with PCR. The E1 region, transgene and fiber were checked in the final virus with sequencing and PCR, which was then taken to the clean lab for production. To this end, viral DNA was extracted by over night (ON) incubation with appropriate buffer solution and after PCR and sequence was performed to analyze the integrity of the fiber as well as the GMCSF cDNA. All phases of the virus production, including transfection, were done on A549 cells to avoid risk of wild type recombination as described before (Kanerva A et al. 2003, Mol Ther 8, 449-58; Baeurschmitz GJ et al. 2006, Mol Ther 14, 164-74). GM-CSF is under the E3 promoter (specifically under endogenous viral E3A gene expression control elements), which results in replication associate transgene expression, which starts about 8h after infection. E3 is intact except for deletion of 6.7K/gp19K.

[0100] Ad5/3-D24-GM-CSF (SEQ ID NO 7) and Ad5-RGD-D24-GM-CSF (SEQ ID NO 9: E1A region with D24 deletion in nucleotide positions 580-1541, a fiber region in nucleotide positions 30514-32286 and RGD-modification in nucleotide positions 32128-32183) were constructed identically, except a rescue plasmid featuring either a knob from serotype 3, or RGD-4C in the Ad5 fiber HI-loop were used. Ad5-pK7-D24-GMCSF (SEQ ID NO 10: E1A region with D24 deletion in nucleotide positions 561-1526, a fiber region in nucleotide positions 30499-32255 and pK7-modification in nucleotide positions 32247-32378) was also created similarly. (Figures 2-4)

[0101] Ad5/3-D24-GM-CSF was constructed as follows. A pAdEasy-1-derived plasmid containing a chimeric 5/3 fiber, pAdEasy5/3, was created by homologous recombination in *E.coli* of Ad5/3luc1 viral genome and *BstXI*-digested 8.9 kb fragment of pAdEasy-1. Next, a shuttle vector containing a 24-bp deletion in *E1A* (pShuttleD24) was linearized with *PmeI* and recombined with pAdEasy5/3 resulting in pAd5/3-D24. In order to insert human GMCSF

gene into *E3* region, an *E3*-cloning vector pTHSN was created by inserting *SpeI* to *NdeI* fragment from Ad5 genome into the multi-cloning site of pGEM5Zf+ (Promega, Madison, WI). pTHSN was further digested with *SunI/MunI* creating a 965-bp deletion in *E3* region (6.7K and *gp19K* deleted). The 432bp cDNA encoding human GMCSF (Invitrogen, Carlsbad CA) was amplified with primers featuring specific restriction sites *SunI/MunI* flanking the gene and then inserted into *SunI/MunI*-digested pTHSN (pTHSN-GMCSF). pAd5/3-D24-GMCSF was generated by homologous recombination in *E.coli* between *FspI*-linearized pTHSN-GMCSF and *SrfI*-linearized pAd5/3-D24. Ad5/3-D24-GMCSF virus genome was released by *PacI* digestion and transfection to A549 cells for amplification and rescue (Figures 13 and 14, SEQ ID NO 7).

Example 2. In vitro analysis of D24-GM-CSF type viruses

[0102] In vitro efficacy of D24-GM-CSF type viruses was studied in lung cancer cells (A549), breast cancer stem cell derived explant cells (JIMT-1) and breast cancer cells (MDA-MB-436) by utilizing MTS cell killing assays. MTS assay is currently the standard method to assess cell viability in cancer gene therapy publications. Ad5Luc1 is a replication deficient virus and acts as a negative control. Ad5wt is a wild type Ad5 virus (strain Ad300wt) and was used as a positive control. Ad5-d24-E3 harbors an isogenic 24bp deletion in *E1* but is intact in *E3*. VP indicates virus particles.

[0103] In summary, Ad5-D24-GMCSF had oncolytic activity similar to positive controls in vitro, and therefore transgene production did not compromise the oncolytic potency of the virus (Figures 5a-c). Similar data was shown for Ad5/3-D24-GM-CSF and Ad5-RGD-D24-GM-CSF (Figure 5d).

[0104] To test whether Ad5D24-GMCSF was able to express the transgene, A549 cell line was infected with 1000 VP/cell and media was collected over time. Concentration of GMCSF (Figure 6a) in the media was measured by FACSARRAY (BD Biosciences, San Diego, CA USA) according to manufacturer's instructions. In addition to that, we also analyzed whether the virus-expressed GMCSF retained its biological function. To this end TF1 cell line, whose growth and survival is strictly dependent on human GMCSF were treated with media collected from A549 cell line previously infected with Ad5D24-GMCSF. TF1 viability was assessed over time by MTS assay. The result of this experiment was that the virus expressed-GMCSF was able to stimu-

late growth of such cell line, and no difference was found with the same cell line treated with human recombinant GMCSF (Sigma) (Figure 6b).

Example 3. Pretreatment analysis of transduction

I. Infection of tumor cells with Ad5Luc1

[0105] To check that a tumor could be infected with Ad5 based viruses, biopsies taken from tissues were homogenized, and infected with luciferase coding Ad5Luc1 according to standard protocol of infection. Briefly, cells seeded in wells were washed twice with PBS, virus was thawed and re-suspended in a minimum amount of growth media and gently poured on the cells. The infection proceeded for 30 minutes and afterwards the cells were washed again in PBS and appropriate amount of complete growth media was added. Luciferase quantification was assessed 24 hours later. Please note that only a minute amount of tissue was obtained and therefore the number of cells could not be calculated, nor the amount of virus normalized to amount of tissue. Thus, no quantitative analyses were made, but qualitative data showed successful gene transfer in patients O12 and C3 (Figures 7a-b). Background luciferase values (circa 200 RLU) were subtracted.

II. Immunohistochemical staining of CAR

[0106] Available archival specimens of patient's tumors (patients for Ad5-D24-GM-CSF treatment) were collected and analyzed for CAR (the adenovirus serotype 5 receptor) expression by immunohistochemistry. Adenovirus receptor CAR stainings of cancer cell cytoplasms (M3), jejunum adenocarcinoma (C3), pancreatic carcinoma (H7), carcinoma lobulare infiltration (R8), liver metastasis of ovarian cancer (O12) and lung metastasis of synovialsarcoma (S23) are shown in Figure 7c.

III. Neutralizing antibody titers against the Ad5/3 capsid

[0107] 293 cells were seeded on 96-well plates at 1×10^4 cells/well and cultured overnight. Next day, cells were washed with DMEM without FCS. To inactivate complement, human serum samples were incubated at 56 °C for 90 min. A four-fold dilution series (1:1 to 1:16 384) was prepared in serum-free DMEM (Sarkioja M et al. 2008, Gene Ther 15(12): 921-9). Ad5/3luc1 was mixed with serum dilutions and incubated at room temperature for 30 min. Next, cells in triplicates were infected with 100 VP/cell in 50 µl of mix, and 1h later 100 µl of growth medium with 10% FCS was added. 24h post-infection, cells were lysed and luciferase activity was measured with Luciferase Assay System (Promega, Madison, WI) utilizing TopCount luminometer (PerkinElmer,

Waltham, MA). Luciferase readings were plotted relative to gene transfer achieved with Ad5/3luc1 alone in order to evaluate the effect of neutralizing antibodies in the serum of patients treated with Ad5/3-d24-GMCSF. The neutralizing antibody titer was determined as the lowest degree of dilution that blocked gene transfer more than 80%.

Example 4. Ex vivo analysis of Ad5/3-D24-GMCSF efficacy in ascites and pleural samples

[0108] Fresh ascites/pleural effusion sample was stored at +4°C over night. The sample was divided into 50ml falcon tubes and the cells were isolated by centrifugation 900rpm, 8min, +4°C. To lyse red blood cells the sample was incubated 5-10min in room temperature with 25ml ACK Lysis Buffer (Invitrogen, Carlsbad, CA). The falcons were filled up with 2%DMEM and the cells were centrifugated (900rpm, 8min, +4°C). Cell suspension of 100 000 cells/ml in 2%DMEM-fungizone was prepared (50ml 2%DMEM + 200µl Fungizone (BMS, Espoo, Finland)).

[0109] For luciferase-assay, to test the effect of capsid modification on transductional efficacy, cells were seeded into two 24-well plates, 50 000 cells/well. 24h later the cells in triplicates were infected with Ad5luc1 or Ad5/3luc1 in concentrations of 500vp/cell and 5000/vp/cell in 2% DMEM. Luciferase expression was analyzed similarly as in Example 3 III (determining neutralizing antibody titers).

[0110] Fresh pre-treatment samples of ascites and pleural effusion for patients K75 and V136, respectively, were analyzed, and in both samples high transduction with Ad5/3 was seen.

[0111] For MTS-assay, to test the potency of Ad5/3-d24-GMCSF on clinical samples, cells were seeded into two 96-well plates, 10 000 cells/well. The cells were infected after 24h incubation. Infections were carried out in 2%DMEM. Next day 10%DMEM was added. The cells were checked daily and the culture medium was changed every other day. Before measuring, the medium was aspirated and 100µl of fresh 10% DMEM was pipetted to the wells. 20µl of MTS- Assay Buffer (Promega, Madison, WI) was added and the cells were incubated for 1.5 - 4 hours. The absorbance was measured with Multiscan Ascent and Ascent Software v2.6 (Thermo Labsystems, Helsinki, Finland) at 490 nm and background absorbance was subtracted from the absorbance of the samples.

[0112] Pre-treatment samples of pleural effusion from V136 and M137 were assessed for oncolytic potency of Ad5/3-d24-GMCSF. Six days after infection there was 62% and 29% less viable cells ($p < 0.001$), respectively, than in an uninfected control sample suggesting that Ad5/3-d24-GMCSF was able to kill tumor cells present in the effusion.

[0113] For assessing the presence of virus in samples obtained after treatment, cells were resuspended in 3ml 2%DMEM after lysing red blood cells and freeze-thawed four times in -80°C . The 293 cells were seeded in 96-well plates 10 000cells/well and incubated for 24h hours. The sample was centrifugated 15min, 4000rpm, $+4^{\circ}\text{C}$ and the supernatant was collected. 293 cells were infected with 100 μl /well of the supernatant. After 10 days of incubation the wells were assessed for the presence of cytopathic effect.

[0114] To assess replication of Ad5/3-d24GMCSF at the tumor, we also analyzed an ascites sample taken from patient O82 7 days after treatment. This resulted in 70% of the cells showing cytopathic effect, while uninfected control cells did not show similar effects.

Example 5. In vivo analysis of D24-GM-CSF type viruses in animals

[0115] In vivo efficacy of Ad5-D24-GM-CSF was tested in immune competent Syrian hamsters, which are semipermissive for human adenovirus replication (mice are non-permissive) (Ying B. et al. 2009, Cancer Gene Ther doi:10.1038/cgt.2009.6.). 7×10^6 HapT1 pancreatic cancer cells were injected subcutaneously and 1×10^9 virus particles (VP) of Ad5-D24-GM-CSF or Ad5D24E3 (which does not express GM-CSF) were injected intratumorally on day 0, 2 and 4. The mock group was injected with the same volume of growth media at the same indicated time points. Figure 8b shows that intratumoral injections of Ad5-D24-GMCSF resulted in high levels of hGM-CSF in serum of Syrian hamsters. Human GM-CSF is known to be active in Syrian hamsters (Cho, Exp Toxicol Pathol 2006 vol. 57 (4) pp. 321-8). Interestingly, all animals were tumor-free by day sixteen except for mock group (Figure 8a). Tumor scars were still analyzed for two additional weeks to assess whether reappearance of the tumor might have occurred. However, on day 32 there was still no sign of tumor in these animals so that the first part of the experiment was terminated and animals of the Mock group were euthanized. The remaining treated animals were at this point challenged with the same tumor in the right side of the upper body by subcutaneous injection of 7×10^6 HapT1 cells while on the left side were challenged with a different tumor (1×10^6 of HaK tumor) for

which the animals were naïve. Tumor growth was measured over time and is reported in Figures 8c-d. Interestingly the animals that were previously treated with Ad5D24GMCSF completely rejected the HapT1 tumor challenge while Hak tumors grew normally, while the animals that were previously treated with Ad5D24E3 grew independently HapT1 and HaK tumors (Figures 8c-d).

[0116] In summary, the data indicates that Ad5-D24-GM-CSF has antitumor activity in immune competent tumor bearing animals, and it is able to elicit tumor-specific immunity to the degree of rejecting subsequent challenge of the same tumor.

Example 6. In vivo analysis of D24-GM-CSF type viruses in human patients

I. Patients

[0117] Patients with advanced and treatment refractory solid tumors were enrolled in a government-approved compassionate treatment protocol. Information of patients receiving Ad5-D24-GM-CSF is listed in Table 1.

[0118] 22 patients with advanced solid tumors refractory to standard therapies (Table 6) were treated with a single round of Ad5/3-d24-GMCSF intravenously and intratumorally (Table 7). Intratumoral injection was performed intraperitoneally or intrapleurally in the case of carcinomatosis or pleural metastases, respectively. Inclusion criteria were solid tumors refractory to conventional therapies, WHO performance score 3 or less and no major organ function deficiencies. Exclusion criteria were organ transplant, HIV, severe cardiovascular, metabolic or pulmonary disease or other symptoms, findings or diseases preventing oncolytic virus treatment. Written informed consent was obtained and treatments were administered according to Good Clinical Practice and the Declaration of Helsinki.

II. Treatments with adenoviral vector encoding GM-CSF

a) Ad5-D24-GM-CSF, Ad5/3-D24-GM-CSF or Ad5-RGD-D24-GM-CSF treatments

[0119] Ad5-D24-GM-CSF, Ad5/3-D24-GM-CSF and Ad5-RGD-D24-GM-CSF were then produced according to clinical grade and treatment of patients was initiated. This "phase 0" compassionate use program has been discussed at the HUCH Surgical Ethics committee. The program has also been discussed at FinOHTA (national evaluation of medical technologies) and Ethics Negotiation Board of the Finnish Medical Association. Legal aspects have been checked with Ministry of Social Affairs and Health, the National Agency of

Medicines, the legal council at the Finnish Medical Association, the National authority for Medicolegal affairs and the Parliamentary Board of Social Affairs and Health. The treatments have been approved by the Finnish Gene Technology Board.

[0120] Patients received a single round of treatment on day 0. Virus administration was performed by ultrasound-guided intratumoral injection and circa one fifth of the dose was given intravenously. The starting dose of 8×10^{10} VP was chosen based on safety results published by others.

[0121] Virus was diluted in sterile saline solution at the time of administration under appropriate condition. Following virus administration all patients were monitored overnight at the hospital and subsequently for the following 4 weeks as outpatients. Physical assessment and medical history were done at each visit and clinically relevant laboratory values were followed. Side effects of treatment were recorded and scored according to Common Terminology for Adverse Events v3.0 (CTCAE).

[0122] Because many cancer patients have symptoms due to disease, pre-existing symptoms were not scored if they did not become worse. However, if the symptom became more severe, e.g. pre-treatment grade 1 changed to grade 2 after treatment, it was scored as grade 2. Serum levels of GMCSF and four other cytokines (IL-6, IL-8, IL-10 and TNF-alpha) were analyzed by BD Cytometric Bead Array (CBA) Human Soluble Protein Flex Set (Becton Dickinson, Franklin Lakes, NJ, US). Tumor size was assessed by contrast-enhanced computer tomography (CT) scanning. Maximum tumor diameters were obtained. Response Evaluation Criteria in Solid Tumors (RECIST1.1) criteria were applied to overall disease, including injected and non-injected lesions. These criteria are: partial response PR (>30% reduction in the sum of tumor diameters), stable disease SD (no reduction/increase), progressive disease PD (>20% increase). Clear tumor decreases not fulfilling PR were scored as minor responses (MR). Serum tumor markers were also evaluated when elevated at baseline, and the same percentages were used.

[0123] Blood samples were collected before and after the treatment for analyses. In Table 1 is summarized the viral load in the serum of patients treated with Ad5-D24-GMCSF. Quantitative PCR (qPCR) was used for that analysis (see section III for description of the methods).

[0124] Tables 2, 3 and 4 summarize all the adverse events that were recorded during and after the Ad5-D24-GM-CSF treatment. All the ad-

verse events have been graded according to Common Terminology for Adverse Events v3.0 (CTCAE). Of note is that all the patients manifested grade 1 or/and 2 flu-like symptoms, but only two grade 3 symptoms were observed, one case of constipation in an ovarian cancer patients who had suffered of constipation before and one grade 3 hyponatremia.

[0125] In Table 5 there is reported the efficacy evaluation of Ad5-D24-GM-CSF according to RECIST criteria (Therasse P et al. 2000, J Natl Cancer Inst 92, 205-16). Interestingly two complete response (CR) and five stable diseases (SD) were observed in 14 analyzable patients for a 50% clinical benefit rate.

b) Safety of Ad5/3-d24-GMCSF in Cancer Patients

[0126] Treatments were well tolerated up to the highest used: 4×10^{11} VP/patient. No grade 4-5 adverse events were seen. Grade 1-2 flu-like symptoms were common with 19/22, 17/22 and 8/22 patients experiencing fever, fatigue or upper respiratory symptoms, respectively. Pain in the injection site (6 patients), abdominal pain (10 patients) and nausea (9 patients) were also common grade 1-2 adverse events as well as (Table 8). Asymptomatic and self-limiting grade 3 hematological side effects were seen in 4 patients: anemia (grade 2 at baseline), neutropenia, aspartate aminotransferase elevation and hyponatremia. The only non-hematological grade 3 side effect was cholecystitis seen three week after treatment in pancreatic cancer patient H83. He also had grade 3 alanine aminotransferase and bilirubin elevations. Taken together, these symptoms suggest tumor mediated biliary duct compression. It is unclear if this was treatment mediated inflammatory swelling or tumor growth caused by disease progression.

III. Detection of the virus from blood

[0127] Serum samples were collected from patients treated with Ad5-D24-GM-CSF or Ad5/3-D24-GM-CSF (see example 6, I.) and conventional PCR was carried out with primers and conditions according to Takayama et al. 2007, J. Med. Virol. 79:278-284. Briefly, total DNA was extracted by adding 3 µg of carrier DNA (polydeoxyadenylic acid; Roche, Mannheim, Germany) to 400 µl of serum and using the QIAamp DNA mini kit. Extracted DNA was eluted in 60 µl nuclease-free water and DNA concentration was measured by spectrophotometry. PCR amplification was based on primers and probe targeting the E1A region flanking the 24-bp deletion (forward primer 5'-TCCGGTTTCTATGCCAAACCT-3' (SEQ ID NO 1), reverse primer 5'-

TCCTCCGGTGATAATGACAAGA-3' (SEQ ID NO 2) and probe onco 5'-FAM-TGATCGATCCACCCAGTGA-3'-MGBNFQ (SEQ ID NO 3)). In addition, a probe complementary to a sequence included in the 24-bp region targeted for deletion was used to test the samples for the presence of wild-type adenovirus infection (probe wt 5'-VIC-TACCTGCCACGAGGCT-3'-MGBNFQ (SEQ ID NO 4)).

[0128] The real-time PCR conditions for each 25 μ l reaction were as follows: 2X LightCycler480 Probes Master Mix (Roche, Mannheim, Germany), 800 nM each forward and reverse primer, 200 nM each probe and 250 ng extracted DNA. PCR reactions were carried out in a LightCycler (Roche, Mannheim, Germany) under the following cycling conditions: 10 min at 95°C, 50 cycles of 10 s at 95°C, 30 s at 62°C and 20 sec at 72°C and 10 min at 40°C. All samples were tested in duplicate. TaqMan exogenous internal positive control reagents (Applied Biosystems) were used in the same PCR runs to test each sample for the presence of PCR inhibitors.

[0129] A regression standard curve was generated using DNA extracted from serial dilutions of Ad5/3-D24-Cox2L (1×10^8 – 10 vp/ml) in normal human serum. The limit of detection and limit of quantification for the assay were 500 vp/ml of serum.

[0130] Positive samples were confirmed by real-time PCR using LightCycler480 SYBR Green I Master mix (Roche, Mannheim, Germany) and primers specific for adenovirus and GM-CSF sequences (forward primer 5'-AAACACCACCCTCCTTACCTG -3' (SEQ ID NO 5) and reverse primer 5'-TCATTCATCTCAGCAGCAGTG -3' (SEQ ID NO 6)).

IV. Presence of Ad5/3-d24-GMCSF in Serum after Treatment

[0131] All patients were negative for Ad5/3-d24-GMCSF prior to the treatment with Ad5/3-d24-GMCSF. On day 1 17/19 patients had measurable levels of virus genomes in the serum, with the highest titer being 2061 VP/ml serum. From samples taken during days 3-7 12/15 patients were positive, with the highest titer of $3,36 \times 10^5$ vp/ml serum. Positive samples were seen up to day 58 after treatment. (Table 9)

V. GMCSF and neutralizing antibody titers in serum after treatment

[0132] There was no significant change in the systemic levels of GMCSF after Ad5/3-d24-GMCSF treatment, which correlated well with no significant effects seen in the levels of total white blood cell counts. This suggests a general restriction of GMCSF production to the local sites of virus replication in the tumors. In one patient, S70, a transient increase in serum GMCSF was

seen on day 4 accompanied by a transient elevation of the leukocyte count. This might have been related to effective virus replication as the patient simultaneously had fever and 3.36×10^5 vp/ml of virus in serum (Table 9). This patient did not experience any serious adverse events during follow-up. However, a post treatment CT scan suggested anti-tumor activity (SD) and for 4 weeks after treatment she was feeling better and her persistent chest pains were gone. The highest GMCSF concentration measured in this patient's blood was 115pg/ml, which is approximately 10-fold lower than the toxic levels of GMCSF in humans.

[0133] At baseline 4/15 patients were completely negative for neutralizing antibodies against Ad5/3. Another 2 patients had barely detectable titers (1-4) while 8 patients had low neutralizing titer (16-64). No patients had medium or high neutralizing titers against Ad5/3 at baseline. After treatment the titer increased in all patients ($p < 0.005$) (Table 9). No clear correlation was seen between neutralizing antibody titers and viral dose, antitumor activity or toxicity. Intriguingly, with regard to virus load in serum two patients, Y62 and O79, were positive for neutralizing antibodies at baseline and had high titers during weeks 2-4 but still had measureable loads of virus present in their serum at least 28 and 58 days, respectively (Table 9). This indicates that even high antibody titers cannot hinder virus replication in the tumors. Interestingly, the antibody titer did not reach maximum in all patients. For example, S70, X122 and H83 had large amounts of virus circulating during week 1 and their antibody titer rose slowly.

VI. Killing of differentiated tumor cells

[0134] CT scans of a patient suffering from ovarian cancer and a patient suffering from mesothelioma (see Table 1) before and after Ad5-D24-GM-CSF treatment are shown in Figures 9a-b.

VII. Efficacy of Ad5/3-D24-GMCSF

[0135] All patients had progressing tumors prior to treatment. 12 patients could be assessed for radiological benefit according to RECIST1.1 (Table 9). 2 patients had a minor response, 6 patients had stable disease, and 4 patients had progressive disease (PD). Therefore, the radiological clinical benefit rate was 67%. Of note, the rapidly growing pancreatic tumor in H96 stopped growing but a metastatic lesion appeared in the lungs and was thus scored PD. Similarly, patient O129 had a 6% reduction of the injected tumor

but had a new metastasis. In patient V136, who had two metastatic cancers, a non-injected liver lesion disappeared, while the other tumors remained SD.

[0136] With regard to tumor markers, assessed for patients who had elevated markers at baseline, 2/6 had some lowering of marker levels and 4/6 had elevation of marker levels (Table 9).

[0137] Overall survival of patients after treatment is shown in Figure 14.

[0138] In addition to objective measurements of anti-tumor activity we also saw clinical and/or subjective benefit in several cases (Table 9). These included the patients overall wellbeing being improved, a palpable tumor feeling softer and/or smaller and symptoms caused by the tumor being relieved. Of importance is that two patients, previously suffering from rapid accumulation of ascites and/or pleural effusion, had a clear reduction of their accumulation after the virus treatment, this effect lasted for several months in both cases.

[0139] Overall, signs of antitumor efficacy were seen in 13/21 patients (62%).

VIII. Hematological effects of the treatment

[0140] Levels of leucocytes, erythrocytes, Hb, thrombocytes, bilirubin, INR, ALT, AST, ALP, creatinine, K, Na, CRP, CA19-9, GT, Fibrin D-dimers and CEA were studied after Ad5/3-D24-GM-CSF treatment (Tables 3-4).

IX. Immune response to virus

a) IL-6, IL-10, TNF- α and IL-8

[0141] One potential drawback of adenovirus gene therapy is its early toxicity due to viral components which may be immunogenic and can lead to a sepsis-like shock and even death (Brunetti-Pierri et al. Hum Gene Ther 15 (2004) 35-46; Raper et al. Mol Gen Metab 80 (2003) 148-158). It is, therefore, extremely important to monitor signs for a possible cytokines storm which may later evolve in organ failure. To this end, soon after the treatment and at indicated time point blood was drawn from patients and pro-inflammatory cytokines were analyzed by FACSARRAY as described in the article of Cerullo V et al. (2007, Mol Ther 15, 378-85). No significant changes were seen in the patients treated with Ad5-D24-GM-CSF indicating lack of early innate toxicity.

[0142] For the results of lack of early innate toxicity related to Ad5/3-D24-GM-CSF see Table 10.

b) Induction of cytotoxic T-cells against tumors and specific immunity against tumor epitope

[0143] Oncolytic cell death allows the immune system to gain the capacity for recognizing and killing tumor cells. This is potentially beneficial for tumor eradication and may facilitate cures. Adenovirus is cleared out from the body in a relative short time following the administration; hence it becomes of key importance to stimulate the immune system to be able to recognize specific tumor-antigen so that the treatment can result in a sustained beneficial effect for the patient. In addition, in the presence of antibody, the virus is neutralized so that it can lose its efficacy of infecting metastasis. However, effector T or NK cells induced against the tumor are free to circulate and eventually kill metastasis far from the injected tumor. In order to demonstrate that the GMCSF-expressing adenovirus is able to elicit adenovirus- and tumor specific immunity, PBMCs collected from treated patients were analyzed by INF-gamma ELISPOT. The ELISPOT was performed in a blinded fashion way by an external company which was not provided of information on any kind of treatment the patients underwent through (Proimmune). In figures 10a-d are illustrated the results from such analysis. It is clear that in same patients, when the T cells were stimulated with a pool of peptide derived from either tumor antigen (survivin) or adenovirus (penton) these cells were activated hence produce INF-gamma (INF-gamma is a specific activation marker of stimulated T cells).

[0144] Figure 11 shows induction of Adenovirus Hexon-specific T cells. Leukocytes harvested from patients treated with Ad5-D24-GMCSF were stained with a CD3, CD8 and hexon-specific tetramer antibodies and analyzed by flow cytometry before and after the treatment. Treatment increased hexon specific cytotoxic T-cells from 0.21 to 2.72%.

c) Reduction of regulatory T-cells

[0145] Previous data has demonstrated that metronomic administration of cyclophosphamide reduces regulatory T cells (T-Reg) in laboratory animals.

[0146] This approach was utilized in patients who received metronomic administration of cyclophosphamide before and after the Ad5-D24-GM-CSF treatment and T reg analysis was performed on PBMCs harvested from these patients. In the example illustrated in Figure 12 it is shown one example from patient R73 and it shows a reduction in circulating T reg. Total PBMCs

were harvested from the patients and frozen in appropriate media. At the time of analysis all the samples were thawed and stained first with CD4 and CD127 antibodies, following the cells were permeabilized and stained for the transcription factor Foxp3. Cells that resulted positive for CD4, negative for CD127 but high in Foxp3 are considered effective T regulatory cells (T reg) (Figure 12).

Example 7. Statistical analysis

[0147] Two tailed Student's t-test was used to compare luciferase activity and pre- and post-treatment neutralizing antibody titers, cytokine levels and GM-CSF concentrations. Survival data was processed with Kaplan-Meier analysis.

Table 1. Table showing the presence of Ad5-D24-GMCSF in the serum of treated patients

Patient (Code)	Primary Tumor	Virus Dose (total VP)	Pre-	Days post-treatment					
			Treatment	1	2	3-7	8-12	13-20	21-40
			Viral Load in serum (VP/ml)						
C3	Jejunum ca	8x10 ⁹	0	0	500	500	NA	NA	0
M3	Hepatocellular ca	1x10 ¹⁰	0	0	4896	0	0	0	0
O12	Ovarian ca	3.6x10 ¹⁰	0	0	0	0	0	0	0
O14	Ovarian ca	1x10 ¹¹	0	0	0	500	0	0	0
G15	Gastric ca	1x10 ¹¹	0	0	565	500	0	0	0
K18	Non-small cell lung ca	2x10 ¹¹	0	500	NA	0	0	0	856
T19	Medullar thyroid ca	2x10 ¹¹	0	765	500	500	0	0	0
U89	Renal ca	2x10 ¹¹	0	0	NA	NA	NA	NA	0
S100	Leiomyosarcoma	2x10 ¹¹	0	500	NA	500	NA	NA	NA
S108	Synovial Sarcoma	2x10 ¹¹	0						
M50	Mesothelioma	2.5x10 ¹¹	0	0	NA	500	NA	0	0
R8	Breast ca	3x10 ¹¹	0	500	NA	500	NA	0	0
M32	Mesothelioma	3x10 ¹¹	0	0	0	NA	NA	0	0
X49	Cervical ca	3x10 ¹¹	0	4290	NA	NA	37975	6706	1211
I52	Melanoma	3x10 ¹¹	0	576					
I78	Choroideal ca	3x10 ¹¹	0	44867	NA	NA	NA	NA	500
C58	Colon ca	4x10 ¹¹	0	1978	NA	4236	878	NA	NA
R73	Breast ca	4x10 ¹¹	0						
O88	Ovarian ca	4x10 ¹¹	0						

Table 2. Table that summarizes the side effects reported by patients treated with Ad5-D24-GMCSF

Reported symptoms	No. of patients				Dose Range
	Grade 1	Grade 2	Grade 3	Grade 4	
Fever	3				L
		1			M
	6	4			H
Pain in injection site					L
		1			M
					H
Muscular Pain	1				L
					M
	1	1			H
Headache		1			L
		1			M
	1				H
Fatigue		1			L
	1				M
	2	3			H
Dyspnea					L
		1			M
	2				H
Diarrhea					L
	1				M
					H
Hypotension					L
	1				M
	1				H
Nausea					L
	2				M
	3				H
Vomiting					L
					M
	3				H
Vertigo					L
					M
	2				H
Cough	1				L
	1	1			M
	3	3			H
Chills					L
	4				M
	1				H

L (Low Dose) = Cohort 1 Dose Range $8 \times 10^9 \leq D \leq 3.6 \times 10^{10}$

M (Medium Dose) = Cohort 2 Dose Range $1 \times 10^{11} \leq D \leq 2.5 \times 10^{11}$

H (High Dose) = Cohort 3 Dose Range $3 \times 10^{11} \leq D \leq 4 \times 10^{11}$

Table 3. Hematological side effects following administration of Ad5-D24-GMCSF

Effect	Early Toxicity (1-7 Days)				Late Toxicity (>7 Days)				Dose Range
	Grade	Grade	Grade	Grade	Grade	Grade	Grade	Grade	
	1	2	3	4	1	2	3	4	
Hyponatremia	1								L
	1				1	1			M
	6		3						H
Hypokalemia	1				2				L
	2				1				M
	1								H
Anemia	1								L
	1								M
	2	4				3			H
Thrombocytopenia						1			L
									M
	1								H
Leucopenia		1							L
									M
									H

L (Low Dose) = Cohort 1 Dose Range $8 \times 10^9 \leq D \leq 3.6 \times 10^{10}$

M (Medium Dose) = Cohort 2 Dose Range $1 \times 10^{11} \leq D \leq 2.5 \times 10^{11}$

H (High Dose) = Cohort 3 Dose Range $3 \times 10^{11} \leq D \leq 4 \times 10^{11}$

Table 4. Liver enzymes following administration of Ad5-D24-GMCSF

		Early Toxicity (1-7 Days)				Late Toxicity (> 7 Days)				
		Grade				Grade				Dose Range
		I	II	III	IV	I	II	III	IV	
ALT			1				1			L
	No. of Patients	3				1				M
										H
AST			1				1			L
	No. of Patients	1	1			1	1			M
										H
Hyperbilirubinemia										L
	No. of Patients	2				1				M
										H

L (Low Dose) = Cohort 1 Dose Range $8 \times 10^9 \leq D \leq 3.6 \times 10^{10}$

M (Medium Dose) = Cohort 2 Dose Range $1 \times 10^{11} \leq D \leq 2.5 \times 10^{11}$

H (High Dose) = Cohort 3 Dose Range $3 \times 10^{11} \leq D \leq 4 \times 10^{11}$

Table 5. Efficacy of the treatment with Ad5-D24-GMCSF in patients treated with different doses of Ad5-D24-GMCSF. Analysis was performed according to RECIST criteria. CR = complete response, PR = partial response, SD = stable disease, PD = progressive disease, NA = not available.

Virus Dose (total VP)	No. of patients	RECIST					Survival (Days)			
		CR	PR	SD	PD	NA	S>300	300<S>200	200<S>100	S<100
8x10 ⁹ VP	1					1			1	
1x10 ¹⁰ VP	1				1		1			
3.6x10 ¹⁰ VP	1			1					1	
1x10 ¹¹ VP	2	1				1	1	1		
2x10 ¹¹ VP	4			1	4		1		2	1
2.5x10 ¹¹ VP	1			1				1		
3x10 ¹¹ VP	5	1			3	1	1		2	
3.6x10 ¹¹ VP	1									
4x10 ¹¹ VP	3		1	1		1			3	

Table 6. Summary of characteristics of patients for Ad5/3-D24-GM-CSF treatment at baseline. The numbers indicate number of patients out of 22.

Sex	N. of Patients
Male	11
Female	11
WHO performance score	
Median	1
Range	0-3
Tumor Type	
Cholangio carcinoma	1
Pancreatic cancer	3
Colorectal cancer	2
Prostatic cancer	2
Lung cancer	1
Ovarian cancer	4
Melanoma (choroidea or skin)	3
Head and neck squamous cell cancer	1
Sarcoma (bone, synovia or chondro)	3
Uterus cancer	1
Bladder cancer	1
Mesothelioma	1
Previous treatments	
Chemotherapy	22
Median chemo regimens per patient	
Radiotherapy	6
Hormone treatments	1
Surgical treatments	15
Ad5/3 Neutralizing antibodies (baseline)	
Positive	11
Negative	4
Age	Years
Median	61
Mean	56
Range	17-78

Table 7. Characteristics of patients for Ad5/3-D24-GM-CSF treatment at baseline and description of treatment

ID	Age Sex	Diagnosis	Prior therapies	WHO ^a	Cyclo- fosfamide ^d	Dose (VP) ^b	Route ^c
Y62	55 M	Cholangiocarcinoma and clear cell hypernephroma. Metastases to liver and neck.	Gemcitabine	2	+	1×10^{11}	$\frac{3}{4}$ i.t. liver met., neck, kidney $\frac{1}{4}$ i.v.
H64	54 F	Pancreatic carcinoma. Metastases to liver.	Operation, gemcitabine.	0	-	8×10^{10}	$\frac{3}{4}$ i.t. liver met. $\frac{1}{4}$ i.v.
C66	63 F	Colon carcinoma. Metastases to liver, lungs, lymph nodes.	Operation, leucovorin, oxaliplatin, 5-fluorouracil and bevacizumab	0	+	2×10^{11}	$\frac{2}{3}$ i.t. liver met. $\frac{1}{3}$ i.v.
S67	55 M	Chondrosarcoma of hip.	Operation, irradiation, cisplatin.	1	+	2×10^{11}	$\frac{2}{3}$ i.t. hip $\frac{1}{3}$ i.v.
S70	24 F	Synovial sarcoma of thigh. Metastases to lungs	Operation x2, ifosfamide, doxorubicin, uromifexan, cisplatin, gemcitabine, sorafenib, etoposide.	2	-	1×10^{11}	$\frac{1}{10}$ i.pl $\frac{6}{10}$ i.t. lungs $\frac{3}{10}$ i.v.
P74	55 M	Prostate carcinoma. Metastases to bones	LHRH-analogues, docetaxel, irradiation, orchiectomy operation.	3	+	1×10^{11}	i.v.
K75	64 M	Lung adenocarcinoma. Metastases in pleura and peritoneum	Operation, cisplatin, vinorelbine, erlotinib.	1	+	3×10^{11}	$\frac{9}{16}$ i.p $\frac{4}{16}$ i.v. $\frac{3}{16}$ i.pl
O79	70 F	Ovarian carcinoma. Carcinomatosis.	Operation x3, docetaxel, carboplatin, paclitaxel, bevacizumab, gemcitabine, topotecan, talidomide, vinorelbine, doxorubicin.	1	+	3×10^{11}	$\frac{1}{12}$ s.c. $\frac{4}{12}$ i.t. $\frac{3}{12}$ i.p. $\frac{4}{12}$ i.v.

I80	38 F	Conjunctival melanoma. Metastases to liver	Dacarbazine, interferon, paclitaxel, carboplatin, angioembolisation, irradiation.	0	+	2×10^{11}	2/3 i.t. 1/3 i.v.
O82	56 F	Ovarian carcinoma. Carcinomatosis.	Paclitaxel, carboplatin, gemcitabine, topotecan, docetaxel, doxorubicine.	2	+	3×10^{11}	10/31 i.v. 20/31 i.t. 1/31 s.c.
H83	64 M	Pancreatic carcinoma	Gemcitabine	1	+	4×10^{11}	2/5 i.v. 3/5 i.t.
I87	64 M	Melanoma Metastases to neck, parotis gland, liver.	Operation, irradiation, interferon, bevacizumab, dacarbazine, vincristine, lomustine, bleomycin	1	+	2×10^{11}	2/3 i.t. 1/3 i.v.
C95	63 M	Rectum carcinoma. Metastases to liver.	Operation, bevacizumab, cabecitabine, irinotecan, cetuximab, oxaliplatin	1	-	3×10^{11}	4/5 i.t. liver met 1/5 i.v.
H96	64 M	Pancreatic carcinoma.	Gemcitabine, erlotinib	2	+	3×10^{11}	2/3 i.t. pancreas 1/3 i.v.
I98	38 F	Choroideal melanoma. Metastases to liver.	Rutonium-plate, dacarbazine, vincristine, lomustine, bleomycin, interferon, cisplatin, chemo-embolization	1	+	3×10^{11}	1/2 i.t. liver met. 1/2 i.v.
N110	60 M	Head and neck carcinoma.	Operation, cisplatin, 5- fluorouracil, boron neutron capture therapy.	2	+	2×10^{11}	3/10 i.v. 7/10 i.t.
O113	67 F	Ovarian carcinoma. Carcinomarsinosis.	Operation, paclitaxel, carcinomarboplatin, cisplatin, doxorubicine, gemcitabine, topotecarcinomane, vinorelbine, docetaxel.	1	+	3×10^{11}	3/4 i.t. pelvic tumor + met 3/4 i.v.

S119	17 M	Sarcoma of limb. Metastases to lungs.	Docetaxel, cisplatin, gemcitabine, ifosfamide, doxorubicine, uromitexane.	1	+	4×10^{11}	2/3 i.t. limb 1/3 i.v.
X122	77 F	Uterus carcinoma. Peritoneal metastases.	Operation, carboplatin, epirubicin, doxorubicin, brachytherapy.	1	-	3×10^{11}	3/4 i.t. 1/4 i.v.
O129	48 F	Ovarian carcinoma. Carcinomatous.	Paclitaxel, docetaxel, carboplatin, cisplatin, gemcitabine, doxorubicin.	0	-	3×10^{11}	1/4 i.v. 1/4 i.t. 2/4 i.p.
V136	78 M	Bladder and prostate carcinomas. Lymph node, bone and lung metastases.	Operation, irradiation, cisplatin, gemcitabine, procarbazine, vincristin, ranimustine.	2	+	3×10^{11}	1/2 i.v. 1/2 i.pl
M137	65 F	Mesothelioma	Carboplatin, pemetrexed.	1	+	3×10^{11}	5/20 i.v. 13/20 i.pl 2/20 i.t.

^aPerformance status at the time of treatment, scale 0-5.

^bTotal dose; vp=viral particles

^ci.v.=intravenous, i.p.=intraperitoneal, i.t.=intratumoral, i.pl.= intrapleura, met.=metastases

^dconcurrent metronomic cyclophosphamide 50 mg/d was given orally in the absence of contraindications

Table 8. Adverse events. All 22 patients were evaluated for adverse events (AE) according to CTCEA v.3.0 criteria for 4 weeks after Ad5/3-D24-GMCSF treatment. Grade 1 AE reported only if present in 2 or more patients. All grade 2-5 AE are reported. Numbers indicate the number of patients out of 22.

	Grade 1	Grade 2	Grade 3	Grade 4-5
Constitutional				
Chills	3	1	0	0
Fatigue	5	12	0	0
Fever	13	6	0	0
Sweating	1	1	0	0
Gastrointestinal				
Anorexia	1	1	0	0
Nausea	8	1	0	0
Vomiting	4	0	0	0
Heartburn	2	0	0	0
Haematological				
Anemia	3	0	1	0
Neutropenia	0	0	1	0
Thrombocytopenia	0	1	0	0
Infection				
Cholecystitis	0	0	1	0
Lymphatics				
Limb edema	0	2	0	0
Metabolic / Laboratory				
ALT increased	2	0	1	0
AST increased	2	2	1	0
Hyperbilirubinemia	2	0	1	0
Hypokalemia	3	0	0	0
Hyperkalemia	0	1	0	0
Hyponatremia	5	0	1	0
Glucose imbalance	0	1	0	0
Neurology and Ocular				
Dizziness	2	0	0	0
Pain				
Injection site	4	2	0	0
Abdominal	4	6	0	0
Joints	1	2	0	0
Lower extremity	1	1	0	0
Back	1	1	0	0
Chest wall	2	0	0	0
Head ache	1	2	0	0
Others	0	1	0	0
Pulmonary / Upper respiratory				
Nasal dripping	3	0	0	0
Hoarseness	0	1	0	0
Cough	1	2	0	0
Other *				
Erythrocytopenia	5	0	0	0
Relative lymphocyto-	3	0	0	0
penia				
Relative lymphocy-	2	0	0	0
tosis				
Leucosytosis	3	0	0	0
Thrombocytosis	3	0	0	0

ALT, alanine aminotransferase; AST, aspartate aminotransferase;

* Not gradeable as adverse events in CTCEA v3.0 criteria

Table 9. Neutralizing antibody titers, virus load in serum and response after Ad5/3-D24-GMCSF treatment

Patient ID	Dose (V ₂)	Tumor type	Neutralizing Antibody Titer					Virus Load in Serum							Response					
			Week post-treatment					Days post treatment							RECIST1.1 (% change)	Marker	Other benefit	Survival		
			0	1	2	3-4	> 4	0	1	2	3-7	8-14	15-21	22-30	31-46	47-63				
H64	8 x 10 ¹⁰	Pancreatic ca.	64	16384	16384	16384	16384	0	<500	<500	<500	0	0	0	0	0	PD (+78%)	MR (+9.0%)		155
Y62	1 x 10 ¹¹	Cholangio- and Renal ca.	64	16384	16384	16384	16384	0	0	<500	<500	714	<500	<500	<500	<500	PD	PD		52
S70	1 x 10 ¹¹	Sarcoma	0	0	0	4096	4096	0	571	336196	336196	743	<500	<500	<500	<500	SD (-5.9%)	PD		77
C66	2 x 10 ¹¹	Colon ca.	64	16384	16384	16384	10240	0	0	<500	<500	0	0	0	0	0	PD	Tumor softer		103
S67	2 x 10 ¹¹	Sarcoma	0	1024	1024	1024	1024	0	<500	<500	<500	0	0	0	0	0	SD (+4.7%)			221
I80	2 x 10 ¹¹	Melanoma	0	64	64	16384	16384	0	2061	1155	0	0	<500	<500	<500	<500	MR (-9.4%)	PD		42
I87	2 x 10 ¹¹	Melanoma	16	16384	16384	16384	16384	0	<500	0	0	<500	<500	<500	<500	<500	MR (-9.4%)	CR: ascites and pleural effusion		265
N110	2 x 10 ¹¹	Head and neck ca.	4	16	16	16384	16384	0	<500	19797	19797	0	0	0	0	0	MR (-9.4%)			27
K75	3 x 10 ¹¹	Lung adenoca.	0	0	0	4096	2560	0	<500	0	<500	<500	<500	<500	<500	<500	MR (-9.4%)	PD		312
O79	3 x 10 ¹¹	Ovarian ca.	64	16384	16384	16384	16384	0	<500	0	<500	0	0	0	0	0	MR (-8.6%)	MR (-8.6%)		84
O82	3 x 10 ¹¹	Ovarian ca.	1	4096	4096	1024	1024	0	<500	0	<500	0	0	0	0	0	PD	PD		95
C95	3 x 10 ¹¹	Rectum ca.	16	16384	16384	16384	16384	0	<500	21964	755	<500	<500	0	0	0	PD (new)	PD	1% reduction in injected tumor	111
H96	3 x 10 ¹¹	Pancreatic ca.	64	256	256	16384	16384	0	<500	0	<500	2412	<500	0	0	0	MR (-15.1%)			139
I98	3 x 10 ¹¹	Melanoma	0	0	0	4096	4096	0	1054	868	868	0	<500	<500	<500	<500	MR (-15.1%)	PD		325*
O113	3 x 10 ¹¹	Ovarian ca.	4	1024	1024	16384	16384	0	<500	868	868	0	<500	<500	<500	<500	SD (+10.7%)	PD		273
X122	3 x 10 ¹¹	Uterus ca.	0	0	0	4096	4096	0	<500	11891	11891	0	<500	<500	<500	<500	PD			93
O129	3 x 10 ¹¹	Ovarian ca.	64	16384	16384	16384	16384	0	<500	0	0	0	<500	<500	<500	<500	PD (new)	PD	injected tumor 5% smaller	234*
V135	3 x 10 ¹¹	Bladder and prostate ca.	4	16394	16394	16394	16394	0	<500	0	0	0	<500	<500	<500	<500	SD (+0.8%)	SD (+0.8%)	CR: non-injected liver metastasis	74
M137	3 x 10 ¹¹	Mesothelioma	0	0	0	0	0	0	<500	0	0	0	<500	<500	<500	<500	SD (+18.9%)		CR: pleural effusion	230*
H83	4 x 10 ¹¹	Pancreatic ca.	4	64	64	16384	16384	0	<500	1744	1744	0	<500	<500	<500	<500	SD (+6.6%)	PD	Tumor softer	59
S119	4 x 10 ¹¹	Sarcoma	54	4096	4096	4096	4096	0	0	0	0	0	0	0	0	0	SD (+6.6%)	PD	Tumor softer	283*

Table 10. Immune response to Ad5/3-D24-GM-CSF

Patient code	Dose (VP)	Days post-treatment																			
		IL-5 (pg/ml)					IL-8 (pg/ml)					IL-10 (pg/ml)					TNF-alpha (pg/ml)				
		0	1	2-4	6-12	13-40	0	1	2-4	6-12	13-40	0	1	2-4	6-12	13-40	0	1	2-4	6-12	13-40
Y62	1 x 10 ¹¹	27	36	40	42	77	157	114	113	135	226	16	18	30	29	38	29	30	51	48	56
S70	1 x 10 ¹¹	29	44	13		7	28	71	16		26	14	8	3		10	35	24	0		23
P74	1 x 10 ¹¹																				
C66	2 x 10 ¹¹	19			32	11	124		114	336		16			19	4	28			32	0
S67	2 x 10 ¹¹	28	39		28	25	17	21	26	22		17	19		19	19	35	37		33	29
I80	2 x 10 ¹¹	293	88		65	102	93	101	52	115		20	28		23	22	36	39	35	43	
I87	2 x 10 ¹¹	26	70	48		72	31	49	39	54		61	85		56	89	80	124	65	105	
N110	2 x 10 ¹¹																				
K75	3 x 10 ¹¹	30	28			33	51	67		63		27	25			47	43	42		72	
O79	3 x 10 ¹¹	39	46		10	37	46	55	38	119		22	25		18	49	41	46	32	95	
O82	3 x 10 ¹¹	0	7		9		10	17	24			4	16		7		16	26	18		
C95	3 x 10 ¹¹	101		63	40	43	320		309	462		100		43	32	39	106		42	20	69
H96	3 x 10 ¹¹		99	18		28		65	3	21			134			36		187	6	43	
I98	3 x 10 ¹¹	38	12	7			31	10	5			16	3	41			50	2	13		
O113	3 x 10 ¹¹																				
X122	3 x 10 ¹¹																				
O129	3 x 10 ¹¹																				
V136	3 x 10 ¹¹																				
M137	3 x 10 ¹¹																				
H83	4 x 10 ¹¹	23	30	20		73	37	49	33	166		24	11	16		58	42	21	30	86	
S119	4 x 10 ¹¹																				
H64	8 x 10 ¹⁰	21	48	26	0	22	35	59	41	32		22	31	26	1	23	47	37	55	9	34

Claims

1. An oncolytic adenoviral vector, characterized by comprising an adenovirus serotype 5 (Ad5) nucleic acid backbone, a 24 bp deletion (D24) in the Rb binding constant region 2 of E1 and a nucleic acid sequence encoding a human granulocyte-macrophage colony-stimulating factor (GM-CSF) in the place of the deleted gp19k/6.7K in the E3 region.
2. An oncolytic adenoviral vector according to claim 1, further characterized by comprising one or more regions selected from a group consisting of E2, E4, and late regions.
3. An oncolytic adenoviral vector according to claim 1 or 2, characterized by comprising the following regions: a left ITR, partial E1, pIX, pIVa2, E2, VA1, VA2, L1, L2, L3, L4, partial E3, L5, E4, and a right ITR.
4. An oncolytic adenoviral vector according to claim 3, characterized in that the regions are in a sequential order in the 5' to 3' direction.
5. An oncolytic adenoviral vector according to any one of claims 1 to 4, characterized in that a wild type region is located upstream of the E1 region.
6. An oncolytic adenoviral vector according to any one of claims 1 to 5, characterized in that the E1 region comprises a viral packaging signal.
7. An oncolytic adenoviral vector according to any one of claims 1 to 6, characterized in that nucleic acid sequence encoding GM-CSF is under the control of the viral E3 promoter.
8. An oncolytic adenoviral vector according to any one of claims 1 to 7, characterized in that a nucleic acid sequence encoding GM-CSF is of a wild type.
9. An oncolytic adenoviral vector according to any one of claims 1 to 8, characterized in that the E4 region is of a wild type.
10. An oncolytic adenoviral vector according to any one of claims 1 to 9, characterized by comprising a capsid modification.
11. An oncolytic adenoviral vector according to claim 10, characterized in that the capsid modification is Ad5/3 chimerism, insertion of an integrin binding (RGD) region and/or heparin sulphate binding polylysine modification into the fiber.
12. An oncolytic adenoviral vector according to claim 10 or 11, characterized in that the capsid modification is a RGD-4C modification.

13. An oncolytic adenoviral vector according to any one of claims 1 to 12, characterized by comprising at least one expression cassette.

14. An oncolytic adenoviral vector according to any one of claims to 13, characterized by only one expression cassette.

15. An oncolytic adenoviral vector according to any one of claims 1 to 14, characterized in that the vector is capable of selectively replicating in cells having defects in the Rb-pathway.

16. A cell, characterized by comprising the adenoviral vector according to any one of claims 1-15.

17. A pharmaceutical composition, characterized by comprising the adenoviral vector according to any one of claims 1-15.

18. An oncolytic adenoviral vector according to any one of claims 1 to 15, characterized in that it acts as an *in situ* cancer vaccine.

19. A pharmaceutical composition according to claim 17, characterized in that it acts as an *in situ* cancer vaccine.

20. A use of the adenoviral vector according to any one of claims 1-15 in the manufacture of a medicament for treating cancer in a subject.

21. The use according to claim 20, characterized in that the cancer is selected from a group consisting of nasopharyngeal cancer, synovial cancer, hepatocellular cancer, renal cancer, cancer of connective tissues, melanoma, lung cancer, bowel cancer, colon cancer, rectal cancer, colorectal cancer, brain cancer, throat cancer, oral cancer, liver cancer, bone cancer, pancreatic cancer, choriocarcinoma, gastrinoma, pheochromocytoma, prolactinoma, T-cell leukemia/lymphoma, neuroma, von Hippel-Lindau disease, Zollinger-Ellison syndrome, adrenal cancer, anal cancer, bile duct cancer, bladder cancer, ureter cancer, brain cancer, oligodendroglioma, neuroblastoma, meningioma, spinal cord tumor, bone cancer, osteochondroma, chondrosarcoma, Ewing's sarcoma, cancer of unknown primary site, carcinoid, carcinoid of gastrointestinal tract, fibrosarcoma, breast cancer, Paget's disease, cervical cancer, colorectal cancer, rectal cancer, esophagus cancer, gall bladder cancer, head cancer, eye cancer, neck cancer, kidney cancer, Wilms' tumor, liver cancer, Kaposi's sarcoma, prostate cancer, lung cancer, testicular cancer, Hodgkin's disease, non-Hodgkin's lymphoma, oral cancer, skin cancer, mesothelioma, multiple myeloma, ovarian cancer, endocrine pancreatic can-

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22. The use according to claims 19-21, characterized in that the subject is a human or an animal.

23. The use according to any one of claims 20-22, characterized in that the medicament is to be administered through an intratumoral, intramuscular, intra-arterial, intravenous, intrapleural, intravesicular, intracavitary or peritoneal injection, or an oral administration.

24. The use according to any one of claims 20-23, characterized in that the oncolytic adenoviral vector having a different fiber knob of the capsid compared to the vector of the earlier treatment, is administered to a subject.

25. The use according to any one of claims 20-24, characterized in that the medicament is to be combined with administration of concurrent radiotherapy and or chemotherapy.

26. The use according to any one of claims 20-25, characterized in that the medicament is to be combined with administration of verapamil or another calcium channel blocker, and/or autophagy inducing agents, and/or temozolomide, and/or chemotherapy or anti-CD20 therapy or other approaches for blocking of neutralizing antibodies, substances capable to downregulating regulatory T-cells in a subject, and/or cyclophosphamide to a subject.

27. A method of producing GM-CSF in a cell, characterized in that the method comprises:

- a) carrying a vehicle comprising an oncolytic adenoviral vector according to any one of claims 1-15 to a cell, and
- b) expressing GM-CSF of said vector in the cell.

28. A use of the oncolytic adenoviral vector according to any one of claims 1-15 for producing GM-CSF in a cell.

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seqlisting2090342PC title amend_ST25.txt

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