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(54) Title: ENGINEERED VACCINIA VIRUS

(57) Abstract: An engineered vaccinia virus, a pharmaceutical composition containing the same, and methods for use in treating a subject in need using the same are provided. The engineered vaccinia virus includes a mutated viral sequence and a heterologous sequence. The mutated viral sequence is used for selective replication in tumor cells and/or activation of immune cells. The heterologous sequence encodes an immune co-stimulatory pathway activating molecule, immunomodulator gene, a truncated viral envelope gene, and/or a tumor suppressor. The heterologous sequence is stably incorporated into the genome of the engineered vaccinia virus. The pharmaceutical composition includes an effective amount of the engineered vaccinia virus and a pharmaceutical acceptable vehicle. The methods for use in treating the subject in need include administering the engineered vaccinia virus to the subject.

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ENGINEERED VACCINIA VIRUS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of the filing date of U.S. provisional patent application Ser. No. 62/873,221, filed on Jul. 12, 2019, the disclosure of which is incorporated herein by reference.

FIELD OF INVENTION

[0002] The present disclosure relates to an engineered vaccinia virus for the treatment of cancer.

BACKGROUND OF INVENTION

[0003] Cancer is a serious and dangerous disease. There are nearly 5 million new cancer cases in China each year, and the number of patients dying from cancer is close to 3 million. These figures have been increasing year by year. It shows that traditional cancer treatments such as surgery, chemotherapy and radiotherapy are not effective in helping most cancer patients. Therefore, cancer patients are in a great need of more effective treatments. Despite some progress in new cancer immunotherapy, how to treat patients with solid tumor more effectively remains one of the biggest challenges. In recent years, oncolytic viruses, which are one of the types of cancer immunotherapy, have received increasing attention in the industry.

[0004] Oncolytic viruses are viruses that target and kill tumor cells by selectively infecting tumor cells or selectively replicating in tumor cells. In addition, oncolytic viruses can effectively provide the risk signals necessary to induce and amplify the host's anti-tumor immune response, thereby allowing the body's immune system to produce a strong and specific anti-tumor immune response.

[0005] Vaccinia virus (VV) is a double-stranded DNA virus, its unique properties make it an advantageous option among oncolytic viruses. 1) VV has demonstrated good safety in the process of preventing smallpox as a vaccine against infectious diseases, so its safety as an oncolytic virus can be guaranteed. In addition, it has been further confirmed in many clinical trials that it is safe to use VV as an oncolytic virus. 2) It can rapidly self-replicate in cells, and new virus particles can be produced in about 6-8 hours, so that infected cells can be rapidly lysed. 3) The genome of VV is about 200Kbp,

and this larger genome has a large capacity to carry foreign genes. 4) Vaccinia virus does not require specific receptors to infect cells, therefore it has a wide tropism for different type of tumors. 5) It can be administered in a variety of ways, including topical intratumoral injection, intraperitoneal injection, intrathoracic injection and systemic intravenous injection. This diversity of administering method has made it possible to treat tumors in any part of the body. 6) In addition, the hypoxic microenvironment normally found in solid tumors has a negative effect on the replication and efficacy of many types of oncolytic viruses. However, VV can replicate efficiently in hypoxic environment (Hiley et al, Gene Therapy 17, 281-287). (2010). 7) There are a variety of natural and synthetic promoters that can be applied to VV, which makes it ideal for carrying transgenes. In recent years, clinical results using vaccinia virus have demonstrated that VV has a good anti-tumor effect and is safe (Haddad et al, Annals of Surgical Oncology 19 Suppl 3, S665-674 (2012); Park et al, Lancet Oncol 9: 533-542, (2008); Breitbach et al, Nature 477: 99-102, (2011)).

[0006] Various deletion mutants of vaccinia virus have been reported so far. A Western Reserve strain mutant having a thymidine kinase (TK) gene and a viral growth factor (VGF) gene deletion is capable of efficiently eliciting an immune response against a tumor antigen (McCart et al, Cancer Res 61, 8751-8757 (2001)). Furthermore, viruses inserted with a heterologous gene, such as a cytokine encoding gene, can further activate an anti-tumor immune response.

[0007] After VV infects tumor cells, VV is released by lysis of infected cells so that the virus can infect tumor cells that are localized or infect tumor cells that are far away from the infected site via circulating blood. The vaccinia virus mainly has two forms of infectious virus particles, namely Intracellular Mature Virus (IMV) and Extracellular Enveloped Virus (EEV) (Appleyard et al., J. Gen. Virology 13, 9-17 (1971)). IMV is a major infectious form of viral particles. EEV is enveloped by the host cell membrane and thus can antagonize host systemic innate (complement) and adaptive (neutralizing antibodies) immune attack, allowing VV to spread widely and over long distances in the host (Smith, GL & Vanderplasschen, A and Law, M J. Gen. Virol. 83, 2915-2931 (2002); Payne, LG & Kristensson, K, J. Gen. Virol. 66 (c), 643-646 (1985)). However, most VV strains produce only a small amount of EEV (only less than 1% of all infectious viruses).

[0008] VV has six genes encoding EEV-specific proteins. They are A33R, A34R, A36R, F13L, B5R and A56R. The B5R gene encodes a 42 kDa glycoprotein containing four copies of a 50-70-amino acid repeat, termed a "short consensus repeat" (SCR). Deletion of the B5R gene results in smaller plaques and a significant reduction in EEV formation (≤10-fold) (Blasco, R. & Moss, B., J. Virol. 65, 5910-5920 (1991); Engelstad, M. & Smith, GL , Virology 194, 627-637 (1993)). The transmembrane and cytoplasmic tail sequences of B5R are important for targeted packaging of viral proteins (Katz et al, J. Virol. 71, 3178-3187 (1997)). VV carries B5R gene mutants lacking SCR4, SCR3, 4 or SCR 2, 3, 4 produces smaller plaques, but produces infectious EEVs that are dozens of times more than wild-type viruses and form a comet tail plaque distribution (Sanderson et al, J. Gen Virol. 79 (f), 1415-1425 (1998); Mathew et al, J. Virol. 72, 2429-2438 (1998)).

[0009] In the past 10 years or so, despite the progress in the field of oncolytic virus research, vaccinia virus-based therapeutic products have not yet entered the clinic, the difficulty is how to scientifically modify the vaccinia virus to have better anti-tumor effect. Therefore, there is an urgent need to develop better oncolytic vaccinia virus products to meet the needs of treating cancer.

[0010] There are a variety of vaccinia strains that have varying degrees of virulence to humans and animals. As part of the 1970s smallpox eradication program, many different strains were used around the world. For example, the New York City Department of Health (NYCBOH) strain and its derivatives Wyeth are popular in the United States, while Copenhagen (CPN) and Lister strain are dominant in Europe. Previous study found that Lister strain has a good anti-tumor effect. This project selected Lister strain as an oncolytic virus for development.

SUMMARY OF INVENTION

[0011] The present disclosure relates to an oncolytic virus, and more particularly to an engineered oncolytic vaccinia virus. The engineered oncolytic vaccinia virus of the present disclosure is capable of selectively infecting cancer cells, which can be beneficially used in the treatment of cancer.

[0012] The present disclosure provides an engineered vaccinia virus comprising a mutated viral sequence and a heterologous sequence, wherein the mutated viral sequence promotes selective replication in tumor cells, and/or activation of immune

cells, the heterologous sequence encodes an immune co-stimulatory pathway activating molecule, immunomodulator gene, a truncated viral envelope gene, and/or a tumor suppressor, and the heterologous sequence is stably incorporated into the genome of the engineered vaccinia virus.

[0013] In an embodiment of the engineered vaccinia virus, the heterologous sequence is stably incorporated into the mutated viral sequence of the engineered vaccinia virus.

[0014] In an embodiment of the engineered vaccinia virus, the mutated viral sequence comprises at least one of:

[0015] (a) a mutation(s) in L025, TK, A46R, or any combination thereof;

[0016] (b) partially deleted L025, TK, A46R, or any combination thereof;

[0017] (c) deleted L025, TK, A46R, or any combination thereof;

[0018] (d) a portion or all of the L025, TK, or A46R, which is replaced by one of sequences set forth in SEQ ID NO: 2, 4, 6, 8, 10, 12, or 14;

[0019] (e) a portion or all of the L025, TK, or A46R, which is replaced by a tumor targeting gene;

[0020] (f) a portion or all of the L025, TK, or A46R, which is replaced by a ligand or an antibody that targets T cells;

[0021] (g) a portion or all of the L025, TK, or A46R, which is replaced by a therapeutic gene or a modified version thereof; or

[0022] (h) a portion or all of the L025, TK, or A46R is replaced by a therapeutic antibody.

[0023] In an embodiment of the engineered vaccinia virus, the heterologous sequence comprises at least one of:

[0024] (a) a sequence set forth in SEQ ID NO: 2;

[0025] (b) a sequence set forth in SEQ ID NO: 4;

[0026] (c) a sequence set forth in SEQ ID NO: 6;

[0027] (d) a sequence set forth in SEQ ID NO: 8;

[0028] (e) a sequence set forth in SEQ ID NO: 10;

[0029] (f) a sequence set forth in SEQ ID NO: 12; or

[0030] (g) a sequence set forth in SEQ ID NO: 14.

[0031] In an embodiment of the engineered vaccinia virus, the heterologous sequence encodes at least one of:

[0032] (a) a sequence set forth in SEQ ID NO: 1;

[0033] (b) a sequence set forth in SEQ ID NO: 3;

[0034] (c) a sequence set forth in SEQ ID NO: 5;

[0035] (d) a sequence set forth in SEQ ID NO: 7;

[0036] (e) a sequence set forth in SEQ ID NO: 9;

[0037] (f) a sequence set forth in SEQ ID NO: 11; or

[0038] (g) a sequence set forth in SEQ ID NO: 13.

[0039] In an embodiment of the engineered vaccinia virus, the engineered vaccinia virus comprises a sequence of formula: 5'-A1-X-A2-B1-Y-B2-C1-Z-C2-3', wherein A1 and A2 are a left arm and a right arm of a first viral gene respectively, B1 and B2 are a left arm and a right arm of a second viral gene, respectively, C1 and C2 are a left arm and a right arm of a third viral gene, respectively, wherein X, Y, and Z are heterologous genes, each selected from one of immunomodulatory genes, cytokines, therapeutic genes, truncated viral envelope genes, tumor suppressor genes, genes encoding therapeutic antibodies, and genes encoding ligands of the therapeutic antibodies.

[0040] In an embodiment of the engineered vaccinia virus, the first viral gene is L025, the second viral gene is TK, and the third viral gene is A46R.

[0041] In an embodiment of the engineered vaccinia virus, X is a hybrid gene of IL-21 and modified B5R, Y is 4-1BBL, and Z is HIC1.

[0042] In an embodiment of the engineered vaccinia virus, the mutated viral sequence comprises mutations of deletions in L025, TK, and A46R, and the heterologous sequence comprises IL-21 and 4-1BBL.

[0043] In an embodiment of the engineered vaccinia virus, the mutated viral sequence comprises mutations of deletions in L025, TK, and A46R, and the

heterologous sequence comprises a hybrid gene of IL-21 and modified B5R, and 4-1BBL.

[0044] In an embodiment of the engineered vaccinia virus, the immunomodulator genes is a cytokine gene encoding IL-12, IL-21, IL-2, IL-15, IL-8, or a modified version thereof.

[0045] In an embodiment of the engineered vaccinia virus, the immune co-stimulatory pathway activating molecule comprises CD40 ligand (CD40L), ICOS ligand, GITR ligand, 4-1BB ligand, OX40 ligand, TL1A, CD30 ligand, CD27, Flt3 ligand, or a modified version thereof.

[0046] In an embodiment of the engineered vaccinia virus, the tumor suppressor gene is HIC1.

[0047] In an embodiment of the engineered vaccinia virus, the engineered vaccinia virus is selected from the group consisting of Lister, Western Reserve (WR), Copenhagen (Cop), Bern, Paris, Tashkent, Tian Tan, Wyeth (DRYVAX), IHD-J, IHD-W, Brighton, Ankara, CVA382, modified vaccinia ankara (MVA), Dairen I, LC16m8, LC16M0, LIVP, ACAM2000, WR 65-16, Connaught, New York City Board of Health (NYCBH), EM-63 and NYVAC strain.

[0048] In an embodiment of the engineered vaccinia virus, the truncated viral envelope gene is B5R containing a short consensus repeats (SCR) 2, SCR3, and SCR4 domains deletion.

[0049] In an embodiment of the engineered vaccinia virus, the tumor suppressor is HIC1.

[0050] The present disclosure further provides a pharmaceutical composition comprising an effective amount of the engineered vaccinia virus of any one of the embodiments of the present disclosure and a pharmaceutical acceptable carrier.

[0051] In an embodiment of the pharmaceutical composition, the pharmaceutical composition is formulated for oral, topical, parenteral delivery, or interventional therapy.

[0052] In an embodiment of the pharmaceutical composition, the pharmaceutical composition is formulated for topical intratumoral injection, intraperitoneal injection, intrathoracic injection, systemic intravenous injection, intramuscular injection,

subcutaneous injection, intrathecal injections, direct intraventricular injection, intracardiac injection, intranasal injections.

[0053] In an embodiment of the pharmaceutical composition, the engineered vaccinia virus is used alone as monotherapy; or in combination with one or more anti-cancer agent, immune suppressors, and/or oncolytic virus enhancers.

[0054] In an embodiment of the pharmaceutical composition, the engineered vaccinia virus is used in combination with 5-fluorouracil (FU), folinic acid (FA), methotrexate, capecitabine, oxaliplatin, bevacizumab, cetuximab, immune checkpoint inhibitors (such as anti-PD1, anti-PDL1, anti-CTLA4 agents), other types of oncolytic viruses, pembrolizumab, nivolumab, ipilimumab, atezolizumab, avelumab, adenovirus, or combination thereof.

[0055] The present disclosure further provides a method for use in treating a cancer in a subject in need thereof, comprising administering to the subject an effective amount of the engineered vaccinia virus of any one of the embodiments of the present disclosure or the pharmaceutical composition of any one of the embodiments of the present disclosure.

[0056] In an embodiment of the method, HIC1 is inactivated, underexpressed, or loss in the cancer.

[0057] In an embodiment of the method, the cancer is selected from the group consisting lung cancer, melanoma, pancreatic cancer, liver cancer, colon cancer, breast cancer, glioblastoma, sarcoma, stomach cancer, ovarian cancer, mesothelioma, and leukemia.

[0058] A method of increasing tumor-specific infectivity of vaccinia virus, comprising administering the engineered vaccinia virus of any one of the embodiments of the present disclosure to a subject, wherein the engineered vaccinia virus is administered in an amount effective for invoking anti-tumor immune response in a subject.

[0059] A method of conferring persistent immunity to tumor relapse in a subject in need thereof comprising administering the engineered vaccinia virus of any one of the embodiments of the present disclosure to the subject.

[0060] A method of screening patients based on a percentage of GFP positive cells 48 hours post infection of patient's cancer cells with the engineered vaccinia virus of any one of the embodiments of the present disclosure.

[0061] An engineered vaccinia virus for use in inducing cancer cells death, regulating a biological activity of the cancer cells, regulating immune response, enhancing proliferation of T cells, and/or cytotoxicity of T cells, wherein the engineered oncolytic is as provided in any one of the embodiments of the present disclosure.

[0062] An engineered vaccinia virus for use in inducing cancer cells death, regulating a biological activity of the cancer cells, regulating immune response, enhancing proliferation of T cells, and/or cytotoxicity of T cells, wherein the biological activity of the cancer cells comprises inhibition of cancer cells replication, inhibition of cancer cells division, inhibition of DNA repair of cancer cells, inhibition of cancer cells migration, or promoting cancer death, wherein the engineered oncolytic is as provided in any one of the embodiments of the present disclosure.

[0063] An engineered vaccinia virus for use in the manufacture of a medicament for treating lung cancer, melanoma, pancreatic cancer, liver cancer, colon cancer, breast cancer, glioblastoma, sarcoma, stomach cancer, ovarian cancer, mesothelioma, and leukemia, wherein the engineered oncolytic is as provided in any one of the embodiments of the present disclosure.

[0064] An engineered vaccinia virus for use in the manufacture of a medicament for suppressing cancer cells growth, inducing cancer cells death, and/or regulating a biological activity of the cancer cells, wherein the engineered oncolytic is as provided in any one of the embodiments of the present disclosure.

DESCRIPTION OF DRAWINGS

[0065] In order to illustrate a technical solution in the embodiments of the present application or in the prior art more clearly, the accompanying drawings required in the embodiments are introduced briefly hereafter. The accompanying drawings in the following description are merely part of the embodiments of the present application. Based upon the accompanying drawings, people with ordinary skills in the art can obtain other drawings without making inventive efforts.

[0066] FIG. 1A is a schematic diagram of the product carrying mB5R.

[0067] FIG. 1B is a schematic diagram of the product carrying sB5R.

[0068] FIG. 2A is a schematic diagram of the shuttle vector carrying mouse 4-1BBL for the deletion of TK gene.

[0069] FIG. 2B is a schematic diagram of the shuttle vector carrying human 4-1BBL for the deletion of TK gene.

[0070] FIG. 3A shows the shuttle vector carrying mouse IL-21 and modified B5R (mB5R/sB5R) for deletion of the L025 gene.

[0071] FIG. 3B shows the shuttle vector carrying human IL-21 and modified B5R (mB5R/sB5R) for deletion of the L025 gene.

[0072] FIG. 4 shows the shuttle vector carrying the HIC1 gene for deleting the A46R gene.

[0073] FIG. 5 shows structure diagrams of the mB5R/sB5R gene.

[0074] FIG. 6 shows recombinant virus with L025 gene deletion.

[0075] FIG. 7. Shows recombinant virus with TK gene deletion and hSPD-41BBL gene insertion.

[0076] FIG. 8. Shows recombinant virus deletion of the A46R gene and viral expression of the inserted gene HIC1.

[0077] FIGs. 9A-9B. KM1 killing of a panel of solid tumor cell lines with escalating dosage of the virus in 6-well plate for two days. The amount of virus used to infect the individual well were 0.01pfu/cell, 0.1pfu/cell, and 1pfu/cell. Crystal violet was used to stain the remaining cells in the well when terminated the experiment.

[0078] FIGs. 10A-10C shows examples of EC50 assay on 17 cell lines.

[0079] FIG. 11 shows the EC50 value for different cell lines.

[0080] FIG. 12 shows killing of leukemia cell lines by vaccinia virus. examples of EC50 assay on two cell lines.

[0081] FIG. 13 shows examples of leukemia patients' white blood cells infected by KM1.

[0082] FIG. 14 shows live/dead cells three days post KM1 infection.

[0083] FIG. 15 shows anti-tumor efficacy of KM1 in Syrian hamster pancreatic cancer HPD 1NR model.

[0084] FIG. 16 shows treatment of human breast cancer cell MDA MB-231 using HIC1 armed vaccinia virus and its counterpart control HY3.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

[0085] Referring to the drawings in the accompanying drawings, same reference numerals represent same components. The following description is based on the detailed embodiments of the present disclosure as exemplified, and should not be construed as a limitation to other embodiments of the present application which are not described herein in detail.

[0086] Please refer to FIGs. 1A-1B. FIG. 1A is a schematic diagram of the product carrying mB5R and FIG. 1B is a schematic diagram of the product carrying sB5R.

[0087] The vaccinia virus and the viral vector of the present disclosure comprises a sequence of formula: 5'-A_1-X-A_2-B_1-Y-B_2-C_1-Z-C_2-3', wherein A_1 and A_2 are a left arm and a right arm of a first viral gene respectively, B_1 and B_2 are a left arm and a right arm of a second viral gene respectively, C_1 and C_2 are a left arm and a right arm of a third viral gene respectively, wherein X, Y, and Z are each selected from immunomodulatory genes, cytokines, therapeutic genes, and therapeutic antibodies, ligands of the therapeutic antibodies. Exemplary structures of the obtained product are shown in FIG. 1A and 1B. The first viral gene is L025, the second viral gene is TK, and the third viral gene is A46R. X is a hybrid gene of IL-21 and modified B5R, Y is 4-1BBL, and Z is HIC1.

[0088] The gaps between "A_1-X-A_2" and "B_1-Y-B_2" and between "B_1-Y-B_2" and "C_1-Z-C_2" can contain other viral genes known in vaccinia virus.

[0089] Product design and construction:

[0090] 1. TK gene deletion and integration of 4-1BBL gene into the TK gene site

[0091] Please refer to FIGs. 2A-2B. FIG. 2A is a schematic diagram of the shuttle vector carrying mouse 4-1BBL for the deletion of TK gene. FIG. 2B is a schematic diagram of the shuttle vector carrying human 4-1BBL for the deletion of TK gene.

[0092] In FIG. 2A, the left arm of the TK targets the left side of the TK gene (L089), and the right arm of the TK targets the right side of the TK gene (L091). The Loxp site is flanked by the H5 promoter and the red fluorescent protein RFP, and is located between the TK left arm and the promoter H5 of the mouse 4-1BBL gene.

[0093] In FIG. 2B, the left arm of the TK targets the left side of the TK gene (L089), and the right arm of the TK targets the right side of the TK gene (L091). The Loxp site is flanked by the H5 promoter and the red fluorescent protein RFP, and is located between the TK left arm and the promoter H5 of the human 4-1BBL gene.

[0094] The expression cassette is designed as follows (see FIG. 2A and FIG. 2B for a schematic diagram):

[0095] TK left arm--loxp-H5-RFP-loxp-H5-m/h4-1BBL-TK right arm

[0096] In order to delete two or more genes using the same reporter protein (RFP), it is important to use a suitable homologous recombination system, such as Cre-Lox, or a Flp/FRT system for removal of the reporter gene. The gene insertion elements may be added to the expression cassette around the nucleic acid sequence encoding the protein(s) and upstream of the promoter region driving expression of the protein(s).

[0097] The loxp sites are located upstream of H5 and downstream of the RFP, respectively, so that the C5 recombinase can be used to excise the H5-RFP in the new recombinant virus by acting on the FRET site.

[0098] The H5 promoter drives expression of the 4-1BBL gene. The amino acid sequence of SPD-m4-1BBL is set forth in SEQ ID NO: 5 and the nucleotide sequence of SPD-m4-1BBL of SPD-m4-1BBL is set forth in SEQ ID NO: 6. The amino acid sequence of SPD-h4-1BBL is set forth in SEQ ID NO: 7 and the nucleotide sequence of SPD-h4-1BBL of SPD-h4-1BBL is set forth in SEQ ID NO: 8.

[0099] In order to perform targeted homologous recombination at the TK gene (L090) site of vaccinia virus, it is necessary to provide an additional sequence complementary to the TK gene and/or the gene adjacent to the TK gene for the recombinant region used to produce the vector of the present disclosure. A TK left arm (L-arm) was provided to target the left side of the TK gene (L089) and the right arm of the TK (R-arm) to target the right side of the TK gene (L091). Then, the actual

expression cassette comprising 4-1BBL gene and the reporter gene RFP can be located between the TK-L arm and the TK-R arm, and they can be inserted into the targeted TK region of the vaccinia virus. The expression cassette can be inserted into the recombinant vector prior to transformation of the vaccinia virus vector.

[0100] 2. Deletion of L025 gene and integration of interleukin 21 (IL-21) and mB5R (or sB5R) into the L025 region

[0101] Please refer to FIG. 3A-3B. FIG. 3A shows the shuttle vector carrying mouse IL-21 and modified B5R (mB5R/sB5R) for deletion of the L025 gene. FIG. 3B shows the shuttle vector carrying human IL-21 and modified B5R (mB5R/sB5R) for deletion of the L025 gene.

[0102] In FIG. 3A, the left arm of L025 targets the left side of the L025 gene (L024), and the right arm of L025 targets the right side of the L025 gene (L026). The FRT site is located on both sides of the H5 promoter and the red fluorescent protein RFP, and is located between the left arm of L025 and the right arm of L025.

[0103] In FIG. 3B, the left arm of L025 targets the left side of the L025 gene (L024), and the right arm of L025 targets the right side of the L025 gene (L026). The FRT site is located on both sides of the H5 promoter and the red fluorescent protein RFP, and is located between the left arm of L025 and the right arm of L025.

[0104] The expression cassette is designed as follows (see FIG. 3A and 3B for a schematic diagram):

[0105] L025 left arm- FRT-H5-RFP-FRT-H5- m/sB5R-H5-m/hIL-21-L025 right arm

[0106] Red fluorescent protein (RFP) is used as a reporter protein to facilitate the screening of new recombinant viruses. A promoter is provided upstream of the reporter protein to drive expression of the reporter protein, here using the H5 promoter. The FRT are recognition site for Flipase, so that the RFP can be excised.

[0107] The amino acid sequence of mouse IL-21 is set forth in SEQ ID NO: 9 and the nucleotide sequence of mouse IL-21 is set forth in SEQ ID NO: 10. The amino acid sequence of human IL-21 is set forth in SEQ ID NO: 11 and the nucleotide sequence of human IL-21 is set forth in SEQ ID NO: 12.

[0108] Please refer to FIG. 5. FIG. 5 shows structure diagrams of the mB5R/sB5R gene. mB5R consists of a signal peptide, stalk, a transmembrane region (TM) and an intracellular tail segment (CT) gene fragment. sB5R consists of a signal peptide, short consensus repeat 1 (SCR1), stalk, a transmembrane region (TM) and an intracellular tail segment (CT) gene fragment.

[0109] The H5 promoter drives the expression of the immunotherapeutic gene IL21 and the expression of mB5R or sB5R. See FIG. 5 for schematic structure diagrams of mB5R and sB5R. The amino acid sequence of mB5R is set forth in SEQ ID NO: 1 and the nucleotide sequence of mB5R is set forth in SEQ ID NO: 2. The amino acid sequence of sB5R is set forth in SEQ ID NO: 3 and the nucleotide sequence of sB5R is set forth in SEQ ID NO: 4.

[0110] The above recombinant region needs to be inserted into the L025 gene of vaccinia virus. Here, insertion was performed using a method of homologous recombination, and the integration site was between the left and right arms of L025.

[0111] In order to perform targeted homologous recombination at the L025 gene site of vaccinia virus, it is necessary to provide an additional sequence complementary to the L025 gene and/or the gene adjacent to the L025 gene for the recombinant region used to produce the vector of the present disclosure. The L025 left arm (L-arm) was provided to target the left side of the L025 gene (L024) and the right arm of the L025 (R-arm) to target the right side of the L025 gene (L026). The actual expression cassette comprising the HIC1 gene and the reporter gene GFP can then be placed between the L025-L arm and the L025-R arm, and they can be inserted into the targeted L025 region of the vaccinia virus. The expression cassette can be inserted into the recombinant vector prior to transformation of the vaccinia virus vector.

[0112] 3. The HIC1 gene is inserted into the A46R region.

[0113] Please refer to FIG. 4. FIG. 4 shows the shuttle vector carrying the HIC1 gene for deleting the A46R gene. The left arm of A46R targets the left side of the A46R gene (L163), and the right arm of A46R targets the right side of the A46R gene (L165). The H5 promoter drives the expression of green fluorescent protein GFP. The H5 promoter drives expression of the HIC1 gene.

[0114] The expression cassette is designed as follows (see FIG. 4 for a schematic diagram):

[0115] A46R left arm - H5-GFP-H5-HIC1- A46R right arm

[0116] In order to perform targeted homologous recombination at the A46R gene (L164) site of vaccinia virus, it is necessary to provide an additional sequence complementary to the A46R gene and/or the gene adjacent to the A46R gene for the recombinant region used to produce the vector of the present disclosure. The A46R left arm (L-arm) was provided to target the left side of the A46R gene (L163) and the right arm of the A46R (R-arm) to target the right side of the A46R gene (L165). The actual expression cassette comprising the HIC1 gene and the reporter gene GFP can then be placed between the A46R-L arm and the A46R-R arm, and they can be inserted into the targeted A46R region of the vaccinia virus. The expression cassette can be inserted into the recombinant vector prior to transformation of the vaccinia virus vector.

[0117] Materials and Methods

[0118] Cell lines: All tumor cell lines used were from the ATCC. All human cancer cell lines were genotyped by STR assay. The murine tumor cell lines used in this study included: the colorectal cancer cell line MC38 was derived from C57B/6 mice. CV1 is an African green monkey "normal" kidney cell line obtained from ATCC, Virginia, USA, and is used as a stock cell line to facilitate large-scale production of the virus as well as all virus titration assays.

[0119] Virus: The wild type VV Lister is commercially available from American type culture collection (ATCC) under ATCC accession number VR-1549™. The wild type VV Lister is also deposited at China Center for Type Culture Collection (CCTCC) under CCTCC deposition number: V201937. The date of deposit is July 4, 2019.The strain designation is Lister vaccinia virus 01(VV01). Detailed information can also be obtained from the product sheet of ATCC VR-1549™. The engineered VV Lister is deposited at CCTCC under CCTCC deposition number: V201938. The date of deposit is July 4, 2019. The strain designation is Vaccinia virus Lister strain KM1. Both the wild type VV Lister and the engineered VV Lister are deposited pursuant to the Budapest Treaty. The wild type VV Lister and the engineered VV Lister are deposited at China Center for Type Culture Collection (CCTCC), College of Life Sciences, Wuhan University Wuhan 430072, China. The depositor of the wild type VV Lister

and the engineered VV Lister is Shenzhen Hua Yao Kang Ming Biopharmaceutial Co.,Ltd., 14 Jinhui road, Pingshan District, Shenzhen, China (the Applicant).

[0120] Construction of the TK shuttle vector:

[0121] The TK shuttle vector includes the left side of the TK gene targeting the TK gene (L089) and the right arm of the TK targeting the right side of the TK gene (L091). The Loxp site is flanked by the H5 promoter and the red fluorescent protein RFP and is located between the TK left arm and the promoter H5 of the 4-1BBL gene. All of the above sequences were put together by us, and synthesized by a company and cloned into the PUC57 vector.

[0122] Construction of the L025 shuttle vector:

[0123] The schematic structure diagram of the L025 shuttle vector is shown in FIG. 3A and 3B. The L025 shuttle vector includes the L025 left arm targeting the left side of the L025 gene (L024) and the L025 right arm targeting the right side of the L025 gene (L026). The FRT site is located on both sides of the H5 promoter and the red fluorescent protein RFP and is located between the left arm of L025 and the promoter H5 of the mB5R/sB5R gene. All of the above sequences were spliced together and synthesized by the company and cloned into the PUC57 vector.

[0124] Construction of the A46R shuttle vector:

[0125] The schematic structure diagram of the A46R shuttle vector is shown in FIG. 4. The A46R shuttle vector includes the A46R left arm targeting the left side of the A46R gene (L163) and the A46R right arm targeting the right side of the A46R gene (L165). The H5 promoter drives the expression of green fluorescent protein GFP. The H5 promoter drives expression of the HIC1 gene. All of the above sequences were put together by us, and synthesized by a company and cloned into the PUC57 vector.

[0126] Obtain the mB5R gene:

[0127] mB5R consists of the signal peptide, stalk, transmembrane (TM) and intracellular tail (CT) gene segments. The H5 promoter drives expression of the sB5R gene.

[0128] Obtaining the sB5R gene:

[0129] sB5R consists of the signal peptide, SCR1, stalk, transmembrane (TM) and intracellular tail (CT) gene segments.

[0130] Cas9-mediated homologous recombination:

[0131] 3 x 10^5 CV-1 cells were seeded into one well of a six-well plate one day prior to transfection. A gRNA vector (L025gRNA targeting the L025 region, TKgRNA targeting the TK region, A46RgRNA targeting the A46R region) was co-transfected with Cas9 into CV-1 cells in a six-well plate. The next day, the well transfected with the gRNA vector and the Cas9 gene was infected with 0.01 PFU/cell backbone virus. The shuttle vector for homologous recombination was transfected into the infected wells 2 hours after virus infection. Cells were harvested after 24 hours and frozen at -80 °C for recombinant virus purification.

[0132] Purification of recombinant virus:

[0133] Cell lysates collected from Cas9-mediated homologous recombination were thawed, and 0.5 μl of this lysate was used to infect all 6 wells of a six-well plate containing CV1 cells grown to 80-90% confluence. After 48 hours of infection, each well was examined under a fluorescence microscope for viruses that emitted red or green fluorescence. After identifying positive infection spots, mark them on the lower surface of the plate with a marker. The plaques were then carefully selected with a 20 μl tip after aspirating the medium from the wells in a fume hood for culturing the cells. The tip was then immersed in a cryotube containing 200 microliters of cell culture fluid. After one freeze-thaw cycle, 5-20 μl of this virus solution was added to each well of a new 6-well plate containing CV1 cells. This process is repeated until each plaque expresses red or green fluorescence, i.e., all plaques are formed by recombinant virus. Typically, 3 to 5 rounds of plaque purification are required to obtain a pure recombinant virus. After confirming that the virus has been purified, the infected cells are scraped off and centrifuged to obtain a cell pellet. Then some cells are taken to extract viral DNA. The purity of the virus was confirmed by PCR amplification of the target gene from the extracted viral DNA.

[0134] Amplification of the virus:

[0135] Once the recombinant virus was confirmed to be the desired recombinant virus, 50 μl of the virus lysate was added to a T175 flask containing CV1 cells, and grown to 80-90% confluence in a cell culture medium containing about 30 ml. After 48 hours, the cells and medium were scraped off and "primary virus amplification" was saved.

[0136] Verify the recombinant virus that deleted the L025 gene:

[0137] CV-1 cells were infected with the purified virus. Infected cells were harvested 2 days after infection, and the VV DNA was extracted. To verify the deletion of the L025 gene, a DNA fragment spanning the L025 gene and the L026 gene was amplified by PCR using a forward primer (SEQ ID NO: 15: 5'-TATCTAGCAATGGACCGT-3') (within the L025 gene) and a reverse primer (SEQ ID NO: 16: 5'-CCGAAGGTAGTAGCATGGA -3') (within the L026 gene). Amplification of the control gene A DNA fragment spanning the A46R and A47L genes was obtained by PCR amplification using a forward primer (SEQ ID NO: 17: 5'-TTGGCTATTAAACAGTATGGA-3') and a reverse primer (SEQ ID NO: 18: 5'-GGATCCCGATAACAAATG-3'). The PCR product was analyzed by 1% agarose gel electrophoresis.

[0138] Verify the recombinant virus that expresses mB5R or sB5R gene:

[0139] To verify the incorporation of mB5R gene in the recombinant virus, mB5R was amplified by PCR using a forward primer (SEQ ID NO: 19: 5'-ATGAAAACGATTTCCGTTGTTACGT-3') and a reverse primer (SEQ ID NO: 20: 5'-TCACGGT AGCAATTTATGGAACTT-3').

[0140] To verify the incorporation of sB5R gene in the recombinant virus, sB5R gene was amplified by PCR using a forward primer (SEQ ID NO: 21: 5'-ATGAAAACGATTTCCGTTGTTACGT-3') and a reverse primer (SEQ ID NO: 22: 5'-TCACGGTAGCAATTTATGGAACTT-3').

[0141] Excision of RFP using Flp recombinase:

[0142] pCAG-Flpe (from Addgene) was transfected into one well of a six-well plate in CV-1 cells. CV-1 cells were infected with 100-200 recombinant viruses Flp-RFP VV 24 hours after transfection with pCAG-Flpe. Two days later, RFP-negative plaques were picked and used to infect CV-1 cells in six-well plates to purify RFP-negative plaques. RFP-negative plaques were then picked and infected with CV-1 cells until no RFP-positive plaques were observed under fluorescent microscope every 2 days.

[0143] Verify the recombinant virus that deleted the TK gene:

[0144] CV-1 cells were infected with the purified virus. Infected cells were harvested 2 days after infection, and the VV DNA was extracted. To verify the deletion of the TK gene, use the forward primer (SEQ ID NO: 23: 5'-GTTATAGTAGCCGCACTCGA-3') (within the TK gene) and the reverse primer (SEQ ID NO: 24: 5'-ATTTCAGCTGAATATGAAGGA-3') (within the L091 gene) for PCR amplification. A DNA fragment spanning the TK gene and the L091 gene. Amplification of the control gene A DNA fragment spanning the A46R and A47L genes was obtained by PCR amplification using a forward primer (SEQ ID NO: 17: 5'-TTGGCTATTAAACAGTATGGA-3') and a reverse primer (SEQ ID NO: 18: 5'-GGATCCCGATAACAAATG-3'). The PCR product was analyzed by 1% agarose gel electrophoresis. The PD1 antibody gene was confirmed by PCR amplification of its gene fragment. The primers used were: (SEQ ID NO: 25: 5'-TCATAAATACCCGAGCCACC-3') and (SEQ ID NO: 26: 5'-ACCCATTCAAGACCCTTTCC-3').

[0145] Excision of RFP using Cre recombinase:

[0146] pCAG-Cre (from Addgene) was transfected into CV-1 cells in one well of a six-well plate. CV-1 cells were infected with 100-200 Cre-RFP viruses 24 hours after transfection with pCAG-Cre. Two days later, RFP-negative plaques were selected and used to infect CV-1 cells in six-well plates to purify RFP-negative plaques. RFP-negative plaques were then picked and infected with CV-1 cells until no RFP-positive plaques were observed under fluorescent microscope every 2 days.

[0147] Verify the recombinant virus that deleted the A46R gene:

[0148] CV-1 cells were infected with the purified virus. Infected cells were harvested 2 days after infection, and the VV DNA was extracted. To verify the deletion of the A46R gene, use the forward primer (SEQ ID NO: 17: 5'-TTGGCTATTAAACAGTATGGA-3') (within the A46R gene) and the reverse primer (SEQ ID NO: 18: 5'-GGATCCCGATAACAAATG-3') (within the A47R gene) for PCR amplification. A DNA fragment spanning the A46R gene and the A47R gene. The control gene was amplified by PCR using a forward primer (SEQ ID NO: 27: 5'-TGTTGTTCGCTGCTATGA-3') and a reverse primer (SEQ ID NO: 28: 5'-TGGCACAACCATATCTTGTA-3') to amplify a DNA fragment of the L09 gene. The PCR product was analyzed by 1% agarose gel electrophoresis. Detection of HIC1

expression was confirmed by western blotting by obtaining proteins extracted from cells infected with the recombinant virus.

[0149] Enzyme-linked immunosorbent assay:

[0150] Expression of mIL-21 and hIL-21 was detected by enzyme-linked immunosorbent assay ELISA according to the reagent manufacturer's instructions.

[0151] Large-scale virus production:

[0152] The primary virus amplification from above was rapidly frozen and thawed once and diluted to the volume required for cell culture required to infect 36 T175 flasks (80-90% confluence) containing CV1 cells. After 48 hours, infected CV1 cells were harvested by scraping and collected by repeating centrifugation at 2,000 rpm (4 °C) for several rounds. The precipitate was washed in PBS, resuspended in 12 ml of 10 mM Tris-HCl (pH 9) buffer and stored at -80 °C for later purification.

[0153] Virus purification:

[0154] The concentrated viral lysate suspension from above was thawed once and vortexed for a few seconds. After centrifugation at 2,000 rpm for 5 minutes at 4 °C, the supernatant (containing released virions) was collected and diluted to a total volume of 30 ml with 10 mM Tris-HCl buffer. A 30 ml average was placed in four Beckman ultracentrifuge tubes, followed by gently adding 17 ml of 36% sucrose solution to the virus solution and centrifuging at 13,500 rpm for 80 minutes at 4 °C. The final pellet was resuspended in 1-4 ml of virus resuspension buffer (PBS; 10% glycerol; 138 mM NaCl; pH 7.4). And save at -80°C.

[0155] Determination of viral replication:

[0156] Depending on the growth rate, cells were seeded at 2 to 4 x 10^5 cells per well in 3 wells of a 6-well plate containing cell culture medium, and infected with 1 PFU/cell of vaccinia virus the next day. Infected cells and their culture solutions were collected at 24 hours, 48 hours, and 72 hours after infection, respectively. The virus concentration is then determined.

[0157] Evaluation of viral cytotoxicity in vitro:

[0158] Cells were seeded at 1 x 10^3 and 1 x 10^4 cells/well in 96-well plates according to growth rate and infected with virus after 16-18 hours. Cell viability at day 6 after viral infection was determined by MTS assay and EC50 values were

calculated as previously described (viral dose killed 50% of tumor cells), all assays were performed at least three times.

[0159] Evaluation of solid tumor cells sensitivity to the viral infection in vitro:

[0160] Cells were seeded at 3 x 10^5 cells/well in 6-well plates according to growth rate and infected with virus after 16-18 hours. Cell viability at day 2 after viral infection was determined by crystal violet staining and results were scanned as previously described.

[0161] Evaluation of leukemia cell lines sensitivity to the viral infection in vitro:

[0162] Cells were seeded at 3 x 10^5 cells/well in 6-well plates in triplicate according to growth rate and infected with virus after cell seeding. Cell viability at day 1,2 and 3 after viral infection was determined by cell counting with trypan blue staining to differentiate live and dead cells.

[0163] Evaluation of leukemia patient's sample's sensitivity to the viral infection in vitro:

[0164] Five milliliters blood was drawn into an anticoagulation tube from leukemia patient with consent. Blood sample was span at 2000rpm for 5 minutes, the plasma was removed and 5 milliliters red cell lysis buffer were added into the tube, then was incubated at room temperature for 10 minutes. After lysis of red cells, sample was transferred into a 15ml tube containing 8mls sterile PBS, and span at 2000rpm for 5 minutes. Supernatant was removed after centrifugation, and 5mls RPMI 1640 containing 10% FBS and antibiotics were used to resuspend the cell pellet. Cells were seeded at 1 x 10^6 cells/well in 24-well plates for two wells and one well was infected with 1pfu KM1 virus/cell after cell seeding, the other well was used as control. Three days after virus infection, infected cells were observed and photographed under fluorescence microscope, then infected cells and control cells were collected into 15ml tubes containing 10mls of PBS and span at 2000rpm for 5 minutes. 100uls of antibodies mixture containing L/D, CD14, CD16 and CD33 antibodies in PBS were added into the cell pellet and stain the cells for 15 minutes in dark at room temperature. The stained cells were washed once with PBS after staining, and resuspend the cells in 300uls of PBS, then analyzed on FACs.

[0165] In vivo efficacy experiments for treatment of cancer using KM1.

[0166] By subcutaneously injecting 5 x 10^6 cancer cells, a subcutaneous tumor of the back was established in 10 mice per treatment group and the diameter was 0.4-0.5 cm, and then the mice were regrouped by tumor size and received 1 x 10^8 PFU (immune-competent mice) or PBS on days 1, 2, 3, 4, and 5 days. Tumor volume (volume = (length x width 2 x π) / 6) was measured twice a week until the mice were sacrificed when the tumor area reached 1.69 cm2. The animals used were 4-5 week male Syrian hamsters.

[0167] Statistical Analysis:

[0168] Comparative statistical analysis was performed using Graphpad Prism 5 unless otherwise stated. Dual condition comparisons were performed using unpaired t-tests. For additional variables of more than one condition, 1 or 2 ANOVA is performed separately. Survival data is represented as a Kaplan-Meier plot with log-rank analysis to plot whether any differences between the groups have statistically significant differences.

[0169] An oncolytic vaccinia virus and a viral vector thereof are provided and comprises one or more of the following features:

[0170] (a) Generating deletion of a combination of viral genes and introduces immunoregulatory genes (e.g. IL21 and 4-1BBL), a tumor suppressor gene (e.g. HIC1), and a novel viral spread gene mB5R/sB5R into infected target cells.

[0171] (b) Deletion of TK gene allows the vaccinia virus to be targeted for replication in tumor cells. The thymidine kinase (TK) of vaccinia virus allows quiescent cells (such as the vast majority of normal cells in the body) to produce thymidine for replication. The TK-deficient vaccinia virus is dependent on the thymidine kinase produced by the host cell. Thymidine kinase is naturally produced in tumor cells but not produced or produced in low quantity in normal cells. Therefore, TK-deficient vaccinia virus can selectively replicate in tumor cells, especially tumor cells with activated EGFR / Ras / ERK pathway.

[0172] (c) Deletion of L025 gene. The protein encoded by the L025 gene can suppress the immune response by inhibiting the activation of the NF Kappa B signaling pathway in immune cells. Deletion of the L025 gene can abolish the immune system's inhibition of the gene product, which can improve the anti-tumor immune response.

[0173] (d) Deletion of A46R gene. The protein encoded by the A46R gene inhibits the activation of immune cells by acting upstream of the NF Kappa B signaling pathway. Deletion of this gene helps to improve the anti-tumor immune response.

[0174] (e) The virus carries additional B5R-partial-region-deletion gene (mB5R or sB5R) for vaccinia virus.

[0175] (f) The virus carries interleukin 21 (IL-21) gene. Interleukin 21 also activates NK and killer T cells. The virus carrying interleukin 21 facilitates to enhance immune response against tumor. The therapeutic gene is inserted into the L025 region.

[0176] (g) The virus carries 4-1BBL gene. 4-1BB is expressed on the activated T cells, 4-1BB binding to its ligand 4-1BBL enhances the proliferation and cytotoxicity of T cells. To make this product more effective, 4-1BBL gene is incorporated into the virus. The therapeutic gene is inserted into the TK region.

[0177] (h) The virus carries a tumor suppressor gene HIC1 gene. HIC1 is inactivated or lost in many tumors. In vivo experiments have shown that expression of this gene in tumors that have lost the gene can inhibit tumor progression. This gene is inserted into the A46R gene region.

[0178] (i) The virus carries interleukin 12 (IL-12) gene. Interleukin 12 is known to regulate and activate natural killer cells (NK cells) and killer T cells. The interleukin 12 carried by this product has shown activating NK and T cells in a preliminary efficacy experiment. The therapeutic gene is inserted into the A46R region.

[0179] (j) Provide new strategies for deleting multiple viral genes. In the process of constructing the virus, the inventors are the first to introduce the CRISPR Cas9 system to delete the fluorescent protein for gene screening. The various gRNA sequence designed for CRISPR Cas9 system can be used to repeatedly delete the fluorescent protein, so that the vaccinia virus with any combination of genes can be deleted.

[0180] According to the feature (e) of the present disclosure, a vaccinia virus vector comprising a nucleic acid sequence encoding a domain deleted B5R gene is provided. This sequence was integrated into and replaced the L025 gene of vaccinia virus. This product carries mB5R/sB5R to allow the virus to spread better, thereby increasing its ability to kill tumor cells.

[0181] The B5R gene of vaccinia virus has an open reading frame (ORF) that encodes a membrane protein essential for the formation of EEV. Deletion of the B5R ORF results in a significant decrease in the production of EEV, and therefore, the virus produces small cell-infected plaques in vitro and the ability to spread in vivo is severely degraded. The extracellular portion of B5R consists primarily of four domains that are similar to the short consensus repeats (SCR) present in complement regulatory proteins.

[0182] The above partial mB5R gene or sB5R can be driven to be expressed by its upstream promoter. Thus, the nucleic acid sequence of the promoter can be part of an expression cassette. The expression cassette can be part of a vector comprising a promoter, an open reading frame (and a 3' untranslated region). A promoter is a region of DNA with a specific sequence that initiates transcription of its downstream genes. Promoters for expressing heterologous genes in vaccinia include promoters that control early and late transcriptional activities, such as mH5, H5, P7.5 and pE/L. A heterologous gene is a gene that is not normally found in a virus.

[0183] An embodiment of the present disclosure provides a sequence, comprising at least one of:

[0184] (a) a sequence set forth in SEQ ID NO: 1;

[0185] (b) a sequence set forth in SEQ ID NO: 3;

[0186] (c) a sequence set forth in SEQ ID NO: 5;

[0187] (d) a sequence set forth in SEQ ID NO: 7;

[0188] (e) a sequence set forth in SEQ ID NO: 9;

[0189] (f) a sequence set forth in SEQ ID NO: 11;

[0190] (g) a sequence set forth in SEQ ID NO: 13;

[0191] (h) therapeutic gene or a modified version thereof;

[0192] (i) 4-1BBL gene or a modified version thereof; or

[0193] (j) ligands or antibodies that target T cells.

[0194] An embodiment of the present disclosure provides a virus, comprising at least one of:

[0195] (a) a sequence set forth in SEQ ID NO: 1;

[0196] (b) a sequence set forth in SEQ ID NO: 3;

[0197] (c) a sequence set forth in SEQ ID NO: 5;

[0198] (d) a sequence set forth in SEQ ID NO: 7;

[0199] (e) a sequence set forth in SEQ ID NO: 9;

[0200] (f) a sequence set forth in SEQ ID NO: 11;

[0201] (g) a sequence set forth in SEQ ID NO: 13;

[0202] (h) therapeutic gene or a modified version thereof;

[0203] (i) 4-1BBLgene or a modified version thereof; or

[0204] (j) ligands or antibodies that target T cells.

[0205] The sequence may further comprise therapeutic genes including immunomodulators, immune co-stimulatory pathway activating molecules, checkpoint inhibitors, cytotoxic genes, tumor suppressor genes, anti-angiogenesis genes, etc.

[0206] The immunomodulator genes may include cytokine genes. Examples of such cytokines are lymphokines, monokines, growth factors and traditional polypeptide hormones. Included among the cytokines are growth hormones such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; prostaglandin, fibroblast growth factor; prolactin; placental lactogen, OB protein; tumor necrosis factor-α and -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-β; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4,

IL-5, IL-6, IL-7, IL-8, IL-9, IL-10 IL-11, IL-12; IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-24, LIF, G-CSF, GM-CSF, M-CSF, EPO, kit-ligand or FLT-3. Most preferably, IL-12, IL-21, IL-2, IL-15, IL-8 or a modified version of any of these.

[0207] The immune co-stimulatory pathway activating molecules may include gene encodes CD40 ligand (CD40L), ICOS ligand, GITR ligand, 4-1BB ligand, OX40 ligand, TL1A, CD30 ligand, CD27 or Flt3 ligand or a modified version of any of these

[0208] The checkpoint inhibitors may include PD-1 inhibitor, PD-L1 inhibitor, CTLA-4 inhibitor or a modified version of any of these.

[0209] The tumor suppressor genes may include HIC1, etc. or a modified version of any of these.

[0210] An embodiment of the present disclosure provides a sequence, comprising at least one of:

[0211] (a) a mutation(s) in L025, TK, A46R, or any combination thereof;

[0212] (b) partially deleted L025, TK, A46R, or any combination thereof;

[0213] (c) deleted L025, TK, A46R, or any combination thereof;

[0214] (d) a portion or all of the L025, TK, or A46R is replaced by one of sequences set forth in SEQ ID NO: 1-2;

[0215] (e) a portion or all of the L025, TK, or A46R is replaced by one of sequences set forth in SEQ ID NO: 3-4;

[0216] (f) a portion or all of the L025, TK, or A46R is replaced by 4-1BBL or a modified version thereof;

[0217] (g) a portion or all of the L025, TK, or A46R is replaced by HIC1 or a modified version thereof;

[0218] (h) a portion or all of the L025, TK, or A46R is replaced by a tumor targeting gene;

[0219] (i) a portion or all of the L025, TK, or A46R is replaced by a ligand or an antibody that targets T cells;

[0220] (j) a portion or all of the L025, TK, or A46R is replaced by a therapeutic gene or a modified version thereof; and

[0221] (k) a portion or all of the L025, TK, or A46R is replaced by a therapeutic antibody.

[0222] An embodiment of the present disclosure provides a virus, comprising at least one of:

[0223] (a) L025, TK, or A46R mutation;

[0224] (b) partially deleted L025, TK, or A46R;

[0225] (c) deleted L025, TK, or A46R;

[0226] (d) a portion or all of the L025, TK, or A46R is replaced by one of sequences set forth in SEQ ID NO: 1-2;

[0227] (e) a portion or all of the L025, TK, or A46R is replaced by one of sequences set forth in SEQ ID NO: 3-4;

[0228] (f) a portion or all of the L025, TK, or A46R is replaced by 4-1BBL or a modified version thereof;

[0229] (g) a portion or all of the L025, TK, or A46R is replaced by HIC1 or a modified version thereof;

[0230] (h) a portion or all of the L025, TK, or A46R is replaced by a tumor targeting gene;

[0231] (i) a portion or all of the L025, TK, or A46R is replaced by a ligand or an antibody that targets T cells;

[0232] (j) a portion or all of the L025, TK, or A46R is replaced by a therapeutic gene or a modified version thereof; and

[0233] (k) a portion or all of the L025, TK, or A46R is replaced by a therapeutic antibody.

[0234] An embodiment of the present disclosure provides an expression vector or a host cell comprising any sequence described here above.

[0235] An embodiment of the present disclosure provides a virus used for a method of treating the human or animal body, comprising at least one of:

[0236] (a) used alone as monotherapy; and

[0237] (b) used in combination with one or more anti-cancer agent.

[0238] An embodiment of the present disclosure provides a virus used for use in the manufacture of a medicament for treating the human or animal body.

[0239] An embodiment of the present disclosure provides a virus used for use in inducing cancer cells death, regulating a biological activity of the cancer cells, regulating immune response, enhancing proliferation and/or cytotoxicity of T cells.

[0240] An embodiment of the present disclosure provides a virus used for use in the manufacture of a medicament for suppressing cancer cells growth, inducing cancer cells death, and/or regulating a biological activity of the cancer cells.

[0241] The biological activity of the cancer cells comprises inhibition of cancer cells replication, inhibition of cancer cells division, inhibition of DNA repair of cancer cells, inhibition of cancer cells migration, or promoting cancer death.

[0242] An embodiment of the present disclosure provides a product of manufacture comprising a virus in a sterile vial, ampoule, or syringe.

[0243] An embodiment of the present disclosure provides a pharmaceutical composition comprising a virus of the embodiments of the present disclosure.

[0244] In an embodiment of the present disclosure, the pharmaceutical composition further comprises an anti-cancer agent and/or antibody.

[0245] In an embodiment of the present disclosure, the pharmaceutical composition further comprises a pharmaceutically acceptable carrier, a diluent, and/or an excipient.

[0246] An embodiment of the present disclosure provides a therapeutic method for a disease, comprising administering an effective amount of a sequence, expression vector, host cell, virus, pharmaceutical composition, or medicament.

[0247] As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

[0248] Herein, the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils, and polyethylene glycols.

[0249] Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier", which may be used interchangeably, refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound.

[0250] Techniques for formulation and administration of drugs may be found in the latest edition of "Remington's Pharmaceutical Sciences", Mack Publishing Co., Easton, PA, which is herein fully incorporated by reference (Remington: The Science and Practice of Pharmacy, Gennaro, A., Lippincott, Williams & Wilkins, Philadelphia, Pa., 20th ed, 2000).

[0251] Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes.

[0252] Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations that can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

[0253] In an embodiment of the present disclosure, the pharmaceutical composition further comprises an antioxidant which can protect cells or macromolecules (e.g., the polysaccharide) from oxidative stress (oxidative damage caused by free radicals). Thus, the antioxidant can extend the survival of the macromolecules by preventing their oxidative depolymerization. Non-limiting examples of suitable antioxidants include molecules such as glutathione, vitamin C (sodium ascorbate), vitamin E (tocopherols and tocotrienols), N-Ac-L-cysteine, hydroquinone, glutamate, or enzymes such as catalase, superoxide dismutase, glutathione peroxidase or other

peroxidases, and glucose-6-phosphate dehydrogenase (G6PD) (see Osmen I., Naziroglu M., Okutan R. Comparative study of antioxidant enzymes in tissues surrounding implant in rabbits. Cell. Biochem. Funct. 24:275-281, 2006).

[0254] Pharmaceutical compositions for potential administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water-based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters such as ethyl oleate, triglycerides, or liposomes. Aqueous injection suspensions may contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents that increase the solubility of the active ingredients, to allow for the preparation of highly concentrated solutions.

[0255] The compositions typically include one or more suitable diluents, fillers, salts, disintegrants, binders, lubricants, glidants, wetting agents, controlled release matrices, colorings, flavoring, carriers, excipients, buffers, stabilizers, solubilizers, commercial adjuvants, and/or other additives known in the art.

[0256] Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., a sterile, pyrogen-free, water-based solution, before use.

[0257] The compositions, the pharmaceutical composition of the present disclosure may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, or an article of manufacture (with packaging material), which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration, implantation and/or treating a subject. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. The compositions, matrix or hydrogel of the invention formulated in a compatible

pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

[0258] Suitable routes of administration may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intracardiac, e.g., into the right or left ventricular cavity, into the common coronary artery, intravenous, intraperitoneal, intranasal, or intraocular injections.

[0259] As used herein, the phrase "chemotherapeutic agent" refers to any chemical agent with therapeutic usefulness in the treatment of cancer. Chemotherapeutic agents as used herein encompass both chemical and biological agents. These agents function to inhibit a cellular activity upon which the cancer cell depends for continued survival. Categories of chemotherapeutic agents include alkylating/alkaloid agents, antimetabolites, hormones or hormone analogs, and miscellaneous antineoplastic drugs. Most if not all of these drugs are directly toxic to cancer cells and do not require immune stimulation. Suitable chemotherapeutic agents are described, for example, in Slapak and Kufe, Principles of Cancer Therapy, Chapter 86 in Harrison' s Principles of Internal medicine, 14th edition; Perry et ah , Chemotherapeutic, Ch 17 in Abeloff, Clinical Oncology 2nd ed., 2000 ChrchillLivingstone, Inc.; Baltzer L. and Berkery R. (eds): Oncology Pocket Guide to Chemotherapeutic, 2nd ed. St. Luois, mosby-Year Book, 1995; Fischer D. S., Knobf M. F., Durivage H.J. (eds): The Cancer Chemotherapeutic Handbook, 4th ed. St. Luois, Mosby-Year Handbook.

[0260] The chemotherapeutic agent of the present invention can be, but not limited to, cytarabine (cytosine arabinoside, Ara-C, Cytosar-U), asprin, sulindac, curcumin, alkylating agents including: nitrogen mustards, such as mechlor-ethamine, cyclophosphamide, ifosfamide, melphalan and chlorambucil; nitrosoureas, such as carmustine (BCNU), lomustine (CCNU), and semustine (methyl-CCNU); thylenimines/methylmelamine such as thriethylenemelamine (TEM), triethylene, thiophosphoramide (thiotepa), hexamethylmelamine (HMM, altretamine); alkyl sulfonates such as busulfan; triazines such as dacarbazine (DTIC); antimetabolites including folic acid analogs such as methotrexate and trimetrexate, pyrimidine analogs such as 5-fluorouracil, fluorodeoxyuridine, gemcitabine, cytosine arabinoside (AraC, cytarabine), 5-azacytidine, 2,2 -difluorodeoxycytidine, purine analogs such as

6- mercaptopurine, 6-thioguanine, azathioprine, 2 '-deoxycoformycin (pentostatin), erythrohydroxynonyladenine (EHNA), fludarabine phosphate, and 2- chlorodeoxy adenosine (cladribine, 2-CdA); natural products including antimitotic drugs such as paclitaxel, vinca alkaloids including vinblastine (VLB), vincristine, and vinorelbine, taxotere, estramustine, and estramustine phosphate; epipodophylotoxins such as etoposide and teniposide; antibiotics, such as actimomycin D, daunomycin (rubidomycin), doxorubicin, mitoxantrone, idarubicin, bleomycins, plicamycin (mithramycin), mitomycinC, and actinomycin; enzymes such as L-asparaginase, cytokines such as interferon (IFN)-gamma, tumor necrosis factor (TNF)-alpha, TNF-beta and GM-CSF, anti-angiogenic factors, such as angiostatin and endostatin, inhibitors of FGF or VEGF such as soluble forms of receptors for angiogenic factors, including soluble VGF/VEGF receptors, platinum coordination complexes such as cisplatin and carboplatin, anthracenediones such as mitoxantrone, substituted urea such as hydroxyurea, methylhydrazine derivatives including Nmethylhydrazine (MIH) and procarbazine, adrenocortical suppressants such as mitotane (o,ρ' -DDD) and aminoglutethimide; hormones and antagonists including adrenocortico steroid antagonists such as prednisone and equivalents, dexamethasone and aminoglutethimide; progestin such as hydroxyprogesterone caproate, medroxyprogesterone acetate and megestrol acetate; estrogen such as diethylstilbestrol and ethinyl estradiol equivalents; antiestrogen such as tamoxifen; androgens including testosterone propionate and fluoxymesterone/equivalents; antiandrogens such as flutamide, gonadotropin-releasing hormone analogs and leuprolide; non-steroidal antiandrogens such as flutamide; kinase inhibitors, histone deacetylase inhibitors, methylation inhibitors, proteasome inhibitors, monoclonal antibodies, oxidants, anti-oxidants, telomerase inhibitors, BH3 mimetics, ubiquitin ligase inhibitors, stat inhibitors and receptor tyrosin kinase inhibitors such as imatinib mesylate (marketed as Gleevac or Glivac) and erlotinib (an EGF receptor inhibitor) now marketed as Tarveca; and anti-virals such as oseltamivir phosphate, Amphotericin B, and palivizumab.

[0261] In some embodiments the daily dose the chemo therapeutic agent of the invention (e.g., cytarabine) or the pharmaceutical composition comprising same is ranging between 1 to 10 g per square meter of body area, between 1.5 to 5 g per square meter of body area or between 2 to 4 g per square meter of body area.

[0262] Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

[0263] Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non-limiting fashion.

[0264] Example 1: A virus that obtains L025 gene deletion.

[0265] Please refer to FIG. 6, FIG. 6 shows recombinant virus with L025 gene deletion. M: is a molecular weight marker, 1: is a control virus, and 2 is a recombinant virus with L025 gene deletion. A46R is an amplified control gene fragment. L025 is a fragment of the L025 gene detected for deletion.

[0266] The virus with L025 gene deletion was obtained by homologous recombination using the VV Lister virus as the female parent and the shuttle vector for deleting the L025 gene. After the pure fluorescent infection spot was obtained by red fluorescence screening, small-scale virus amplification was carried out, and the viral DNA was extracted to identify the L025 gene deletion by PCR. A new virus with L025 gene deletion was obtained, as shown in FIG. 6.

[0267] Example 2: A virus that obtains L025 gene deletion and TK gene deletion.

[0268] The new virus with L025 gene deletion obtained in Example 1 contains the FRT site on both sides of the red fluorescent protein RFP. The Flipase was used to cleave the FRT site to delete the RFP gene, thus obtaining a new RFP-negative VV with deletion of the L025 gene. This virus was used as a backbone virus and a shuttle vector with TK gene deletion was used for homologous recombination to obtain a virus with TK gene deletion and simultaneously PD1 antibody gene insertion into this region. Small-scale virus amplification was performed after fluorescent screening to obtain pure virus-infected plaques, and viral DNA was extracted to identify TK gene deletion by PCR. A new virus with deletion in both L025 gene and the TK gene was obtained, and the PD1 antibody gene was inserted into the TK region (FIG.7).

[0269] Example 3: A virus with deletion of L025 gene, deletion of TK gene and deletion of A46R gene.

[0270] The new virus with L025 gene deletion and the TK gene deletion obtained in Example 2 contains Loxp sites on both sides of the red fluorescent protein RFP.

The Loxp site was deleted by Cre recombinase to delete the RFP gene, thus obtaining the RFP-negative new virus with L025 and TK gene deletion. The virus was used as a backbone virus and a shuttle vector with A46R gene deletion was subjected to homologous recombination to obtain a virus with A46R gene deletion and simultaneous HIC1 gene insertion into this region. A small-scale virus amplification was carried out after green fluorescent screening to obtain pure virus-infected plaques, and viral DNA was extracted to identify A46R gene deletion by PCR. A new virus with L025 gene, TK gene and A46R gene deletion was obtained, and the PD1 antibody gene was inserted into the TK region, and the HIC1 gene was inserted into the A46R region, and the virus expressed green fluorescent protein GFP.

[0271] Example 4: A virus contains L025 gene deletion with IL21 and mB5R/sB5R insertion in this region; TK gene deletion with 4-1BBL gene insertion in this region; A46R gene deletion with HIC1 gene insertion in this region.

[0272] Please refer to FIGs. 14-15. FIG. 7 shows recombinant virus with TK gene deletion and hSPD-41BBL gene insertion. mV: is the modified virus, V: is the wild type virus, TK: is the amplified TK gene (negative results indicate that the gene has been deleted in the genome of the detected recombinant virus), hSPD-41BBL: is amplified inserted human SPD-41BBL positive result indicates that the gene has been inserted in the genome of the recombinant virus being tested), and L09: is an amplified control gene fragment.

[0273] FIG. 8. Shows recombinant virus deletion of the A46R gene and viral expression of the inserted gene HIC1. A: PCR was used to identify the deletion of the A46R gene. M: is a molecular weight marker, 1: is a control virus, and 2 is a recombinant virus deleting the A46R gene. L09 is an amplified control gene fragment. B: Western blot analysis was used to identify the expression of HIC1 protein. C is a control virus, and T is a new recombinant virus carrying the HIC1 gene and deleting the A46R gene. HIC1 is the result of antibody detection, and β-actin is a protein loading control.

[0274] The new virus with L025 gene, TK gene and the A46R gene deletion obtained in Example 3 was used as a backbone virus, and a shuttle vector for L025 gene deletion were homologously recombined to obtain the virus with L025 gene deletion and simultaneous IL21 and mB5R/sB5R gene insertion into this region. After

the pure recombinant virus was obtained by fluorescence screening, small-scale virus was amplified, and the viral DNA was extracted and then the L025 gene deletion was identified by PCR. A new virus deleting the L025 gene, the TK gene and the A46R gene was obtained, and the 4-1BBL gene was inserted into the TK region, the HIC1 gene was inserted into the A46R region (FIG. 8), and the IL21 and mB5R/sB5R genes were inserted into the L025 region.

[0275] Example 5: A panel of solid tumor cells infected by KM1 virus.

[0276] Please refer to FIGs. 9A-9B. FIGs. 9A-9B shows that KM1 killing of a panel of solid tumor cell lines with escalating dosage of the virus in 6-well plate for two days. The amount of virus used to infect the individual well were 0.01pfu/cell, 0.1pfu/cell, and 1pfu/cell. Crystal violet was used to stain the remaining cells in the well when terminated the experiment.

[0277] Two days after virus infection at 0.01pfu, 0.1pfu and 1pfu/cell, the cells were stained with crystal violet. The plates were scanned after staining, as shown in FIGs. 9A-9B.

[0278] Example 6: Solid tumor cells sensitivity to KM1. A panel of solid tumor cells infected by KM1 virus in EC50 assay.

[0279] Please refer to FIGs. 10A-11. FIGs. 10A-10C shows examples of EC50 assay on 17 cell lines, in which EC50 was performed in triplicate on the individual cell line. FIG. 11 shows the EC50 value for different cell lines.

[0280] Six days after virus infection cells viability was measured with MTS. The plates were read after incubation with MTS. (FIGs. 10A-10C and 11)

[0281] Example 7: leukemia cell lines sensitivity to KM1. A panel of leukemia cell lines were infected by KM1.

[0282] Please refer to FIG. 12. FIG. 12 shows killing of leukemia cell lines by vaccinia virus. EC50 assay performed on three cell lines (K562, C8166, THP-1) are provided as exemplary examples.

[0283] Subfigures A, B, and C show cell counting at the seeding point, 48 hours, and 72 hours post virus infection in control well (without virus infection, solid line) and virus infected well (donated line). Subfigure D shows the image of infected THP1

cells 48 hours post infection. Cells glowing with green fluorescence represents virus replicating/replicated in the cells.

[0284] The vaccinia virus of the present disclosure infected and killed majority of the leukemia cells in 72 hours. Live/dead cells were counted 24, 48 and 73 hours after virus infection. The results of selective sensitive cells were presented (FIG. 12).

[0285] Example 8: leukemia patient's cells sensitivity to KM1.

[0286] Please refer to FIGs. 13-14. FIG. 13 shows examples of leukemia patients' white blood cells infected by KM1. FIG. 14 shows live/dead cells three days post KM1 infection.

[0287] In FIG. 13, A: sample from patient with CMML; B: sample from patient with M5b; and C: sample from patient with M4.

[0288] In FIG. 14, A: FACs profiles of live/dead cells in control well (without KM1 infection, upper panel) and KM1 infected well of leukemia patients 0518#, 6#, 7#, 8# and 2#. B: dead cell percentages in control well (without KM1 infection, upper panel) and KM1 infected well of leukemia patients 0518#, 6#, 7#, 8# and 2#.

[0289] White blood cells from leukemia patients were infected. 72 hours post virus infection, cells were checked by fluorescence microscope and FACs. (FIGs. 13-14).

[0290] Example 9. KM1 treatment of Syrian golden hamster pancreatic cancer.

[0291] Please refer to FIG. 15, FIG. 15 shows anti-tumor efficacy of KM1 in Syrian hamster pancreatic cancer HPD 1NR model. The left panel shows tumor volume changes after treatment with KM1 and in control group without KM1 treatment. The right panel shows tumor volume changes in individual animal in the KM1 treatment group.

[0292] Example 10. HIC1 armed vaccinia virus treatment of human breast cancer cell MDA MB-231.

[0293] Please refer to FIG. 16, FIG. 16 shows treatment of human breast cancer cell MDA MB-231 using HIC1 armed vaccinia virus and its counterpart control HY3. Top panel shows treatment of human breast cancer cell MDA MB-231 using HIC1 armed vaccinia virus (HIC1) and its counterpart control HY3 on dose escalation in 6-well plate. Bottom panel shows EC50 calculated from the experiment of the top panel.

[0294] Accordingly, the present disclosure provides an engineered vaccinia virus, a pharmaceutical composition containing the same, and methods for use in treating a subject in need using the same. The engineered vaccinia virus includes deletion of a combination of viral genes and introduction of an immune co-stimulatory pathway activating molecule, immunomodulator gene, a truncated viral envelope gene, and/or a tumor suppressor into infected target cells. The oncolytic vaccinia virus of the present disclosure is capable of selectively infecting cancer cells, selectively replicating in tumor cells, selectively targeting tumor cells with activated EGFR/ Ras /ERK pathway. The oncolytic vaccinia virus is also capable of inhibiting tumor-induced immunosuppression and activating of the NF Kappa B signaling pathway in immune cells. The oncolytic vaccinia virus is also capable of invoking anti-tumor immune response, enhancing the proliferation and cytotoxicity of T cells. The oncolytic vaccinia virus is also capable of inhibiting tumor progression. The oncolytic vaccinia virus may integrate a tumor suppressor gene into genome of cancer cells that underexpress or loss the tumor suppressor gene.

[0295] As used herein the term "about" refers to ± 10 %.

[0296] As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

[0297] The term "treating" refers to arresting the development of a pathology (disease, disorder or condition) and/or causing the reduction, remission, or regression of a pathology. Those of skill in the art will understand that various methodologies and assays can be used to assess the reduction, remission or regression of a pathology. It will be appreciated that the treating may be performed alone or in conjunction with other therapies.

[0298] As used herein, the term “preventing” refers to keeping a disease, disorder or condition from occurring in a subject who may be at risk for the disease, but does not yet display symptoms of the disease disorder or condition or has not yet been diagnosed as having the disease, disorder or condition. Those of skill in the art will

understand that various methodologies and assays can be used to assess the development of a pathology.

[0299] As used herein, the terms "subject" or "subject in need thereof' include mammals, preferably human beings at any age or gender. The subject may be showing preliminary signs of a pathology, e.g. a disease, disorder or condition associated with a mutant or a nonfunctional HIC1 protein, e.g., hyperproliferative disease.

[0300] Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); “Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., Eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course

Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

[0301] It is understood that any Sequence Identification Number (SEQ ID NO) disclosed in the instant application can refer to either a DNA sequence or a RNA sequence, depending on the context where that SEQ ID NO is mentioned, even if that SEQ ID NO is expressed only in a DNA sequence format or a RNA sequence format. For example, SEQ ID NO:3 is expressed in a DNA sequence format (e.g., reciting T for thymine), but it can refer to either a DNA sequence that corresponds to a WNT3A nucleic acid sequence, or the RNA sequence of an RNA molecule nucleic acid sequence. Similarly, though some sequences are expressed in a RNA sequence format (e.g., reciting U for uracil), depending on the actual type of molecule being described, it can refer to either the sequence of a RNA molecule comprising a dsRNA, or the sequence of a DNA molecule that corresponds to the RNA sequence shown. In any event, both DNA and RNA molecules having the sequences disclosed with any substitutes are envisioned.

[0302] It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

[0303] In summary, people with ordinary skills in the art may make various changes and modifications according to the technical solutions and technical concept of the present application, and all such changes and modifications fall into the protection scope of claims appended to the present application.

WHAT IS CLAIMED IS:

1. An engineered oncolytic virus, comprising

a mutated viral sequence for selective replication in tumor cells and/or activation of immune cells; and

a heterologous sequence for encoding an immune co-stimulatory pathway activating molecule, immunomodulator gene, a truncated viral envelope gene, and/or a tumor suppressor,

wherein the heterologous sequence is stably incorporated into the genome of the engineered oncolytic virus.

2. The engineered vaccinia virus according to claim 1, wherein the heterologous sequence is stably incorporated into the mutated viral sequence of the engineered vaccinia virus.

3. The engineered vaccinia virus according to claim 1, wherein the mutated viral sequence comprises at least one of:

(a) a mutation(s) in L025, TK, A46R, or any combination thereof;

(b) partially deleted L025, TK, A46R, or any combination thereof;

(c) deleted L025, TK, A46R, or any combination thereof;

(d) a portion or all of the L025, TK, or A46R, which is replaced by one of sequences set forth in SEQ ID NO: 2, 4, 6, 8, 10, 12, or 14;

(e) a portion or all of the L025, TK, or A46R, which is replaced by a tumor targeting gene;

(f) a portion or all of the L025, TK, or A46R, which is replaced by a ligand or an antibody that targets T cells;

(g) a portion or all of the L025, TK, or A46R, which is replaced by a therapeutic gene or a modified version thereof; or

(h) a portion or all of the L025, TK, or A46R is replaced by a therapeutic antibody.

4. The engineered vaccinia virus according to claim 1, wherein the heterologous sequence comprises at least one of:

(a) a sequence set forth in SEQ ID NO: 2;

(b) a sequence set forth in SEQ ID NO: 4;

(c) a sequence set forth in SEQ ID NO: 6;

(d) a sequence set forth in SEQ ID NO: 8;

(e) a sequence set forth in SEQ ID NO: 10;

(f) a sequence set forth in SEQ ID NO: 12; or

(g) a sequence set forth in SEQ ID NO: 14.

5. The engineered vaccinia virus according to claim 1, wherein the heterologous sequence encodes at least one of:

(a) a sequence set forth in SEQ ID NO: 1;

(b) a sequence set forth in SEQ ID NO: 3;

(c) a sequence set forth in SEQ ID NO: 5;

(d) a sequence set forth in SEQ ID NO: 7;

(e) a sequence set forth in SEQ ID NO: 9;

(f) a sequence set forth in SEQ ID NO: 11; or

(g) a sequence set forth in SEQ ID NO: 13.

6. The engineered vaccinia virus according to claim 1, wherein the engineered vaccinia virus comprises a sequence of formula: 5'-A_1-X-A_2-B_1-Y-B_2-C_1-Z-C_2-3', wherein A_1 and A_2 are a left arm and a right arm of a first viral gene respectively, B_1 and B_2 are a left arm and a right arm of a second viral gene, respectively, C_1 and C_2 are a left arm and a right arm of a third viral gene, respectively, wherein X, Y, and Z are heterologous genes, each selected from one of immunomodulatory genes, cytokines, therapeutic genes, truncated viral envelope genes, tumor suppressor genes, genes encoding therapeutic antibodies, and genes encoding ligands of the therapeutic antibodies.

7. The engineered vaccinia virus according to claim 5, wherein the first viral gene is L025, the second viral gene is TK, and the third viral gene is A46R.

8. The engineered vaccinia virus according to claim 5, wherein X is a hybrid gene of IL-21 and modified B5R, Y is 4-1BBL, and Z is HIC1.

9. The engineered vaccinia virus according to claim 1, wherein the mutated viral sequence comprises mutations of deletions in L025, TK, and A46R, and the heterologous sequence comprises IL-21 and 4-1BBL.

10. The engineered vaccinia virus according to claim 1, wherein the mutated viral sequence comprises mutations of deletions in L025, TK, and A46R, and the heterologous sequence comprises a hybrid gene of IL-21 and modified B5R, and 4-1BBL.

11. The engineered vaccinia virus according to claim 1, wherein the immunomodulator genes is a cytokine gene encoding IL-12, IL-21, IL-2, IL-15,

IL-8, or a modified version thereof.

12. The engineered vaccinia virus according to claim 1, wherein the immune co-stimulatory pathway activating molecule comprises CD40 ligand (CD40L), ICOS ligand, GITR ligand, 4-1BB ligand, OX40 ligand, TL1A, CD30 ligand, CD27, Flt3 ligand, or a modified version thereof.

13. The engineered vaccinia virus according to claim 1, wherein the tumor suppressor gene is HIC1.

14. The engineered vaccinia virus according to claim 1, wherein the engineered vaccinia virus is selected from the group consisting of Lister, Western Reserve (WR), Copenhagen (Cop), Bern, Paris, Tashkent, Tian Tan, Wyeth (DRYVAX), IHD-J, IHD-W, Brighton, Ankara, CVA382, modified vaccinia ankara (MVA), Dairen I, LC16m8, LC16M0, LIVP, ACAM2000, WR 65-16, Connaught, New York City Board of Health (NYCBH), EM-63 and NYVAC strain.

15. The engineered vaccinia virus according to claim 1, wherein the truncated viral envelope gene is B5R containing a short consensus repeats (SCR) 2, SCR3, and SCR4 domains deletion.

16. The engineered vaccinia virus according to claim 1, wherein the tumor suppressor is HIC1.

17. A pharmaceutical composition comprising an effective amount of the engineered vaccinia virus of claim 1 and a pharmaceutical acceptable carrier.

18. The pharmaceutical composition according to claim 17, wherein the pharmaceutical composition is formulated for oral, topical, parenteral delivery, or interventional therapy.

19. The pharmaceutical composition according to claim 19, wherein the pharmaceutical composition is formulated for topical intratumoral injection, topical intra-artery (blood vessel supplying tumor) injection, intraperitoneal injection, intrathoracic injection, systemic intravenous injection, intramuscular injection, subcutaneous injection, intrathecal injections, direct intraventricular injection, intracardiac injection, intranasal injections.

20. The pharmaceutical composition according to claim 17, wherein the engineered vaccinia virus is used alone as monotherapy; or in combination with anti-cancer agent, immune suppressors, and/or oncolytic virus enhancers.

21. The pharmaceutical composition according to claim 17, wherein the engineered

vaccinia virus is used in combination with 5-fluorouracil (FU), folinic acid (FA), methotrexate, capecitabine, oxaliplatin, bevacizumab, cetuximab, immune checkpoint inhibitors, other types of oncolytic viruses, or any combination thereof.

22. A method for use in treating a cancer in a subject in need thereof, comprising administering to the subject an effective amount of the engineered vaccinia virus of claim 1.

23. The method according to claim 22, wherein HIC1 is inactivated, underexpressed, or loss in the cancer.

24. The method according to claim 22, wherein the cancer is selected from the group consisting lung cancer, melanoma, pancreatic cancer, liver cancer, colon cancer, breast cancer, glioblastoma, sarcoma, stomach cancer, ovarian cancer, mesothelioma, and leukemia.

25. A method of using an effective amount of the engineered vaccinia virus of claim 1 to screen cancer tissues or cells.

26. A method of increasing tumor-specific infectivity of vaccinia virus, comprising administering the engineered vaccinia virus of claim 1 to a subject, wherein the engineered vaccinia virus is administered in an amount effective for invoking anti-tumor immune response in a subject.

27. A method of conferring persistent immunity to tumor relapse in a subject in need thereof comprising administering the engineered vaccinia virus of claim 1 to the subject.

28. A method of screening patients based on a percentage of GFP positive cells 48 hours post infection of patient's cancer cells with the engineered vaccinia virus of claim 1.

29. An engineered vaccinia virus for use in inducing cancer cells death, regulating a biological activity of the cancer cells, regulating immune response, enhancing proliferation of T cells, and/or cytotoxicity of T cells, wherein the engineered oncolytic is as provided in claim 1.

30. An engineered vaccinia virus for use in inducing cancer cells death, regulating a biological activity of the cancer cells, regulating immune response, enhancing proliferation of T cells, and/or cytotoxicity of T cells of claim 28, wherein the biological activity of the cancer cells comprises inhibition of cancer cells replication, inhibition of cancer cells division, inhibition of DNA repair of cancer

cells, inhibition of cancer cells migration, or promoting cancer death, wherein the engineered oncolytic is as provided in claim 1.

31. An engineered vaccinia virus for use in the manufacture of a medicament for treating lung cancer, melanoma, pancreatic cancer, liver cancer, colon cancer, breast cancer, glioblastoma, sarcoma, stomach cancer, ovarian cancer, mesothelioma, and leukemia, wherein the engineered oncolytic is as provided in claim 1.

32. An engineered vaccinia virus for use in the manufacture of a medicament for suppressing cancer cells growth, inducing cancer cells death, and/or regulating a biological activity of the cancer cells, wherein the engineered oncolytic is as provided in claim 1.

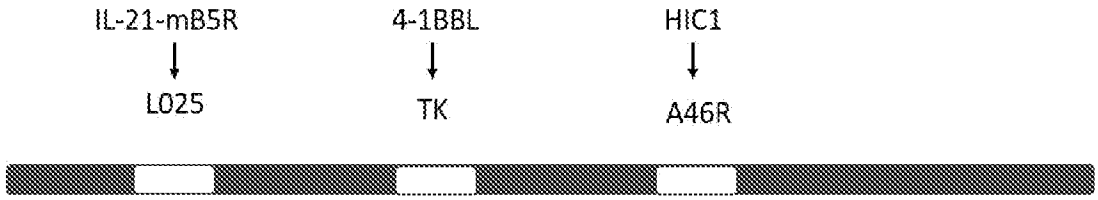


FIG. 1A

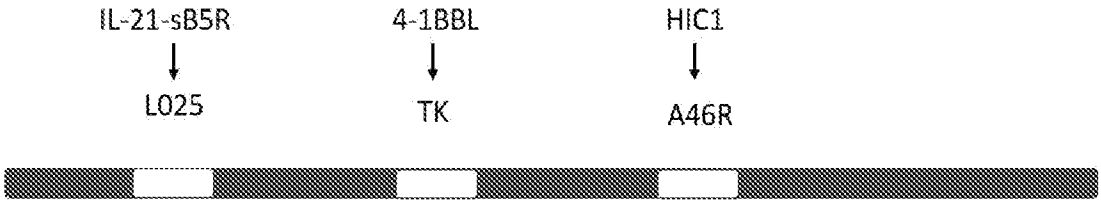


FIG. 1B

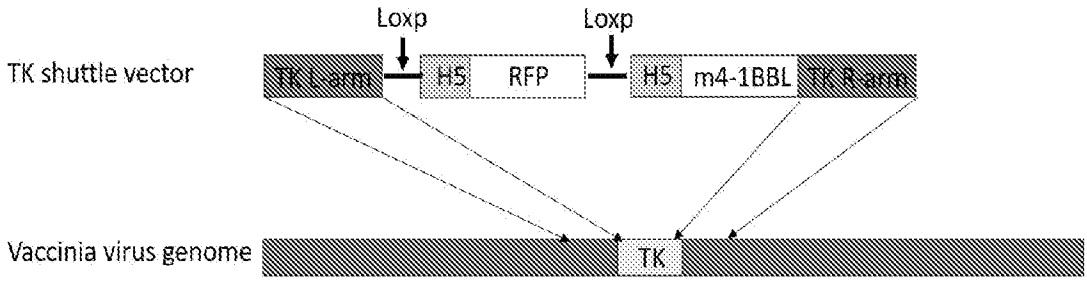


FIG. 2A

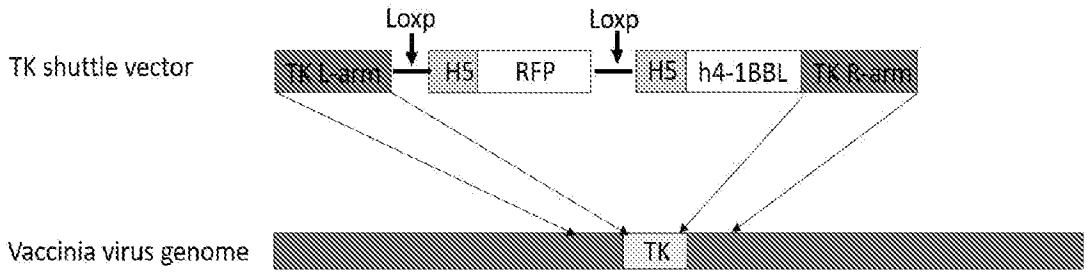


FIG. 2B

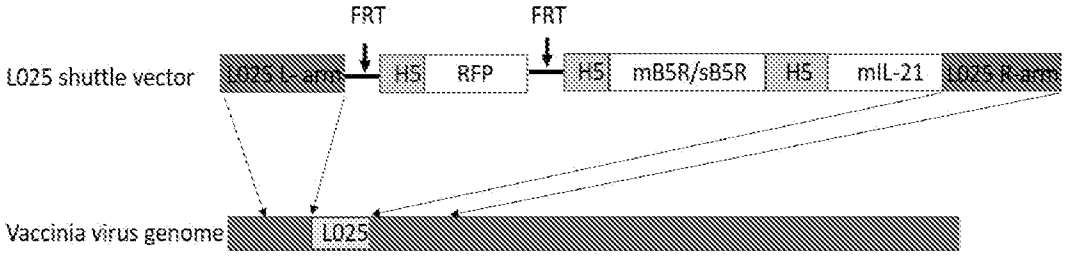


FIG. 3A

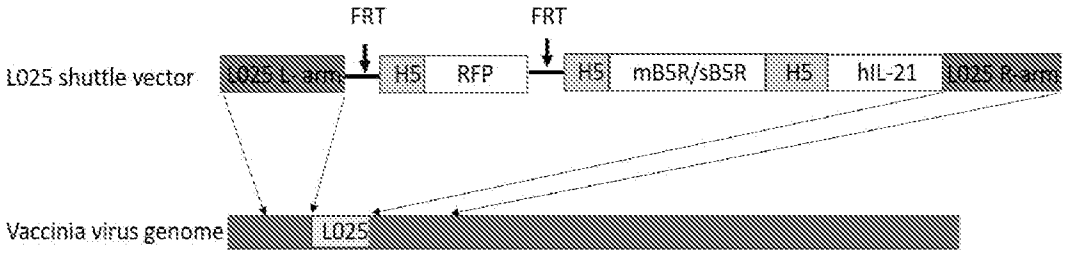


FIG. 3B

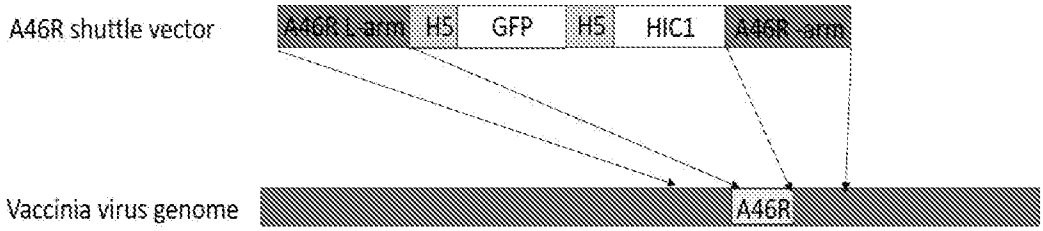


FIG. 4

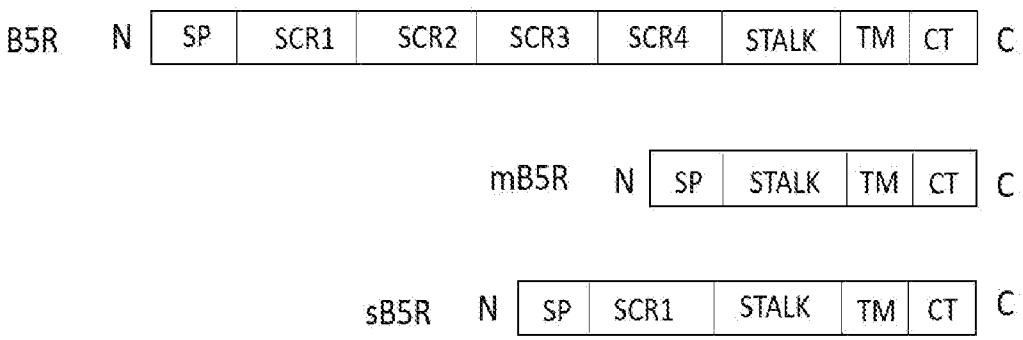


FIG. 5

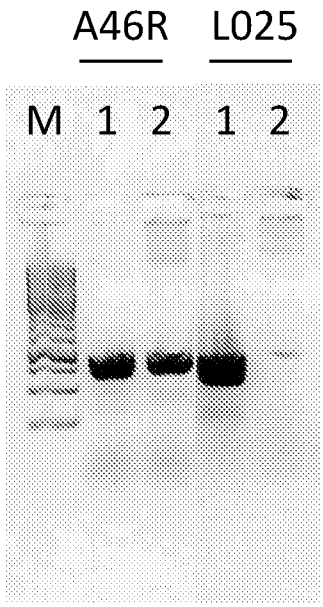


FIG. 6

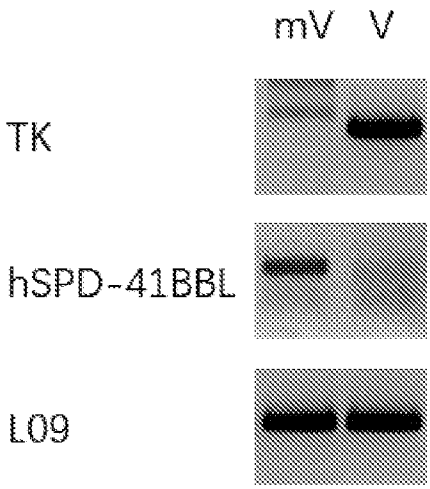


FIG. 7

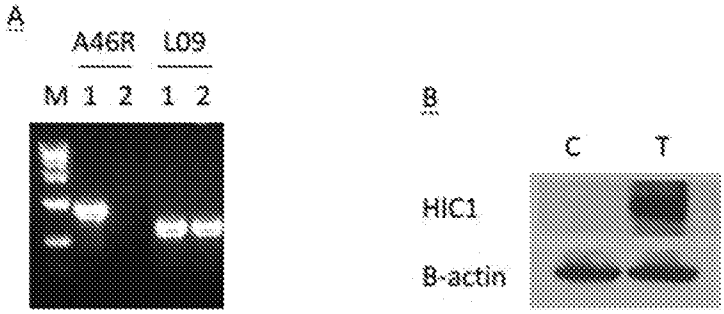


FIG. 8

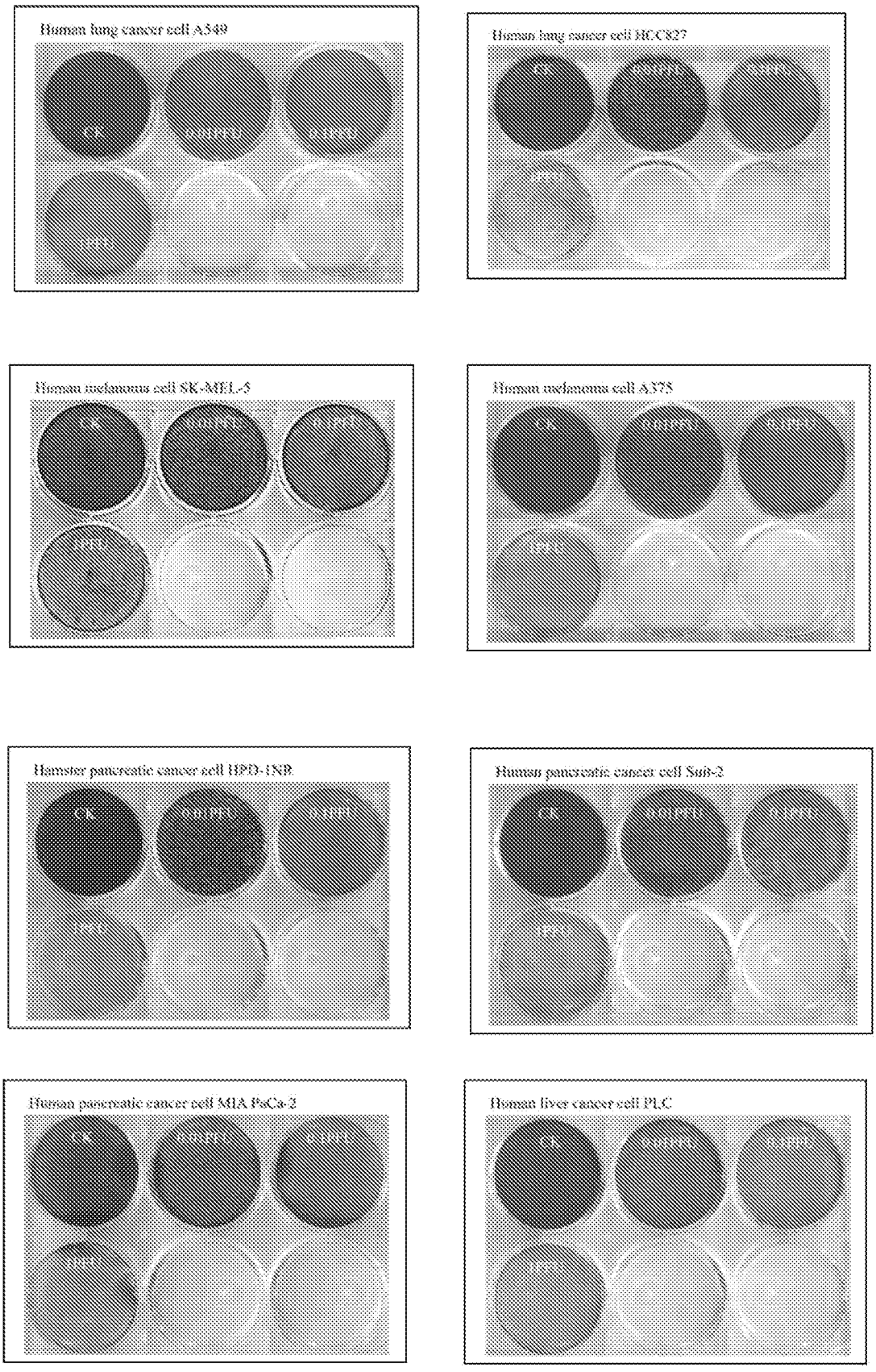

Human lung cancer cell A549
Human lung cancer cell HCC827
Human melanoma cell SK-MEL-5
Human melanoma cell A375
Hamster pancreatic cancer cell HPD-1NR
Human pancreatic cancer cell Su8-2
Human pancreatic cancer cell MIA PaCa-2
Human liver cancer cell PLC

FIG. 9A

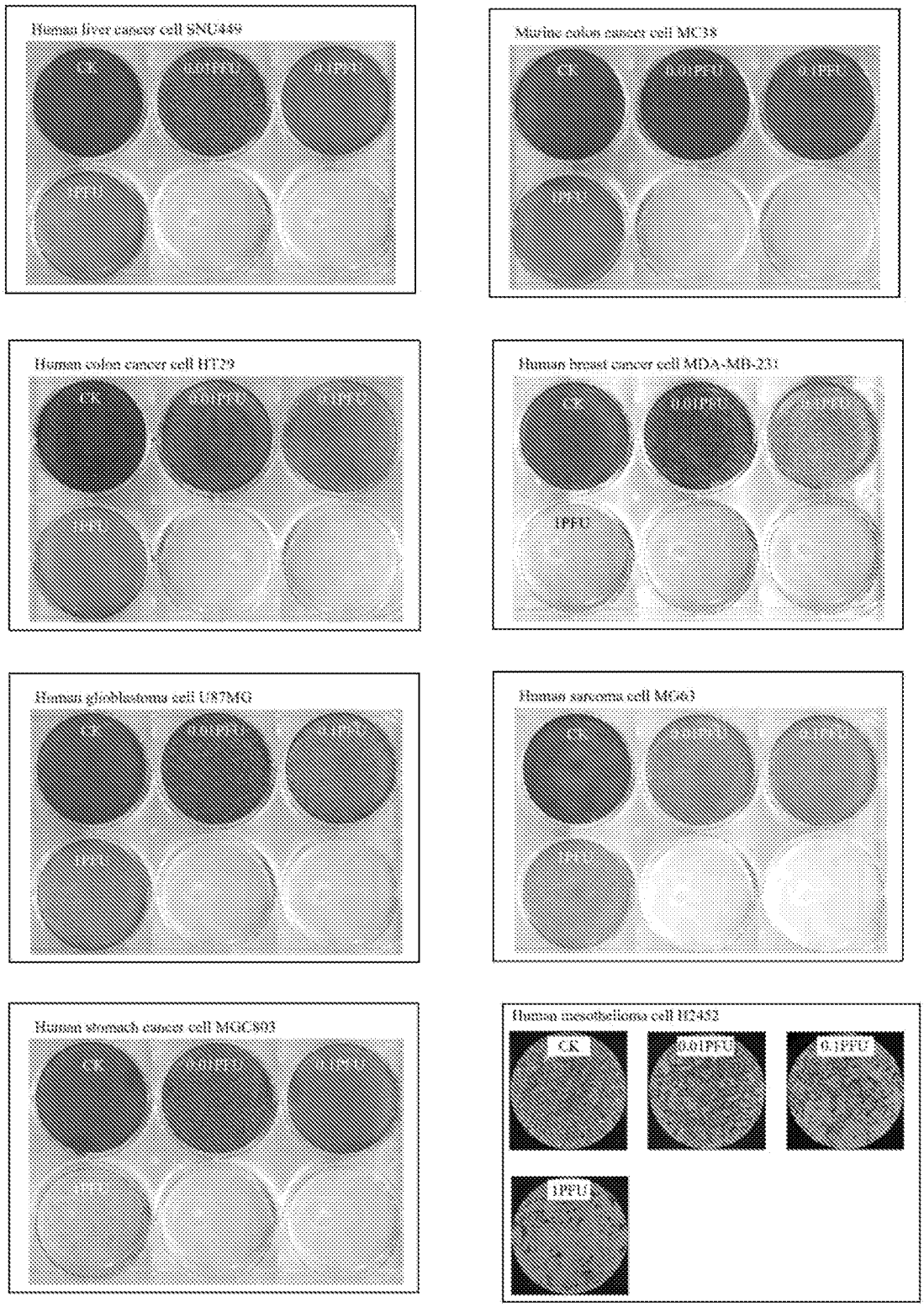
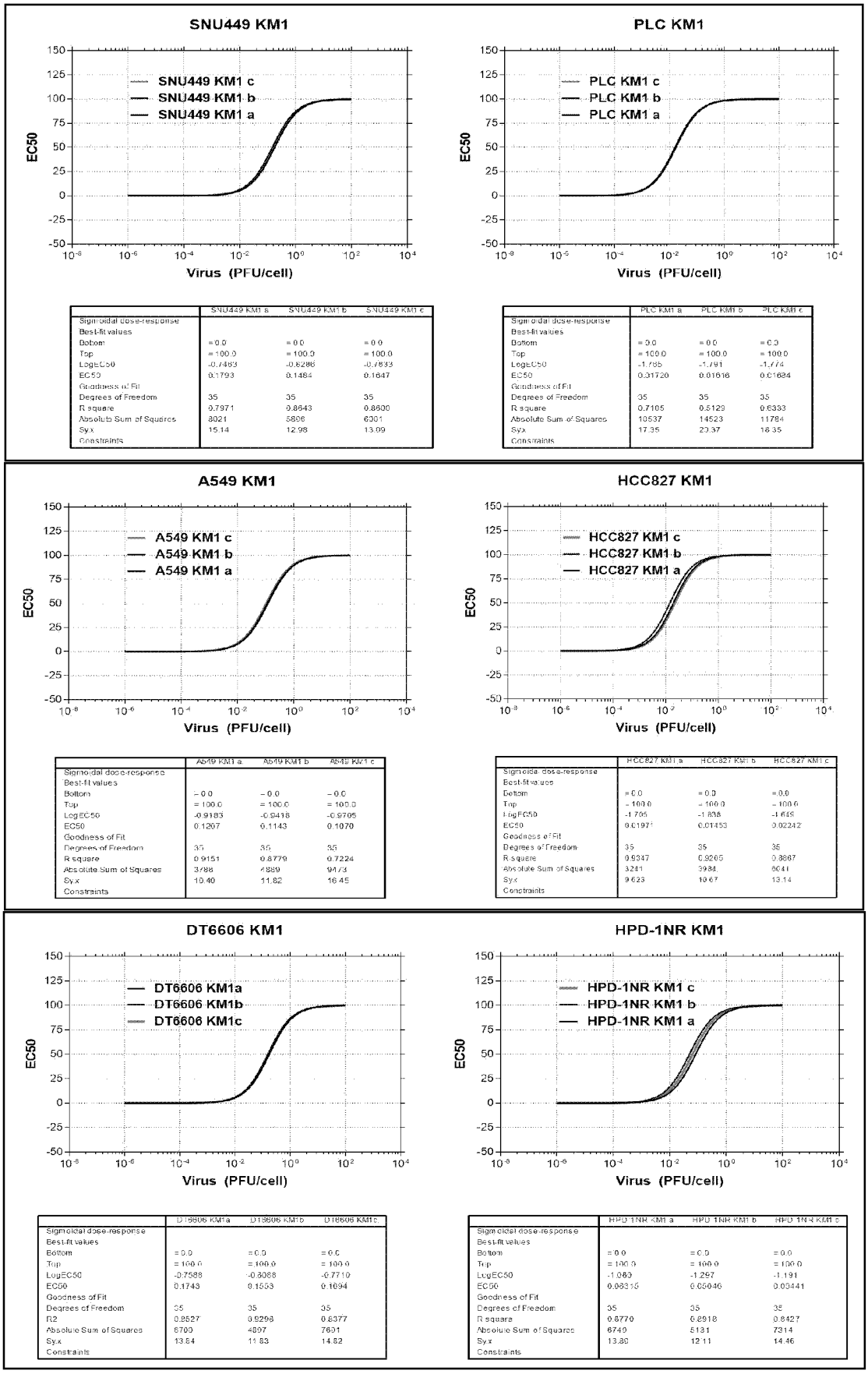

Human liver cancer cell SNU449
CK
0.01PFU
0.1PFU
1PFU
Murine colon cancer cell MC38
CK
0.01PFU
0.1PFU
1PFU
Human colon cancer cell HT29
CK
0.01PFU
0.1PFU
1PFU
Human breast cancer cell MDA-MB-231
CK
0.01PFU
0.1PFU
1PFU
Human glioblastoma cell U87MG
CK
0.01PFU
0.1PFU
1PFU
Human sarcoma cell MG63
CK
0.01PFU
0.1PFU
1PFU
Human stomach cancer cell MGC803
CK
0.01PFU
0.1PFU
1PFU
Human mesothelioma cell H2452
CK
0.01PFU
0.1PFU
1PFU

FIG. 9B



	SNU449 KM1 a	SNU449 KM1 b	SNU449 KM1 c
Sigmoidal dose-response			
Best-fit values			
Bottom	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0
LogEC50	-0.7483	-0.8286	-0.7833
EC50	0.1793	0.1484	0.1647
Goodness of Fit			
Degrees of Freedom	35	35	35
R square	0.7971	0.8643	0.8600
Absolute Sum of Squares	8021	5806	6301
Sy.x	15.14	12.98	13.09
Constraints			

	PLC KM1 a	PLC KM1 b	PLC KM1 c
Sigmoidal dose-response			
Best-fit values			
Bottom	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0
LogEC50	-1.795	-1.791	-1.774
EC50	0.01720	0.01616	0.01684
Goodness of Fit			
Degrees of Freedom	35	35	35
R square	0.7105	0.5129	0.6333
Absolute Sum of Squares	10537	14523	11784
Sy.x	17.35	20.37	18.35
Constraints			

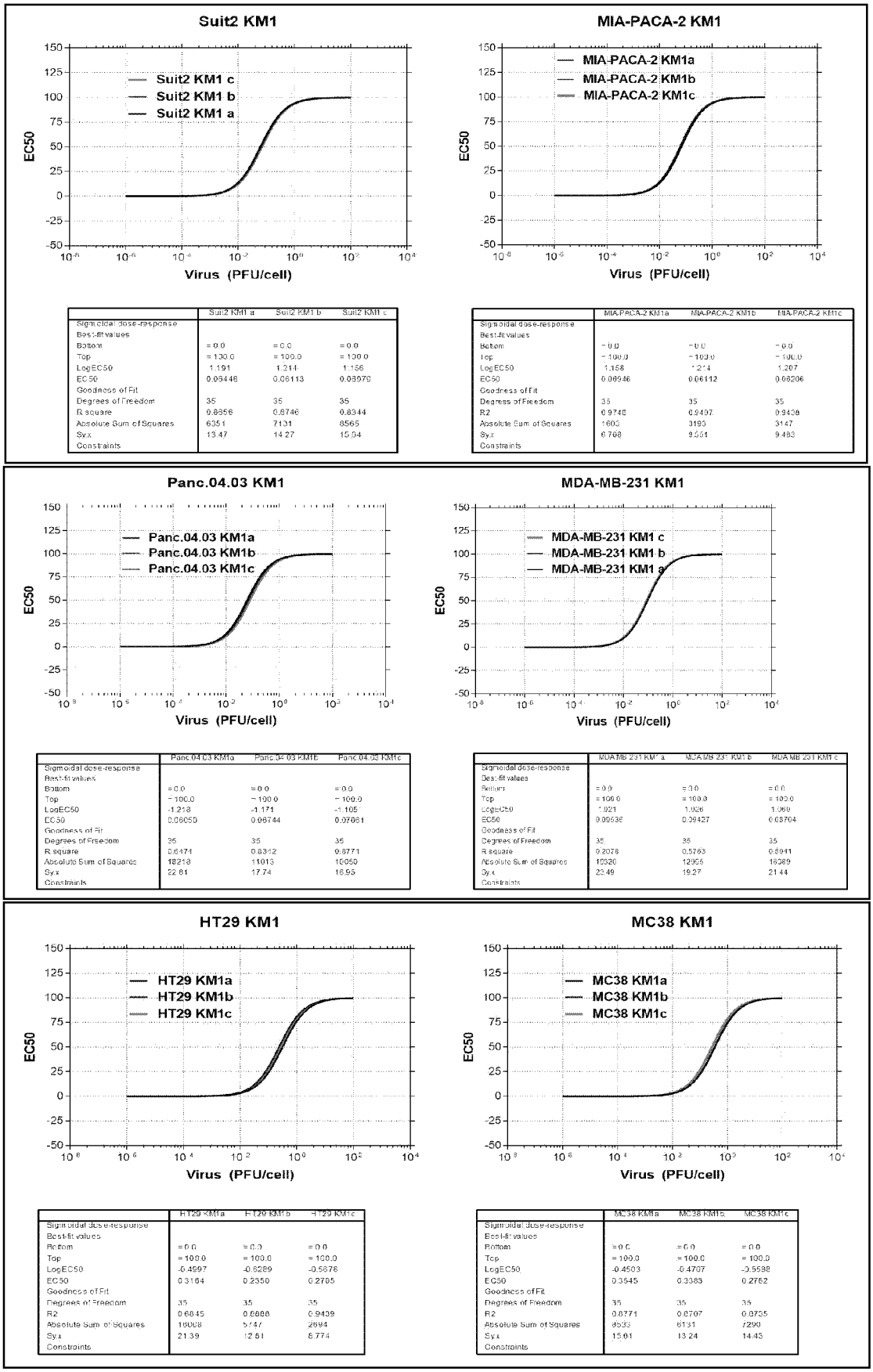
	A549 KM1 a	A549 KM1 b	A549 KM1 c
Sigmoidal dose-response			
Best-fit values			
Bottom	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0
LogEC50	-0.9183	-0.9418	-0.9705
EC50	0.1207	0.1143	0.1070
Goodness of Fit			
Degrees of Freedom	35	35	35
R square	0.9151	0.8779	0.7224
Absolute Sum of Squares	3788	4889	9473
Sy.x	10.40	11.82	16.45
Constraints			

	HCC827 KM1 a	HCC827 KM1 b	HCC827 KM1 c
Sigmoidal dose-response			
Best-fit values			
Bottom	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0
LogEC50	-1.705	-1.858	-1.649
EC50	0.0197	0.01453	0.02242
Goodness of Fit			
Degrees of Freedom	35	35	35
R square	0.9347	0.9265	0.8867
Absolute Sum of Squares	3241	3984	6041
Sy.x	9.623	10.67	13.14
Constraints			

	DT6606 KM1a	DT6606 KM1b	DT6606 KM1c
Sigmoidal dose-response			
Best-fit values			
Bottom	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0
LogEC50	-0.7588	-0.8088	-0.7710
EC50	0.1743	0.1553	0.1694
Goodness of Fit			
Degrees of Freedom	35	35	35
R2	0.8527	0.9298	0.8377
Absolute Sum of Squares	6700	4897	7601
Sy.x	13.84	11.83	14.82
Constraints			

	HPD-1NR KM1 a	HPD-1NR KM1 b	HPD-1NR KM1 c
Sigmoidal dose-response			
Best-fit values			
Bottom	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0
LogEC50	-1.060	-1.297	-1.191
EC50	0.08715	0.05046	0.06441
Goodness of Fit			
Degrees of Freedom	35	35	35
R square	0.8770	0.8918	0.8427
Absolute Sum of Squares	6749	5131	7314
Sy.x	13.88	12.11	14.46
Constraints			

FIG. 10A



	Suit2 KM1 a	Suit2 KM1 b	Suit2 KM1 c
Sigmoidal dose-response			
Best-fit values			
Bottom	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0
LogEC50	-1.191	-1.214	-1.156
EC50	0.06446	0.06113	0.06970
Goodness of Fit			
Degrees of Freedom	35	35	35
R square	0.8856	0.8746	0.8944
Absolute Sum of Squares	6351	7131	8565
Sy.x	13.47	14.27	15.04
Constraints			

	MIA-PACA-2 KM1a	MIA-PACA-2 KM1b	MIA-PACA-2 KM1c
Sigmoidal dose-response			
Best-fit values			
Bottom	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0
LogEC50	-1.158	-1.214	-1.207
EC50	0.06946	0.06112	0.06206
Goodness of Fit			
Degrees of Freedom	35	35	35
R2	0.9748	0.9497	0.9438
Absolute Sum of Squares	1603	3193	3147
Sy.x	6.768	9.551	9.483
Constraints			

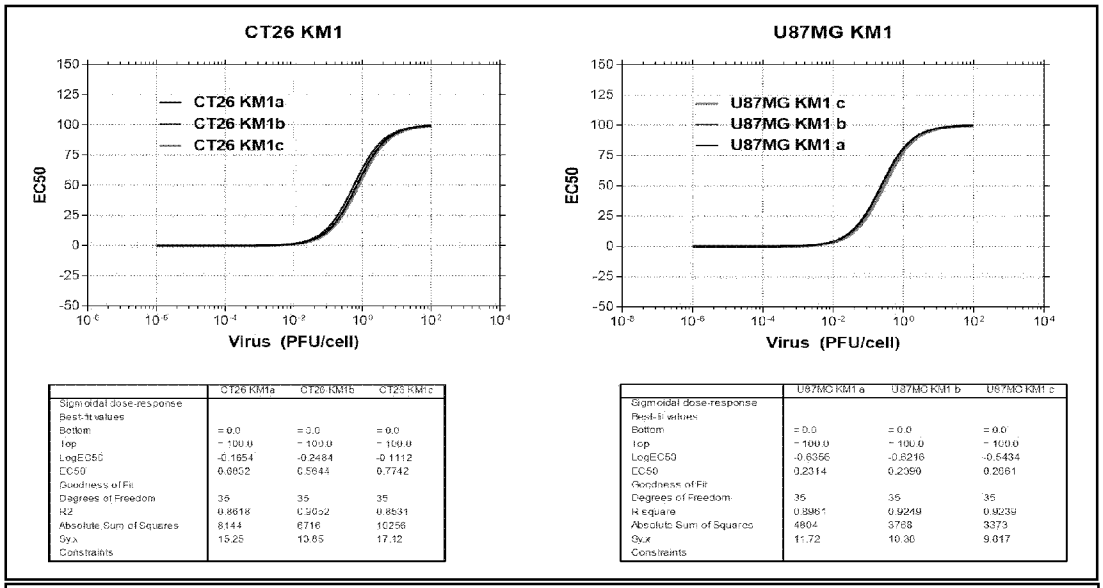
	Panc.04.03 KM1a	Panc.04.03 KM1b	Panc.04.03 KM1c
Sigmoidal dose-response			
Best-fit values			
Bottom	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0
LogEC50	-1.218	-1.171	-1.105
EC50	0.06050	0.06744	0.07861
Goodness of Fit			
Degrees of Freedom	35	35	35
R square	0.8471	0.8342	0.8771
Absolute Sum of Squares	18218	11013	10050
Sy.x	22.81	17.74	16.95
Constraints			

	MDA-MB-231 KM1 a	MDA-MB-231 KM1 b	MDA-MB-231 KM1 c
Sigmoidal dose-response			
Best-fit values			
Bottom	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0
LogEC50	-1.021	-1.026	-1.060
EC50	0.09538	0.09427	0.08704
Goodness of Fit			
Degrees of Freedom	35	35	35
R square	0.2078	0.5763	0.6941
Absolute Sum of Squares	19320	12956	10389
Sy.x	23.49	19.27	21.44
Constraints			

	HT29 KM1a	HT29 KM1b	HT29 KM1c
Sigmoidal dose-response			
Best-fit values			
Bottom	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0
LogEC50	-0.4997	-0.6289	-0.5678
EC50	0.3164	0.2350	0.2705
Goodness of Fit			
Degrees of Freedom	35	35	35
R2	0.6845	0.8888	0.9439
Absolute Sum of Squares	18008	5747	2894
Sy.x	21.39	12.81	8.774
Constraints			

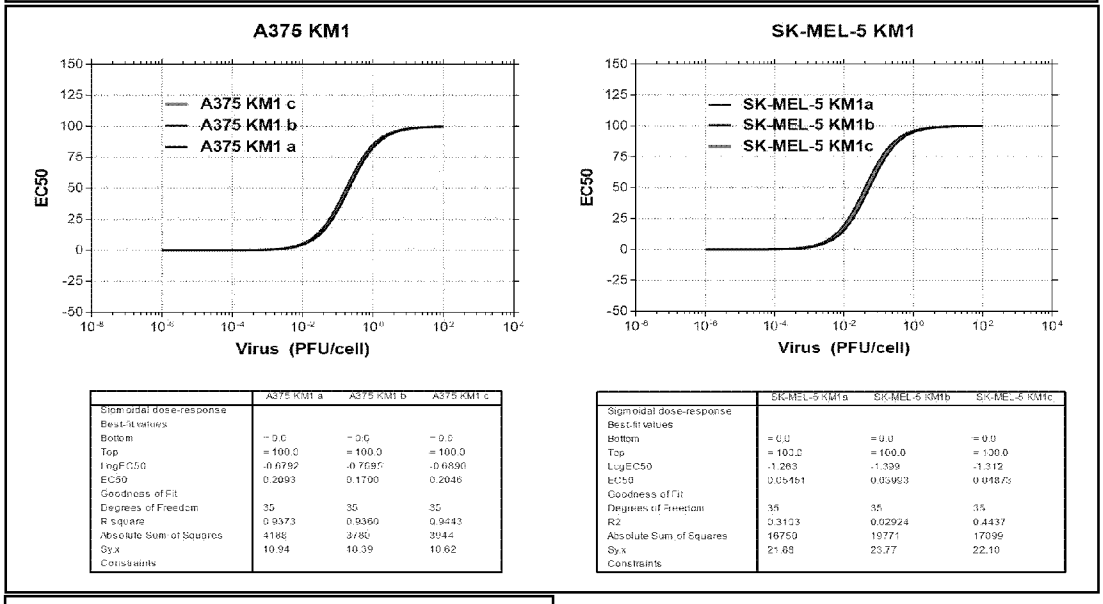
	MC38 KM1a	MC38 KM1b	MC38 KM1c
Sigmoidal dose-response			
Best-fit values			
Bottom	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0
LogEC50	-0.4503	-0.4707	-0.5538
EC50	0.3545	0.3383	0.2782
Goodness of Fit			
Degrees of Freedom	35	35	35
R2	0.8771	0.8707	0.8735
Absolute Sum of Squares	8503	6131	7290
Sy.x	15.61	13.24	14.43
Constraints			

FIG. 10B



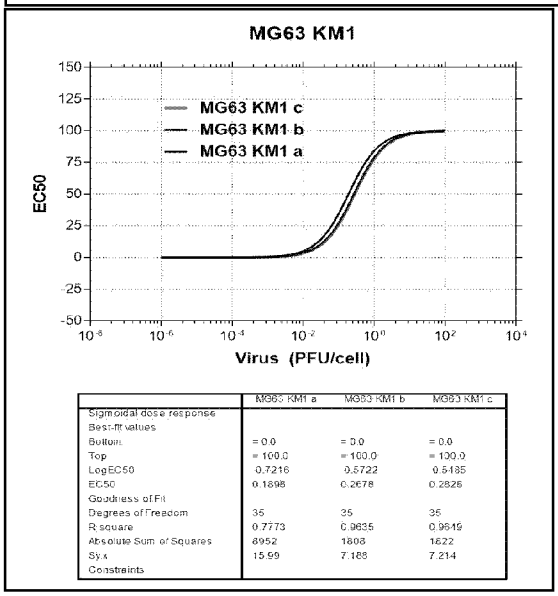
	CT26 KM1a	CT26 KM1b	CT26 KM1c
Sigmoidal dose-response			
Best-fit values			
Bottom	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0
LogEC50	-0.1654	-0.2484	-0.1112
EC50	0.6852	0.5644	0.7742
Goodness of Fit			
Degrees of Freedom	35	35	35
R2	0.8618	0.9052	0.8531
Absolute Sum of Squares	8144	6716	10256
Sy.x	15.25	13.85	17.12
Constraints			

	U87MG KM1 a	U87MG KM1 b	U87MG KM1 c
Sigmoidal dose-response			
Best-fit values			
Bottom	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0
LogEC50	-0.6356	-0.6216	-0.5434
EC50	0.2314	0.2390	0.2861
Goodness of Fit			
Degrees of Freedom	35	35	35
R square	0.8981	0.9249	0.9239
Absolute Sum of Squares	4804	3768	3373
Sy.x	11.72	10.36	9.817
Constraints			



	A375 KM1 a	A375 KM1 b	A375 KM1 c
Sigmoidal dose-response			
Best-fit values			
Bottom	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0
LogEC50	-0.6792	-0.7695	-0.6890
EC50	0.2093	0.1700	0.2046
Goodness of Fit			
Degrees of Freedom	35	35	35
R square	0.9373	0.9360	0.9443
Absolute Sum of Squares	4188	3785	3944
Sy.x	10.94	10.39	10.62
Constraints			

	SK-MEL-5 KM1a	SK-MEL-5 KM1b	SK-MEL-5 KM1c
Sigmoidal dose-response			
Best-fit values			
Bottom	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0
LogEC50	-1.283	-1.339	-1.312
EC50	0.05181	0.04583	0.04873
Goodness of Fit			
Degrees of Freedom	35	35	35
R2	0.3123	0.02924	0.4437
Absolute Sum of Squares	16750	19771	17099
Sy.x	21.88	23.77	22.10
Constraints			



	MG63 KM1 a	MG63 KM1 b	MG63 KM1 c
Sigmoidal dose-response			
Best-fit values			
Bottom	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0
LogEC50	-0.7216	-0.5722	-0.5485
EC50	0.1898	0.2678	0.2826
Goodness of Fit			
Degrees of Freedom	35	35	35
R square	0.7773	0.9635	0.9619
Absolute Sum of Squares	8952	1808	1822
Sy.x	15.99	7.188	7.214
Constraints			

FIG. 10C

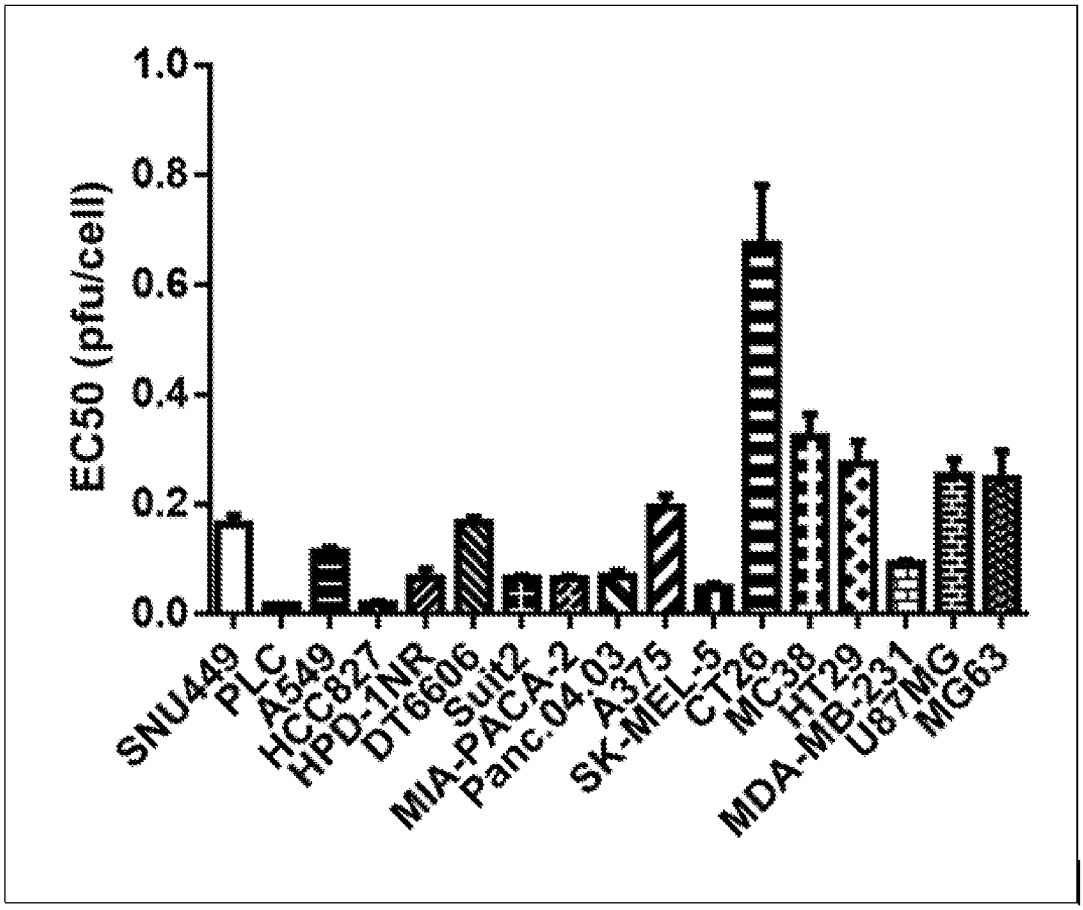

EC50 (pfu/cell)
1.0
0.8
0.6
0.4
0.2
0.0
SNU449
PLC
A549
HCC827
HPD-1NR
DT6606
Suit2
MIA-PACA-2
Panc.04.03
A375
SK-MEL-5
CT26
MC38
HT29
MDA-MB-231
U87MG
MG63

FIG. 11

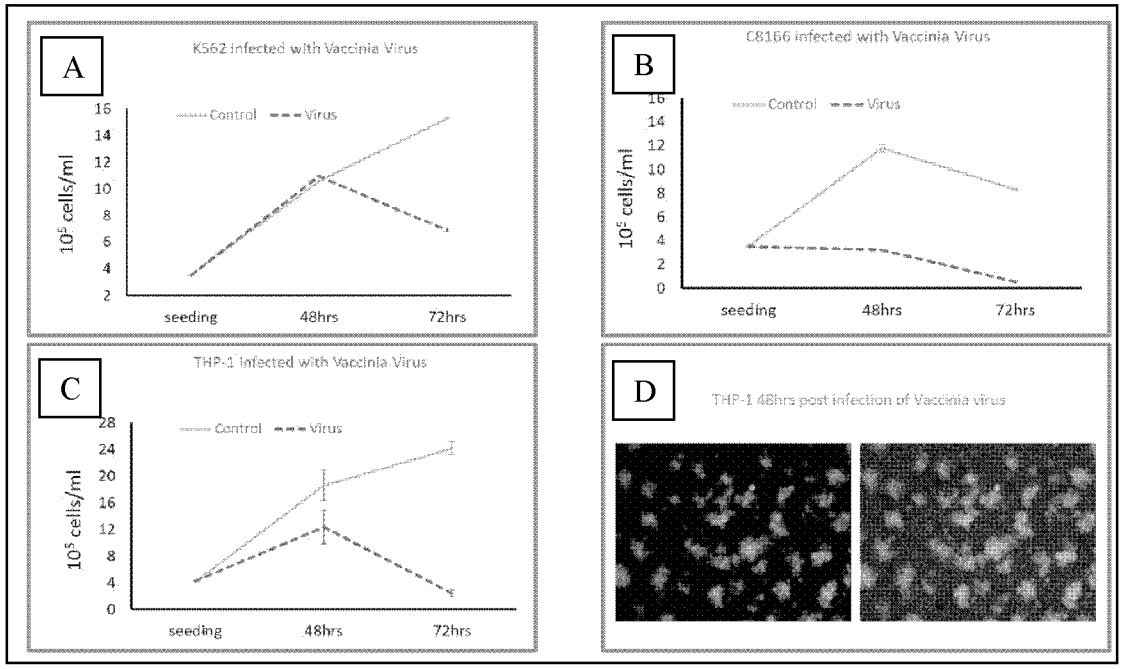

A
K562 infected with Vaccinia Virus
Control
Virus
10^5 cells/ml
16
14
12
10
8
6
4
2
seeding
48hrs
72hrs
B
C8166 infected with Vaccinia Virus
Control
Virus
10^5 cells/ml
16
14
12
10
8
6
4
2
0
seeding
48hrs
72hrs
C
THP-1 infected with Vaccinia Virus
Control
Virus
10^5 cells/ml
28
24
20
16
12
8
4
0
seeding
48hrs
72hrs
D
THP-1 48hrs post infection of Vaccinia virus

FIG. 12

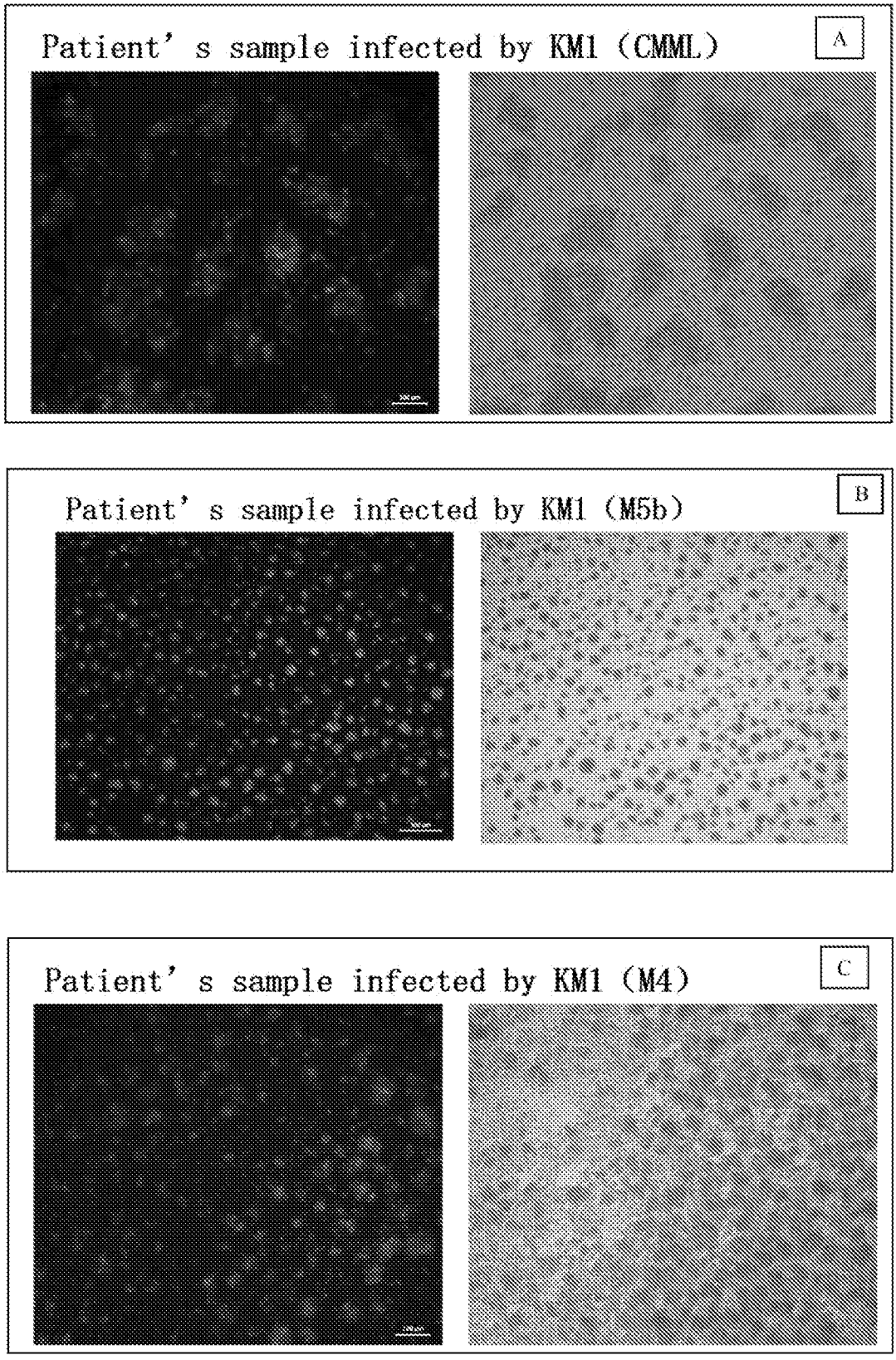
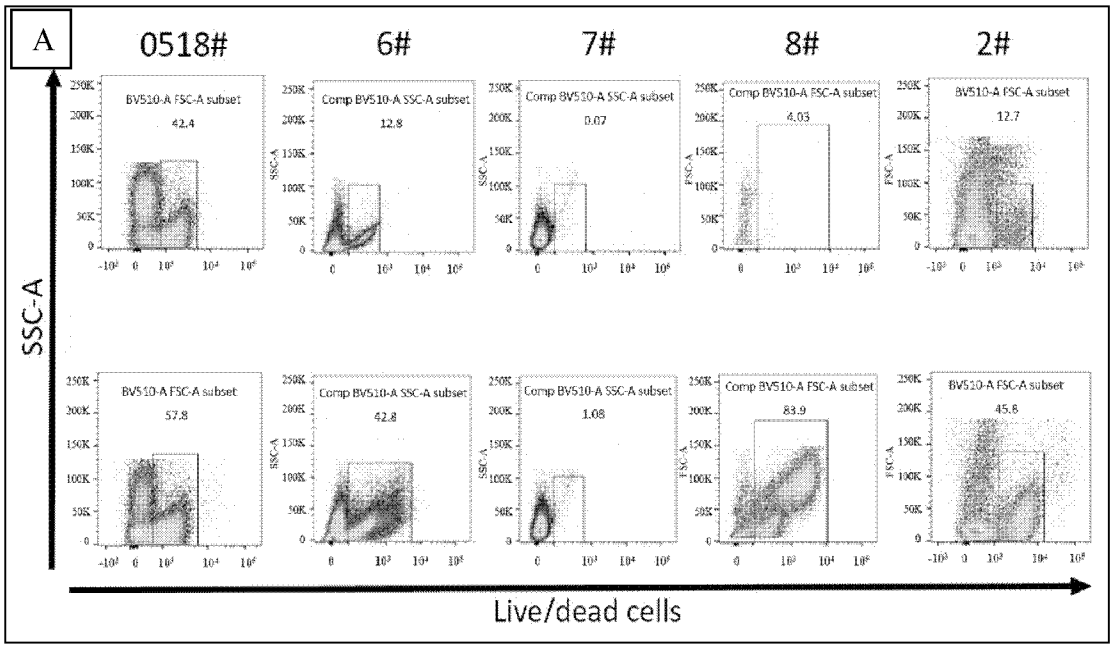

Patient' s sample infected by KM1 (CMML)
A
Patient' s sample infected by KM1 (M5b)
B
Patient' s sample infected by KM1 (M4)
C

FIG. 13


A
0518#
6#
7#
8#
2#
BV510-A FSC-A subset
42.4
Comp BV510-A SSC-A subset
12.8
Comp BV510-A SSC-A subset
0.07
Comp BV510-A FSC-A subset
4.03
BV510-A FSC-A subset
12.7
BV510-A FSC-A subset
57.8
Comp BV510-A SSC-A subset
42.8
Comp BV510-A SSC-A subset
1.08
Comp BV510-A FSC-A subset
83.9
BV510-A FSC-A subset
45.8
SSC-A
Live/dead cells

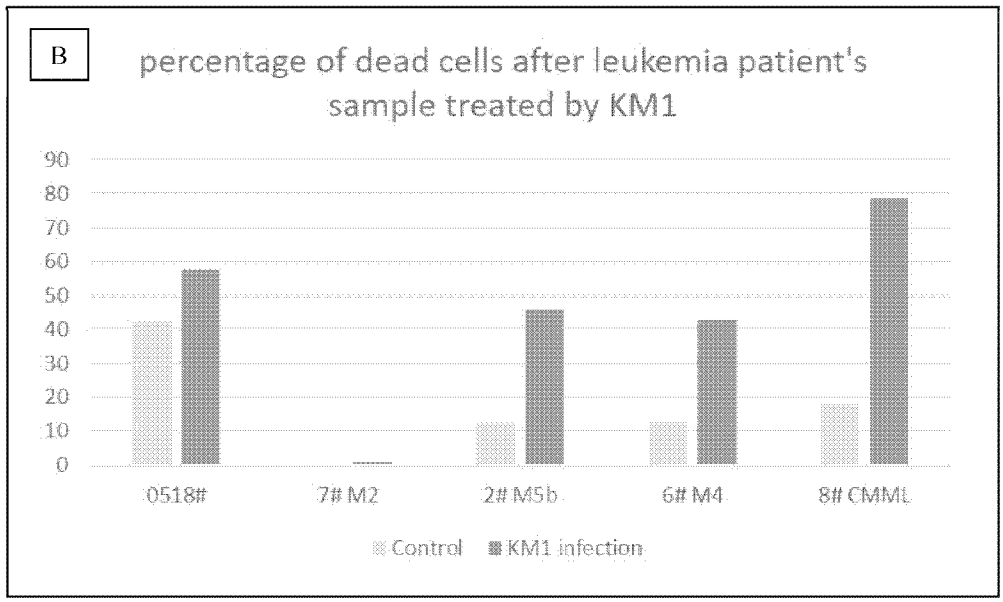

B
percentage of dead cells after leukemia patient's sample treated by KM1
90
80
70
60
50
40
30
20
10
0
0518#
7# M2
2# M5b
6# M4
8# CMML
Control
KM1 infection

FIG. 14

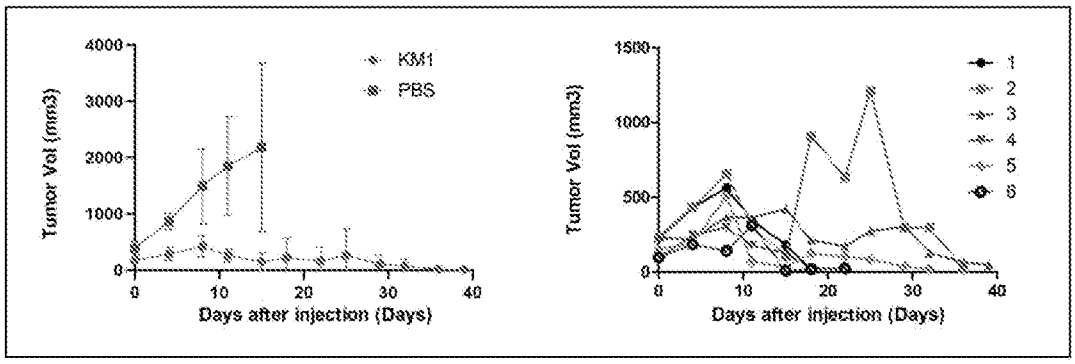

Tumor Vol (mm3)
4000
3000
2000
1000
0
KM1
PBS
0
10
20
30
40
Days after injection (Days)
Tumor Vol (mm3)
1500
1000
500
0
1
2
3
4
5
6
0
10
20
30
40
Days after injection (Days)

FIG. 15

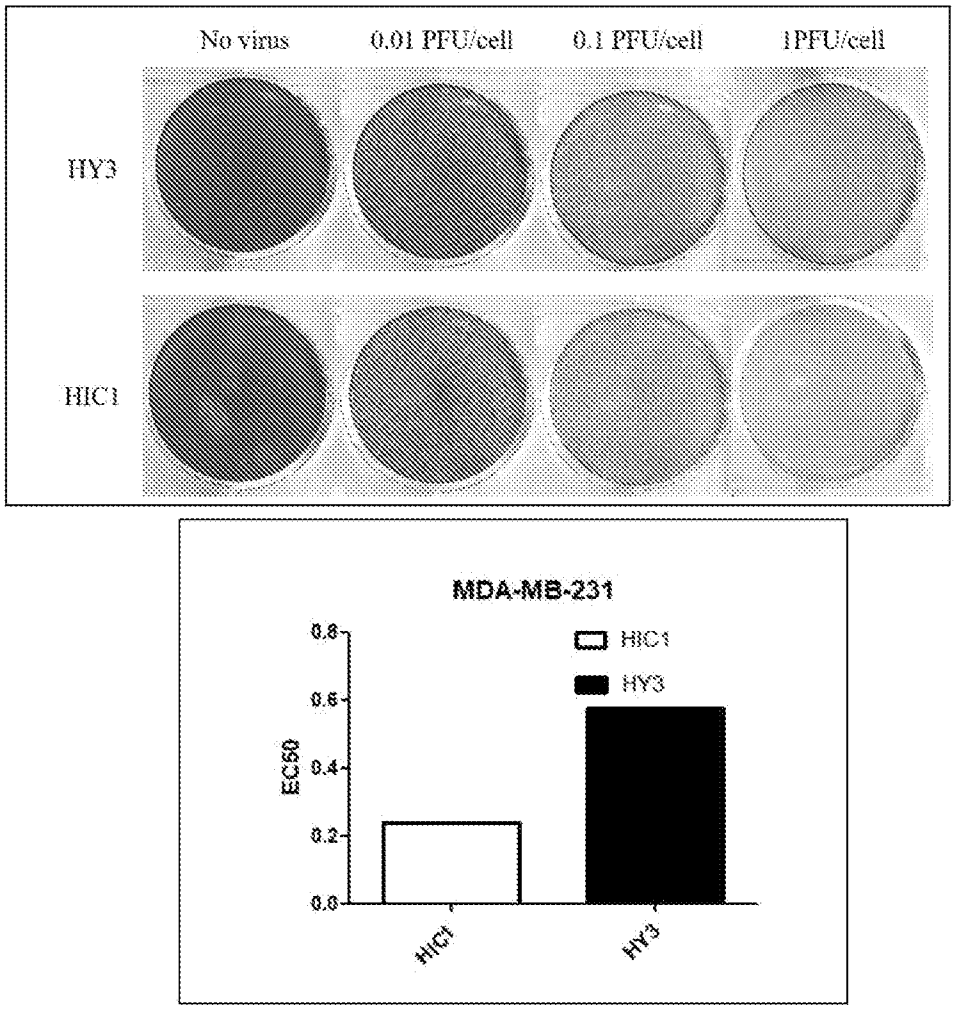

No virus
0.01 PFU/cell
0.1 PFU/cell
1PFU/cell
HY3
HIC1
MDA-MB-231
HIC1
HY3
EC50
0.8
0.6
0.4
0.2
0.0
HIC1
HY3

FIG. 16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2020/101727

A. CLASSIFICATION OF SUBJECT MATTER

A61K 39/21(2006.01)i; A61K 48/00(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CNABS, SIPOABS, DWPI, CNKI, baidu, patentics, shenzhen hua yao kang ming biopharmaceutical, yuan ming, SPD-m4-1BBL, SPD-h4-1BBL, engineered oncolytic virus, mutated viral, selective replication, tumor, immune, immunomodulator gene, truncated viral envelope, immune co-stimulatory pathway activating molecule, tumor suppressor,L025, TK, A46R, SEQ ID NO: 1-14, 5' -A1-X-A2-B1-Y-B2-C1-Z-C2-3' , heterologous, cytokines, therapeutic, genes encoding therapeutic antibodies, ligands of the therapeutic antibodies, L-21, modified B5R, 4-1BBL, HIC1, IL-12, IL-21, IL-2, IL-15, IL-8, CD40 ligand, CD40L , ICOS ligand, GITR ligand, 4-1BB ligand, OX40 ligand, TL1A, CD30 ligand, CD27, Flt3 ligand, Lister, Western Reserve (WR) , Copenhagen (Cop) , Bern, Paris, Tashkent, Tian Tan, Wyeth (DRYVAX) , IHD-J, IHD-W, Brighton, Ankara, CVA382, modified vaccinia ankara (MVA) , Dairen I, LC16m8, LC16M0, LIVP, ACAM2000, WR 65-16, Connaught, New York City Board of Health (NYCBH) , EM-63, NYVAC strain, short consensus repeats (SCR) 2, SCR3, SCR4 domains deletion.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YUAN, M.et al. "A marker-free system for highly efficient construction of vaccinia virus vectors using CRISPR Cas9." *Methods & Clinical Development.*, Vol. 2, 16 September 2015 (2015-09-16), ID 15035, pages 1-9	1-5, 13-32
Y	YUAN, M.et al. "A marker-free system for highly efficient construction of vaccinia virus vectors using CRISPR Cas9." *Methods & Clinical Development.*, Vol. 2, 16 September 2015 (2015-09-16), ID 15035, pages 1-9	6-12
Y	WO 2012/040266 A2 (UNIVERSITY OF MIAMI) 29 March 2012 (2012-03-29) description, paragraphs 0008-0010, 0042, 0063, figure 2	6-12

☑ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 August 2020	**15 October 2020**
Name and mailing address of the ISA/CN **National Intellectual Property Administration, PRC** **6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing 100088** **China** Facsimile No. **(86-10)62019451**	Authorized officer **ZHU,Ning** Telephone No. **86-(10)-53961936**

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2009/263348 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 22 October 2009 (2009-10-22) the whole document	1-32
A	WANG, X.M.et al. "Hypermethylated in cancer 1(HIC1) suppresses non-small cell lung cancer progression by targeting interleukin-6/Stat3 pathway." *Oncotarget.*, Vol. 7, No. 21, 14 April 2016 (2016-04-14), pages 30350-30364	1-32
A	GANGULY, S.et al. "Adjuvantive effects of anti-4-1BB agonist Ab and 4-1BBL DNA for a HIV-1 Gag DNA vaccine: Different effects on cellular and humoral immunity." *Vaccine.*, Vol. 28, No. 5, 03 February 2010 (2010-02-03), ID 1300, pages 1-19	1-32
A	WANG, X.et al. "Membrane-bound interleukin-21 and CD137 ligand induce functional human natural killer cells from peripheral blood mononuclear cells through STAT-3 activation." *Clinical and Experimental Immunology.*, Vol. 172, 31 December 2012 (2012-12-31), pages 104-112	1-32
A	SANCHEZ-PAULETE, A.R.et al. "Deciphering CD137 (4-1BB) signaling in T-cell costimulation for translation into successful cancer immunotherapy." *Eur. J. Immunol.*, Vol. 46, 31 December 2016 (2016-12-31), pages 513–522	1-32
A	GARCEL, A.et al. "plaque-size/host range protein precursor [Vaccinia virus], GenBank: ABD52686.1, 317aa linear." *NCBI genbank.*, 12 June 2007 (2007-06-12), pages 1-2	1-32

INTERNATIONAL SEARCH REPORT

International application No.
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Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
   a. ☑ forming part of the international application as filed:
      ☑ in the form of an Annex C/ST.25 text file.
      ☐ on paper or in the form of an image file.
   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).
      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2020/101727

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☑ Claims Nos.: **22-24, 26-27**
because they relate to subject matter not required to be searched by this Authority, namely:

[1] Claims 22-24, 26-27 relate to a method for treating a cancer, and therefore do not warrant an international search according to the criteria set out in PCT Rule 39.1(iv). An international search is still carried out on the basis of the engineered oncolytic

[2] virus in the manufacture of a medicament for treating a cancer.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2020/101727

Patent document cited in search report	Publication date (day/month/year)	Patent family member(s)	Publication date (day/month/year)
WO 2012/040266 A2	29 March 2012	WO 2012040266 A3	19 July 2012
US 2009/263348 A1	22 October 2009	AU 4016700 A	18 June 2001
		US 7300774 B1	27 November 2007
		DE 60042609 D1	03 September 2009
		EP 1235853 B1	22 July 2009
		ES 2329334 T3	25 November 2009
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		AT 437180 T	15 August 2009
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		AU 785297 B2	04 January 2007
		DK 1235853 T3	23 November 2009
		CA 2393659 C	05 January 2016
		WO 0142298 A1	14 June 2001
		EP 1235853 A4	31 March 2004
		CA 2393659 A1	14 June 2001
		US 2005158831 A1	21 July 2005