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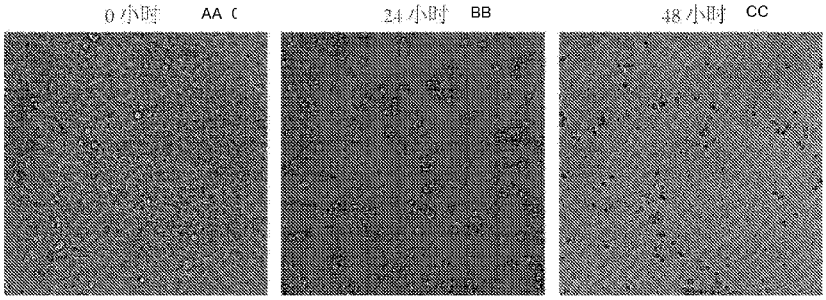
(54) **Title:** VIRUS FOR TREATING TUMORS

(54) **发明名称**: 一种用于治疗肿瘤的病毒



AA 0 hour
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图 4

(57) **Abstract:** Provided are Enterovirus D68 (EV-D68) or a modified form thereof, a nucleic acid molecule containing the genomic sequence or cDNA sequence of the EV-D68 or the modified form thereof or a complementary sequence of the genomic sequence or cDNA sequence, a pharmaceutical composition containing the EV-D68, the modified form thereof, or the nucleic acid molecule, and a use of the EV-D68, the modified form thereof, or the nucleic acid molecule in preparation of the pharmaceutical composition for treating tumors.

(57) **摘要**: 提供了肠道病毒68型 (EV-D68) 或其修饰形式、或包含EV-D68或其修饰形式的基因组序列或cDNA序列、或其互补序列的核酸分子，包含该EV-D68、其修饰形式或核酸分子的药物组合物，以及将其用于制备治疗肿瘤的药物组合物中的用途。

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IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG)。

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A virus for treatment of tumor

Technical Field

The present invention relates to the field of viruses and the field of tumor treatment. Specifically, the present invention relates to use of an Enterovirus D68 (EV-D68) or a modified form thereof, or a nucleic acid molecule comprising a genomic sequence or cDNA sequence of EV-D68 or a modified form thereof, or a complementary sequence of the genomic sequence or cDNA sequence, for treating a tumor in a subject (e.g., a human), and for manufacture of a medicament for treating a tumor in a subject (e.g., a human). The present invention also relates to a method for treating a tumor, which comprises a step of administering to a subject in need thereof EV-D68 or a modified form thereof, or a nucleic acid molecule comprising a genomic sequence or cDNA sequence of EV-D68 or a modified form thereof, or a complementary sequence of the genomic sequence or cDNA sequence.

Background Art

The current methods for treatment of malignant tumors include surgery chemotherapy and radiotherapy. These traditional therapies are not satisfactory for the treatment of metastatic tumors, and may also cause great harm to patients' health. In contrast, as a new type of treatment method, the tumor treatment method using oncolytic virus has high specificity, good effectiveness, and less side effects, and is currently considered as a promising tumor treatment method.

Oncolytic virus is a virus that can self-replicate in tumor cells, thereby killing and lysing the tumor cells, or arresting the growth of the tumor cells. When used in in vivo treatment, oncolytic virus shows specificity for tumor cells, and can directly induce death of tumor cells, but has little or no effect on normal cells. Meanwhile, oncolytic virus can also induce cytotoxic T lymphocyte response in the immune system, thereby indirectly killing tumor cells.

Enterovirus belongs to *Picornaviridae* family, and its genome is single-stranded positive-sense RNA. There are following advantages in using enterovirus as oncolytic virus: firstly, as single-stranded RNA virus, its genome won't undergo any stages of DNA in the host, so that there won't be genotoxicity caused by the insertion of the viral genome into the host's DNA, which has better safety; secondly, the enterovirus genome is relatively small, and a large number of viruses can be replicated in a short period of time to further infect other tumor cells, causing a strong cytopathic effect; next, the enterovirus does not contain oncogenes and therefore does not induce

tumor; and finally, the genome of the enterovirus can be modified by reverse genetics technology to achieve attenuation of virus and reduce its side effects.

At present, the reported enteroviruses with oncolytic activity include chimeric polioviruses for the treatment of human solid tumors such as malignant gliomas (Dobrikova et al., Mol Ther 2008, 16 (11): 1865-1872); Coxsackie viruses A13, A15, A18, and A21 that kill human melanoma cells (Au et al., Virol J 2011, 8: 22); Echo virus ECHO1 that kills human gastric cancer cells and ovarian cancer cells (Shafren et al., Int J Cancer 2005, 115 (2): 320-328; Haley et al., J Mol Med (Berl) 2009, 87 (4): 385-399) and the like. However, it is still necessary to obtain viruses with both tumor-specificity and tumor-killing activity.

Enterovirus D68 (EV-D68) is a kind of Enterovirus D of the genus *Enteroviruses* of *Picornaviridae* family, which was first isolated from children with respiratory infections in California in 1962 (Schieble et al., Am J Epidemiol 1967, 85 (2): 297-310). Unlike most enteroviruses, which are resistant to acids and reproduce in the human gastrointestinal tract, EV-D68 is sensitive to acids and replicates mainly in the respiratory tract. Since there have been few reports of EV-D68 infection for a long time, EV-D68 is considered to be a rare pathogen that mainly causes mild respiratory diseases, including runny nose, sneezing, and cough. At present, there is not report in the art that enterovirus 68 has oncolytic activity.

Contents of the Invention

After a lot of experiments and repeated explorations, it is unexpectedly found that Enterovirus D68 has a broad spectrum and significant tumor cell killing ability. Based on this finding, the inventors of the present invention have developed a new oncolytic virus for treating tumors and a tumor treatment method based on the virus.

Medical use

In a first aspect, the present invention provides use of an Enterovirus D68 (EV-D68) or a modified form thereof, or an isolated nucleic acid molecule, in treatment of a tumor in a subject, or in the manufacture of a medicament for treating a tumor in a subject; wherein the isolated nucleic acid molecule comprises a sequence selected from the following:

(1) a genomic sequence or cDNA sequence of EV-D68 or a modified form thereof; and

(2) a complementary sequence of the genomic sequence or cDNA sequence.

In certain preferred embodiments, the EV-D68 is a wild-type EV-D68. In certain preferred embodiments, the EV-D68 may be a clinical isolate isolated from an individual infected with the

Enterovirus D68.

In certain preferred embodiments, the EV-D68 or a modified form thereof has a genomic sequence that has a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to a nucleotide sequence as shown in SEQ ID NO: 12. In certain preferred embodiments, the genomic sequence of the EV-D68 or a modified form thereof is a nucleotide sequence as shown in SEQ ID NO: 12.

In certain preferred embodiments, the EV-D68 or a modified form thereof has a cDNA sequence that has a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to a nucleotide sequence shown in SEQ ID NO: 1. In certain preferred embodiments, the cDNA sequence of the EV-D68 or a modified form thereof is a nucleotide sequence as shown in SEQ ID NO: 1.

In certain preferred embodiments, the modified form is a modified EV-D68, which has a substitution, insertion, or deletion of one or more nucleotides in the genome as compared to a wild-type EV-D68.

In certain preferred embodiments, as compared to the wild-type EV-D68, the modified EV-D68 has one or more modifications selected from the following:

(1) one or more mutations in an untranslated region (e.g., 5'UTR or 3'UTR);

(2) an insertion of one or more exogenous nucleic acids;

(3) a deletion or mutation of one or more endogenous genes; and

(4) any combination of the above three items.

In certain preferred embodiments, the modified EV-D68 includes one or more mutations in the 5' untranslated region (5'UTR).

In certain preferred embodiments, the modified EV-D68 has a substitution of all or part of the 5'UTR sequence. In certain preferred embodiments, the internal ribosome entry site (IRES) sequence in the 5'UTR of the modified EV-D68 is replaced with an exogenous IRES sequence, such as the internal ribosome entry site sequence of human rhinovirus 2 (HRV2). In certain preferred embodiments, the internal ribosome entry site sequence of the human rhinovirus 2 (HRV2) is shown in SEQ ID NO: 2.

The use of the internal ribosome entry site sequence of human rhinovirus 2 (HRV2) is advantageous in some cases, for example, it is conducive to improvement of the tumor specificity

of oncolytic viruses. It has been previously reported that in normal human nerve cells, the internal ribosome entry site sequence of human rhinovirus 2 is specifically bound by host RNA-binding proteins (DRBP76 and NF45), thereby preventing the recruitment of factors such as elF4G (Merrill et al. J Virol 2006,80 (7): 3147-3156; Merrill and Gromeier, J Virol 2006,80 (14): 6936-6942; Neplioueva et al., PLoS One 2010,5 (7): e11710); meanwhile, due to the lack of support of signaling pathways such as Raf/Erk1/2/MAPK, it is difficult for ribosomes to bind to the internal ribosome entry site sequence of human rhinovirus 2, so that it is impossible to initiate translation of viral protein (Dobrikov et al., Mol Cell Biol 2011, 31(14): 2947-2959; Dobrikov et al., Mol Cell Biol 2013, 33(5): 937-946). In human glioma tumor cells, the internal ribosome entry site of human rhinovirus 2 is not affected by the above two factors, and thus can normally initiate transcription and translation of viral protein. Therefore, in some cases, replacing the internal ribosome entry site sequence of EV-D68 with the internal ribosome entry site sequence of human rhinovirus 2 is beneficial to avoid or reduce the toxic and side effect of the virus of the present invention to normal human nerve cells, without affecting the use of the virus in the treatment of human gliomas.

In certain preferred embodiments, the modified EV-D68 comprises an exogenous nucleic acid.

In certain preferred embodiments, the exogenous nucleic acid encodes a cytokine (e.g., GM-CSF, preferably human GM-CSF), or an antitumor protein or polypeptide (e.g., a scFv against PD-1 or PD-L1, preferably a scFv against human PD-1 or PD-L1). In certain preferred embodiments, the exogenous nucleic acid is inserted between the 5'UTR gene and the VP4 gene, or between the VP1 gene and the 2A gene of the genome of the modified EV-D68.

In certain preferred embodiments, the exogenous nucleic acid comprises a target sequence of microRNA (miRNA) (e.g., miR-133 or miR-206). In certain preferred embodiments, the target sequence of microRNA inserted in the 3' untranslated region (3'UTR) of the genome of the modified EV-D68.

It has been previously reported that the expression level of certain microRNA in tumor cells is significantly lower than normal cells and/or has obvious tissue specificity. Therefore, in some cases, the modified EV-D68 of the present invention containing a target sequence of such microRNA is advantageous, because such microRNA that are highly expressed in normal cells or tissues can reduce or even block the replication of the modified EV-D68 in the normal cells or tissues by the corresponding target sequence, thereby reducing even avoiding the toxic side effects of the modified EV-D68 on non-tumor cells. Such microRNAs include but are not limited to miR-133, miR-206, miR-1, miR-143, miR-145, miR-217, let-7, miR-15, miR-16, etc. (see, for example, PCT International Application WO2008103755A1, US patent application US20160143969A1, or Baohong Zhang et al., Developmental Biology, Volume 302, Issue 1, 1 February 2007, Pages 1-

12; all of these documents are incorporated herein by reference).

In certain preferred embodiments, the exogenous nucleic acid comprises a target sequence of one or more (e.g., 2, 3, or 4) microRNA as described above. In certain preferred embodiments, the exogenous nucleic acid comprises a target sequence of miR-133 and/or miR-206. In certain preferred embodiments, the target sequence of miR-133 is shown in SEQ ID NO: 3. In certain preferred embodiments, the target sequence of miR-206 is shown in SEQ ID NO: 4. In some cases, the insertion of the target sequence of miR-133 and/or miR-206 is advantageous. This is because miR-133 and miR-206 are specifically expressed in muscle tissue, so that the tissue tropism of the oncolytic virus can be changed by inserting the target sequence of miR-133 and/or miR-206 into the modified EV-D68, thereby reducing or avoiding damage to normal muscle tissue.

In certain preferred embodiments, the modified EV-D68 comprises at least one insertion of the exogenous nucleic acid as described above and/or at least one mutation in the untranslated region as described above.

In certain preferred embodiments, the genomic sequence of the modified EV-D68 has a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to a nucleotide sequence selected from: the nucleotide sequences as shown in SEQ ID NOs: 13-16. In certain preferred embodiments, the genomic sequence of the modified EV-D68 is selected from the nucleotide sequences as shown in any one of SEQ ID NOs: 13-16.

In certain preferred embodiments, the cDNA sequence of the modified EV-D68 has a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to a nucleotide sequence selected from: the nucleotide sequences as shown in SEQ ID NOs: 8-11. In certain preferred embodiments, the cDNA sequence of the modified EV-D68 is selected from the nucleotide sequences as shown in any one of SEQ ID NOs: 8-11.

In the present invention, the modified EV-D68 can be obtained by reverse genetics technology, and the reverse genetics technology is known in the art, for example, see Yang LS, Li SX, Liu YJ, et al Virus Res, 2015, 210: 165-168; Hou WH, Yang LS, Li SX, et al. Virus Res, 2015, 205: 41-44; which is incorporated herein by reference in its entirety. In such embodiments, the modified EV-D68 is typically obtained by modifying the cDNA of wild-type EV-D68 (e.g., insertion of an exogenous nucleic acid, deletion or mutation of an endogenous gene, or mutation in a non-translated region).

In the present invention, the EV-D68 or a modified form thereof may be pretreated to reduce

or eliminate the immune response against the virus in a subject, wherein the pretreatment may comprise: packaging the EV-D68 in a lipidosome or micelle, and/or using a protease (e.g., chymotrypsin or trypsin) to remove the capsid protein of the virus to reduce the humoral and/or cellular immunity against the virus in host.

In the present invention, the EV-D68 or a modified form thereof can be serially passaged for adaptation in tumor cells. In certain preferred embodiments, the tumor cells may be tumor cell lines or tumor cell strains known in the art, or may be tumor cells obtained by surgical resection or clinical isolation from an individual (e.g., a subject) having a tumor. In certain preferred embodiments, the EV-D68 or a modified form thereof is serially passaged for adaptation in tumor cells obtained from an individual (e.g., a subject) having a tumor. In certain preferred embodiments, the tumor cells are obtained by surgical resection or clinical isolation from an individual (e.g., a subject) having a tumor. In certain preferred embodiments, the method for serial passaging for adaptation comprises a plurality of (e.g., at least 5, at least 10, at least 15, at least 20) cycles consisting of the following processes: 1) infecting a target tumor cell with a virus; 2) harvesting the virus in a supernatant; and 3) reinfecting a fresh target tumor cell with the obtained virus.

In certain preferred embodiments, the EV-D68 and modified forms thereof as described above can be used in combination. Therefore, the medicament may comprise one or several of EV-D68 and modified forms thereof.

In certain preferred embodiments, the isolated nucleic acid molecule consists of a genomic sequence or cDNA sequence of EV-D68 or a modified form thereof as described above, or a complementary sequence of the genomic sequence or cDNA sequence. In certain preferred embodiments, the isolated nucleic acid molecule has a genomic sequence of EV-D68 or a modified form thereof as described above. In certain preferred embodiments, the isolated nucleic acid molecule is RNA. In certain preferred embodiments, the isolated nucleic acid molecule has a nucleotide sequence as shown in any one of SEQ ID NOs: 12-16.

In certain preferred embodiments, the isolated nucleic acid molecule is a vector (e.g. cloning vector or expression vector) comprising a genomic sequence or cDNA sequence of EV-D68 or a modified form thereof as described above, or a complementary sequence of the genomic sequence or cDNA sequence. In certain preferred embodiments, the isolated nucleic acid molecule is a vector (e.g., cloning vector or expression vector) comprising a cDNA sequence of EV-D68 or a modified form thereof as described above, or a complementary sequence of the cDNA sequence. In certain preferred embodiments, the isolated nucleic acid molecule is a vector comprising a nucleotide sequence as shown in any one of SEQ ID NOs: 1, 8-11 or a complementary sequence thereof.

In certain preferred embodiments, the isolated nucleic acid molecule comprises the complementary sequence of the genomic sequence of EV-D68 or a modified form thereof as described above. In certain preferred embodiments, the complementary sequence is complementary to a nucleotide sequence selected from:

(1) a nucleotide sequence as shown in SEQ ID NO: 12;

(2) a nucleotide sequence having a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to the nucleotide sequence as shown in SEQ ID NO: 12;

(3) a nucleotide sequence as shown in any one of SEQ ID NOs: 13-16; and

(4) a nucleotide sequence having a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to the nucleotide sequence as shown in any of SEQ ID NOs: 13-16.

In certain preferred embodiments, the isolated nucleic acid molecule comprises a complementary sequence to the cDNA sequence of EV-D68 or a modified form thereof as described above. In certain preferred embodiments, the complementary sequence is complementary to a nucleotide sequence selected from:

(1) a nucleotide sequence as shown in SEQ ID NO: 1;

(2) a nucleotide sequence having a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to the nucleotide sequence as shown in SEQ ID NO: 1;

(3) a nucleotide sequence as shown in any one of SEQ ID NOs: 8-11; and

(4) a nucleotide sequence having a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to the nucleotide sequence as shown in any one of SEQ ID NOs: 8-11.

In the present invention, the isolated nucleic acid molecule can be delivered by any means known in the art, for example, a naked nucleic acid molecule (e.g., a naked RNA) can be directly injected, or a non-viral delivery system can be used. The non-viral delivery system can be obtained from a variety of materials well known in the art, including, but not limited to, the materials

described in detail in "Yin H, et al. Nat Rev Genet. 2014 Aug; 15(8): 541-55." and "Riley MK, Vermerris W. Nanomaterials (Basel). 2017 Apr 28; 7(5). Pii: E94.", which are incorporated herein by reference in their entirety, such as liposomes, inorganic nanoparticles (such as gold nanoparticles), polymers (such as PEG), and so on.

In certain preferred embodiments, the medicament comprises a therapeutically effective amount of the EV-D68 and/or a modified form thereof as described above, or a therapeutically effective amount of the isolated nucleic acid molecule as described above. In certain preferred embodiments, the medicament may be in any form known in the medical arts. For example, the medicament may be in the form of a tablet, a pill, a suspension, an emulsion, a solution, a gel, a capsule, a powder, a granule, an elixir, a lozenge, a suppository, or an injection (including injection solution, lyophilized powder) and so on. In some embodiments, the medicament is an injection solution or a lyophilized powder.

In certain preferred embodiments, the medicament further comprises a pharmaceutically acceptable carrier or excipient. In certain preferred embodiments, the medicament comprises a stabilizer.

In certain preferred embodiments, the medicament optionally further comprises an additional pharmaceutically active agent. In a preferred embodiment, the additional pharmaceutically active agent is a medicament having antitumor activity, such as an additional oncolytic virus, chemotherapeutic agent or immunotherapeutic agent.

In the present invention, the additional oncolytic virus includes, but is not limited to, herpesvirus, adenovirus, parvovirus, reovirus, Newcastle disease virus, vesicular stomatitis virus, measles virus, or any combination thereof. The chemotherapeutic agent includes but is not limited to 5-fluorouracil, mitomycin, methotrexate, hydroxyurea, cyclophosphamide, dacarbazine, mitoxantrone, anthracyclines (e.g., epirubicin or doxorubicin), etoposide, platinum compounds (e.g., carboplatin or cisplatin), taxanes (e.g., paclitaxel or taxotere), or any combination thereof. The immunotherapeutic agent includes, but is not limited to, immune checkpoint inhibitors (e.g., PD-L1/PD-1 inhibitors or CTLA-4 inhibitors), tumor-specific targeting antibodies (e.g., rituximab or Herceptin) or any combination thereof.

In certain preferred embodiments, the medicament comprises a unit dose of the EV-D68 and/or a modified form thereof as described above, for example comprising at least 1×10^2 pfu, at least 1×10^3 pfu, at least 1×10^4 pfu, 1×10^5 pfu, 1×10^6 pfu, at least 1×10^7 pfu, at least 1×10^8 pfu, at least 1×10^9 pfu, at least 1×10^{10} pfu, at least 1×10^{11} pfu, at least 1×10^{12} pfu, at least 1×10^{13} pfu, at least 1×10^{14} pfu, or at least 1×10^{16} pfu of the EV-D68 and/or a modified form thereof. In certain preferred embodiments, the medicament comprises 1×10^2 pfu to 1×10^{17} pfu of the EV-D68 and/or

a modified form thereof as described above.

In certain preferred embodiments, the medicament contains a unit dose of an isolated nucleic acid molecule as described above, such as the nucleic acid molecule containing $3x10^{10}$ to $3x10^{14}$ virus genome copies.

In certain preferred embodiments, the medicament may be administered in combination with an additional therapy. This additional therapy may be any therapy known for tumors, such as surgery, chemotherapy, radiation therapy, immunotherapy, hormone therapy or gene therapy. This additional therapy may be administered before, concurrently with, or after the administration of the medicament.

In certain preferred embodiments, the tumor includes, but is not limited to, cervical cancer, ovarian cancer, endometrial cancer, lung cancer, liver cancer, kidney cancer, neuroblastoma, glioma, breast cancer, melanoma, prostate cancer, bladder cancer, pancreatic cancer, gastric cancer, colorectal cancer, esophageal cancer, thyroid cancer, laryngeal cancer, osteosarcoma, hematopoietic malignancy (e.g., lymphoma or leukemia).

In certain preferred embodiments, the subject is a mammal, such as a human.

In another aspect, the invention also relates to use of the EV-D68 and/or a modified form thereof as defined in the first aspect, or the isolated nucleic acid molecule as defined in the first aspect, as a medicament.

Treatment method

In a second aspect, the present invention provides a method for treating a tumor, comprising the step of administering to a subject in need thereof an effective amount of an EV-D68 or a modified form thereof, or an effective amount of an isolated nucleic acid molecule; wherein the isolated nucleic acid molecule comprises a sequence selected from the group consisting of:

(1) a genomic sequence or cDNA sequence of EV-D68 or a modified form thereof; and

(2) a complementary sequence of the genomic sequence or cDNA sequence.

In certain preferred embodiments, EV-D68 is administered to the subject. In certain preferred embodiments, the EV-D68 is wild-type EV-D68. In certain preferred embodiments, the EV-D68 may be a clinical isolate that is isolated from an individual infected with Enterovirus D68.

In certain preferred embodiments, the genomic sequence of the EV-D68 or a modified form

thereof has a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to the nucleotide sequence as shown in SEQ ID NO: 12. In certain preferred embodiments, the genomic sequence of the EV-D68 or a modified form thereof is a nucleotide sequence as shown in SEQ ID NO: 12.

In certain preferred embodiments, the cDNA sequence of the EV-D68 or a modified form thereof has a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to the nucleotide sequence as shown in SEQ ID NO: 1. In certain preferred embodiments, the cDNA sequence of the EV-D68 or a modified form thereof is a nucleotide sequence as shown in SEQ ID NO: 1.

In certain preferred embodiments, a modified form of EV-D68 is administered to the subject. In certain preferred embodiments, as compared to the wild-type EV-D68, the modified form is a modified EV-D68, which has a substitution, insertion, or deletion of one or more nucleotides in the genome.

In certain preferred embodiments, as compared to the wild-type EV-D68, the modified EV-D68 has one or more modifications selected from the following:

(1) one or more mutations in an untranslated region (e.g., 5'UTR or 3'UTR);

(2) an insertion of one or more exogenous nucleic acids;

(3) a deletion or mutation of one or more endogenous genes; and

(4) any combination of the above three items.

In certain preferred embodiments, the modified EV-D68 includes one or more mutations in the 5' untranslated region (5'UTR).

In certain preferred embodiments, the modified EV-D68 has a substitution of all or part of the 5'UTR sequence. In certain preferred embodiments, the internal ribosome entry site (IRES) sequence in the 5'UTR of the modified EV-D68 is replaced with an exogenous IRES sequence, such as the interior ribosome entry site sequence of human rhinovirus 2 (HRV2). In certain preferred embodiments, the internal ribosome entry site sequence of the human rhinovirus 2 (HRV2) is shown in SEQ ID NO: 2.

In certain preferred embodiments, the modified EV-D68 comprises an exogenous nucleic acid.

In certain preferred embodiments, the exogenous nucleic acid encodes a cytokine (e.g., GM-CSF, preferably human GM-CSF), or an antitumor protein or polypeptide (e.g., scFv against PD-

1 or PD-L1, preferably scFv against human PD-1 or PD-L1). In certain preferred embodiments, the exogenous nucleic acid is inserted between the 5'UTR gene and the VP4 gene, or between the VP1 gene and the 2A gene of the genome of the modified EV-D68.

In certain preferred embodiments, the exogenous nucleic acid comprises a target sequence of microRNA (miRNA) (e.g., miR-133 or miR-206). In certain preferred embodiments, the target sequence of microRNA is inserted in the 3' untranslated region (3'UTR) of the genome of the modified EV-D68.

In certain preferred embodiments, the exogenous nucleic acid comprises a target sequence of one or more (e.g., 2, 3, or 4) microRNA as described above. In certain preferred embodiments, the exogenous nucleic acid comprises a target sequence of miR-133 and/or miR-206. In certain preferred embodiments, the target sequence of miR-133 is shown in SEQ ID NO: 3. In certain preferred embodiments, the target sequence of miR-206 is shown in SEQ ID NO: 4.

In certain preferred embodiments, the modified EV-D68 comprises at least one insertion of the exogenous nucleic acid as described above and/or at least one mutation in the untranslated region as described above.

In certain preferred embodiments, the genomic sequence of the modified EV-D68 has a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to a nucleotide sequence selected from: the nucleotide sequences as shown in SEQ ID NOs: 13-16. In certain preferred embodiments, the genomic sequence of the modified EV-D68 is selected from the nucleotide sequence as shown in any one of SEQ ID NOs: 13-16.

In certain preferred embodiments, the cDNA sequence of the modified EV-D68 has a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to a nucleotide sequence selected from: the nucleotide sequences as shown in SEQ ID NOs: 8-11. In certain preferred embodiments, the cDNA sequence of the modified EV-D68 is selected from the nucleotide sequence as shown in any one of SEQ ID NOs: 8-11.

In certain preferred embodiments, the EV-D68 and modified forms thereof as described above can be used in combination. Thus, one or more of the EV-D68 and modified forms can be administered to a subject.

In certain preferred embodiments, the isolated nucleic acid molecule as described above is administered to the subject.

In certain preferred embodiments, the isolated nucleic acid molecule consists of the genomic

sequence or cDNA sequence of the EV-D68 or a modified form thereof as described above, or the complementary sequence of the genomic sequence or cDNA sequence. In certain preferred embodiments, the isolated nucleic acid molecule has the genomic sequence of the EV-D68 or a modified form thereof as described above. In certain preferred embodiments, the isolated nucleic acid molecule is RNA. In certain preferred embodiments, the isolated nucleic acid molecule has a nucleotide sequence as shown in any one of SEQ ID NOs: 12-16.

In certain preferred embodiments, the isolated nucleic acid molecule is a vector (e.g. cloning vector or expression vector) comprising the genomic sequence or cDNA sequence of EV-D68 or a modified form thereof as described above, or the complementary sequence of the genomic sequence or cDNA sequence. In certain preferred embodiments, the isolated nucleic acid molecule is a vector (e.g., cloning vector or expression vector) comprising the cDNA sequence of EV-D68 or a modified form thereof as described above, or the complementary sequence of the cDNA sequence. In certain preferred embodiments, the isolated nucleic acid molecule is a vector comprising the nucleotide sequence as shown in any one of SEQ ID NOs: 1, 8-11 or the complementary sequence thereof.

In certain preferred embodiments, the isolated nucleic acid molecule comprises the complementary sequence of the genomic sequence of EV-D68 or a modified form thereof as described above. In certain preferred embodiments, the complementary sequence is complementary to a nucleotide sequence selected from:

(1) a nucleotide sequence as shown in SEQ ID NO: 12;

(2) a nucleotide sequence having a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to the nucleotide sequence as shown in SEQ ID NO: 12;

(3) a nucleotide sequence as shown in any one of SEQ ID NOs: 13-16; and

(4) a nucleotide sequence having a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to the nucleotide sequence shown in any of SEQ ID NOs: 13-16.

In certain preferred embodiments, the isolated nucleic acid molecule comprises the complementary sequence of the cDNA sequence of EV-D68 or a modified form thereof as described above. In certain preferred embodiments, the complementary sequence is complementary to a nucleotide sequence selected from:

(1) a nucleotide sequence as shown in SEQ ID NO: 1;

(2) a nucleotide sequence having a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to the nucleotide sequence as shown in SEQ ID NO: 1;

(3) a nucleotide sequence as shown in any one of SEQ ID NOs: 8-11; and

(4) a nucleotide sequence having a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to the nucleotide sequence as shown in any one of SEQ ID NOs: 8-11.

In the present invention, the isolated nucleic acid molecule can be delivered by any means known in the art, for example, a naked nucleic acid molecule (e.g., naked RNA) can be directly injected, or a non-viral delivery system can be used. The non-viral delivery system can be obtained from a variety of materials well known in the art, including, but not limited to, the materials described in detail in "Yin H, et al. Nat Rev Genet. 2014 Aug; 15(8): 541-55." and "Riley MK, Vermerris W. Nanomaterials (Basel). 2017 Apr 28; 7(5). Pii: E94.", which are incorporated herein by reference in their entirety, such as liposomes, inorganic nanoparticles (such as gold nanoparticles), polymers (such as PEG), and so on.

In certain preferred embodiments, the EV-D68 and/or a modified form thereof as described above, or the isolated nucleic acid molecule as described above, can be formulated and administered as a pharmaceutical composition. Such a pharmaceutical composition may comprise a therapeutically effective amount of the EV-D68 and/or a modified form thereof as described above, or a therapeutically effective amount of the isolated nucleic acid molecule as described above. In certain preferred embodiments, the pharmaceutical composition may be in any form known in the medical arts. For example, the pharmaceutical composition may be in the form of a tablet, a pill, a suspension, an emulsion, a solution, a gel, a capsule, a powder, a granule, an elixir, a lozenge, a suppository, or an injection (including injection solution, lyophilized powder) and so on. In some embodiments, the medicament is an injection solution or a lyophilized powder.

In certain preferred embodiments, the pharmaceutical composition further comprises a pharmaceutically acceptable carrier or excipient. In certain preferred embodiments, the pharmaceutical composition comprises a stabilizer.

In the present invention, the EV-D68 and/or a modified form thereof, or the isolated nucleic acid molecule as described above can be administered to a subject by any suitable administration

route. In some cases, the route of administration of the EV-D68 and/or a modified form thereof, or the isolated nucleic acid molecules as described above, depends on the location and type of tumor. For example, for a solid tumor that is easily accessible, the virus or nucleic acid molecule is optionally administered by injection directly into the tumor (e.g., intratumoral injection); for a tumor of hematopoietic system, the virus or nucleic acid molecule can be administered by intravenous or other intravascular routes; for a tumor that is not easily accessible in the body (e.g., metastases), the virus or nucleic acid molecule can be administered systematically so that it can run over the whole body and thereby reaching the tumor (e.g., intravenous or intramuscular injection). Optionally, the virus or nucleic acid molecule of the present invention can be administrated via subcutaneous, intraperitoneal, intrathecal (e.g., for brain tumors), topical (e.g., for melanoma), oral (e.g., for oral or esophageal cancer), intranasal or inhalation spray (e.g., for lung cancer) routes and so on. In certain preferred embodiments, the EV-D68 and/or a modified form thereof as described above, or the isolated nucleic acid as described above, can be administered via intradermal, subcutaneous, intramuscular, intravenous, oral routes etc.

In certain preferred embodiments, the method further comprises administering an additional pharmaceutically active agent having antitumor activity. This additional pharmaceutically active agent may be administered before, concurrently with or after the administration of the EV-D68 and/or a modified form thereof, or an isolated nucleic acid molecule as described above.

In certain preferred embodiments, the additional pharmaceutically active agent includes an additional oncolytic virus, chemotherapeutic agent, or immunotherapeutic agent. In the present invention, the additional oncolytic virus includes, but is not limited to, herpesvirus, adenovirus, parvovirus, reovirus, Newcastle disease virus, vesicular stomatitis virus, measles virus, or any combination thereof. The chemotherapeutic agent includes but is not limited to 5-fluorouracil, mitomycin, methotrexate, hydroxyurea, cyclophosphamide, dacarbazine, mitoxantrone, anthracyclines (such as epirubicin or doxorubicin), etoposide, platinum compounds (such as carboplatin or cisplatin), taxanes (such as paclitaxel or taxotere), or any combination thereof. The immunotherapeutic agents include, but are not limited to, immune check point inhibitors (such as PD-L1/PD-1 inhibitors or CTLA-4 inhibitors), tumor-specific targeting antibodies (such as rituximab or Herceptin) or any combination thereof.

In certain preferred embodiments, the EV-D68 and/or a modified form thereof can be administered in any amount from 1 to 1×10^{15} pfu/kg of the subject's body weight, for example, the EV-D68 and/or a modified form thereof is administered in an amount of at least 1×10^{3} pfu/kg, at least 1×10^{4} pfu/kg, 1×10^{5} pfu/kg, 1×10^{6} pfu/kg, at least 1×10^{7} pfu/kg, at least 1×10^{8} pfu/kg, at least 1×10^{9} pfu/kg, at least 1×10^{10} pfu/kg, at least 1×10^{11} pfu/kg, or at least 1×10^{12} pfu/kg of the

subject's body weight. In certain preferred embodiments, the isolated nucleic acid molecule as described above can be administered in any amount of 3×10^{10} to 3×10^{14} virus genome copies per kg of the subject's body weight. In certain preferred embodiments, the EV-D68 and/or a modified form thereof or the isolated nucleic acid molecule as described above can be administered 3 times a day, 2 times a day, 1 time a day, once every 2 days or once a week, optionally the above dosage regimen can be repeated weekly or monthly as appropriate.

In certain preferred embodiments, the method further comprises administering an additional therapy. This additional therapy may be any therapy known for tumors, such as surgery, chemotherapy, radiation therapy, immunotherapy, hormone therapy or gene therapy. This additional therapy may be administered before, concurrently with, or after the administration of the method described above.

In certain preferred embodiments, the subject is a mammal, such as a human.

In certain preferred embodiments, the tumor includes, but is not limited to, cervical cancer, ovarian cancer, endometrial cancer, lung cancer, liver cancer, kidney cancer, neuroblastoma, glioma, breast cancer, melanoma, prostate cancer, bladder cancer, pancreatic cancer, gastric cancer, colorectal cancer, esophageal cancer, thyroid cancer, laryngeal cancer, osteosarcoma, hematopoietic malignancy (e.g., lymphoma or leukemia).

Pharmaceutical composition

In a third aspect, the present invention provides a pharmaceutical composition comprising an EV-D68 and/or a modified form thereof as defined in the first or second aspect, or an isolated nucleic acid molecule as defined in the first or second aspect.

In certain preferred embodiments, the pharmaceutical composition comprises the EV-D68 and/or a modified form thereof as defined in the first or second aspect. In certain preferred embodiments, the EV-D68 and/or modified forms thereof may be used in combination. Therefore, the pharmaceutical composition of the present invention may comprise one or several of the EV-D68 and/or modified forms thereof. In certain preferred embodiments, the pharmaceutical composition comprises a unit dose of the EV-D68 and/or a modified form thereof, for example at least 1×10^{2} pfu, at least 1×10^{3} pfu, at least 1×10^{4} pfu, 1×10^{5} pfu, 1×10^{6} pfu, at least 1×10^{7} pfu, at least 1×10^{8} pfu, at least 1×10^{9} pfu, at least 1×10^{10} pfu, at least 1×10^{11} pfu, at least 1×10^{12} pfu, at least 1×10^{13} pfu, at least 1×10^{14} pfu, or at least 1×10^{16} pfu of the EV-D68 and/or a modified form thereof. In certain preferred embodiments, the pharmaceutical composition comprises 1×10^{2} pfu to 1×10^{17} pfu of the EV-D68 and/or a modified form thereof.

In certain preferred embodiments, the pharmaceutical composition comprises an isolated nucleic acid molecule as defined in the first aspect or the second aspect. In certain preferred embodiments, the isolated nucleic acid molecules can be used in combination. Therefore, the pharmaceutical composition of the present invention may include one or several of the isolated nucleic acid molecules. In certain preferred embodiments, the pharmaceutical composition comprises a unit dose of the isolated nucleic acid molecule, for example 3×10^{10} to 3×10^{14} virus genome copies of the isolated nucleic acid molecule.

In certain preferred embodiments, the pharmaceutical composition may be in any form known in the medical arts. For example, the pharmaceutical composition may be in the form of a tablet, a pill, a suspension, an emulsion, a solution, a gel, a capsule, a powder, a granule, an elixir, a lozenge, a suppository, or an injection (including injection solution, lyophilized powder) and so on. In some embodiments, the medicament is an injection solution or a lyophilized powder.

In certain preferred embodiments, the pharmaceutical composition further comprises a pharmaceutically acceptable carrier or excipient. In certain preferred embodiments, the pharmaceutical composition comprises a stabilizer.

In certain preferred embodiments, the pharmaceutical composition optionally further comprises an additional pharmaceutically active agent. In a preferred embodiment, the additional pharmaceutically active agent is a medicament having antitumor activity, such as an additional oncolytic virus, chemotherapeutic agent or immunotherapeutic agent.

In certain preferred embodiments, the pharmaceutical composition is used to treat a tumor in a subject.

In certain preferred embodiments, the subject is a mammal, such as a human.

In certain preferred embodiments, the tumor includes, but is not limited to, cervical cancer, ovarian cancer, endometrial cancer, lung cancer, liver cancer, kidney cancer, neuroblastoma, glioma, breast cancer, melanoma, prostate cancer, bladder cancer, pancreatic cancer, gastric cancer, colorectal cancer, esophageal cancer, thyroid cancer, laryngeal cancer, osteosarcoma, hematopoietic malignancy (e.g., lymphoma or leukemia).

Modified EV-D68

In a fourth aspect, the present invention provides a modified EV-D68 having a substitution, insertion, or deletion of one or more nucleotides in the genome compared to wild-type EV-D68.

In certain preferred embodiments, the genomic sequence of the wild-type EV-D68 has a

sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to a nucleotide sequence selected from the nucleotide sequence as shown in SEQ ID NO: 12. In certain preferred embodiments, the genomic sequence of the wild-type EV-D68 is a nucleotide sequence as shown in SEQ ID NO: 12.

In certain preferred embodiments, the cDNA sequence of the wild-type EV-D68 has a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to a nucleotide sequence as shown in SEQ ID NO: 1. In certain preferred embodiments, the cDNA sequence of the wild-type EV-D68 is a nucleotide sequence as shown in SEQ ID NO: 1.

In certain preferred embodiments, as compared to the wild-type EV-D68, the modified EV-D68 has one or more modifications selected from the following:

(1) one or more mutations in an untranslated region (e.g., 5'UTR or 3'UTR);

(2) an insertion of one or more exogenous nucleic acids;

(3) a deletion or mutation of one or more endogenous genes; and

(4) any combination of the above three items.

In certain preferred embodiments, the modified EV-D68 includes one or more mutations in the 5' untranslated region (5'UTR).

In certain preferred embodiments, the modified EV-D68 has a substitution of all or part of the 5'UTR sequence. In certain preferred embodiments, the internal ribosome entry site (IRES) sequence in the 5'UTR of the modified EV-D68 is replaced with an exogenous IRES sequence, such as the interior ribosome entry site sequence of human rhinovirus 2 (HRV2). In certain preferred embodiments, the internal ribosome entry site sequence of the human rhinovirus 2 (HRV2) is shown in SEQ ID NO: 2.

In certain preferred embodiments, the modified EV-D68 comprises an exogenous nucleic acid.

In certain preferred embodiments, the exogenous nucleic acid encodes a cytokine (e.g., a GM-CSF, preferably a human GM-CSF), or an antitumor protein or polypeptide (e.g., a scFv against PD-1 or PD-L1, preferably a scFv against human PD-1 or PD-L1). In certain preferred embodiments, the exogenous nucleic acid is inserted between the 5'UTR gene and the VP4 gene, or between the VP1 gene and the 2A gene of the genome of the modified EV-D68.

In certain preferred embodiments, the exogenous nucleic acid comprises a target sequence of

microRNA (miRNA) (e.g., miR-133 or miR-206). In certain preferred embodiments, the target sequence of microRNA is inserted in the 3' untranslated region (3'UTR) of the genome of the modified EV-D68.

In certain preferred embodiments, the exogenous nucleic acid comprises a target sequence of one or more (e.g., 2, 3, or 4) microRNA as described above. In certain preferred embodiments, the exogenous nucleic acid comprises a target sequence of miR-133 and/or miR-206. In certain preferred embodiments, the target sequence of miR-133 is shown in SEQ ID NO: 3. In certain preferred embodiments, the target sequence of miR-206 is shown in SEQ ID NO: 4.

In certain preferred embodiments, the modified EV-D68 comprises at least one insertion of the exogenous nucleic acid as described above and/or at least one mutation in the untranslated region as described above.

In certain preferred embodiments, the genomic sequence of the modified EV-D68 has a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to a nucleotide sequence selected from: the nucleotide sequences as shown in SEQ ID NOs: 13-16. In certain preferred embodiments, the genomic sequence of the modified EV-D68 is selected from the nucleotide sequences as shown in any one of SEQ ID NOs: 13-16.

In certain preferred embodiments, the cDNA sequence of the modified EV-D68 has a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to a nucleotide sequence selected from: the nucleotide sequences as shown in SEQ ID NOs: 8-11. In certain preferred embodiments, the cDNA sequence of the modified EV-D68 is selected from the nucleotide sequences as shown in any one of SEQ ID NOs: 8-11.

In the present invention, the modified EV-D68 can be obtained by reverse genetics technology, and the reverse genetics technology is known in the art, for example, see Yang LS, Li SX, Liu YJ, et al Virus Res, 2015, 210: 165-168; Hou WH, Yang LS, Li SX, et al. Virus Res, 2015, 205: 41-44; which are incorporated herein by reference in their entirety. In such embodiments, the modified EV-D68 is typically obtained by modifying the cDNA of wild-type EV-D68 (e.g., insertion of an exogenous nucleic acid, deletion or mutation of an endogenous gene, or mutation in a non-translated region).

In the present invention, the modified EV-D68 may be pretreated to reduce or eliminate the immune response against the virus in a subject, wherein the pretreatment may comprise: packaging

the EV-D68 in a lipidosome or micelle, and/or using a protease (e.g., chymotrypsin or trypsin) to remove the capsid protein of the virus to reduce the humoral and/or cellular immunity against the virus in host.

In the present invention, the modified EV-D68 can be serially passaged for adaptation in tumor cells. In certain preferred embodiments, the tumor cells may be tumor cell lines or tumor cell strains known in the art, or may be tumor cells obtained by surgical resection or clinical isolation from an individual (e.g., a subject) having a tumor. In certain preferred embodiments, the modified EV-D68 is serially passaged for adaptation in tumor cells obtained from an individual (e.g., a subject) having a tumor. In certain preferred embodiments, the tumor cells are obtained by surgical resection or clinical isolation from an individual (e.g., a subject) having a tumor. In certain preferred embodiments, the method for serial passaging for adaptation comprises a plurality of (e.g., at least 5, at least 10, at least 15, at least 20) cycles consisting of the following processes: 1) infecting a target tumor cell with a virus; 2) harvesting the virus in a supernatant; and 3) reinfecting a fresh target tumor cell with the obtained virus.

In certain preferred embodiments, the modified EV-D68 is used to treat a tumor in a subject, or to prepare a medicament for treating a tumor in a subject.

In certain preferred embodiments, the tumor includes, but is not limited to, cervical cancer, ovarian cancer, endometrial cancer, lung cancer, liver cancer, kidney cancer, neuroblastoma, glioma, breast cancer, melanoma, Prostate cancer, bladder cancer, pancreatic cancer, gastric cancer, colorectal cancer, esophageal cancer, thyroid cancer, laryngeal cancer, osteosarcoma, hematopoietic malignancy (such as lymphoma or leukemia).

In certain preferred embodiments, the subject is a mammal, such as a human.

In certain preferred embodiments, the modified EV-D68 of the present invention has the internal ribosome entry site (IRES) sequence in the 5'UTR replaced with the internal ribosome entry site sequence of human rhinovirus 2 (HRV2) compared to wild type EV-D68.

In certain preferred embodiments, the modified EV-D68 further comprises an exogenous nucleic acid.

In certain preferred embodiments, the exogenous nucleic acid encodes a cytokine (eg, GM-CSF, preferably human GM-CSF), or an antitumor protein or polypeptide (e.g., a scFv against PD-1 or PD-L1, preferably a scFv against human PD-1 or PD-L1). In certain preferred embodiments, the exogenous nucleic acid is inserted between the 5'UTR and the VP4 gene, or between the VP1 gene and the 2A gene of the genome of the modified EV-D68.

In certain preferred embodiments, the exogenous nucleic acid comprises a target sequence of microRNA (microRNA, miRNA) (eg, miR-133 or miR-206). In certain preferred embodiments, the target sequence of the microRNA is inserted in the 3' untranslated region (3'UTR) of the genome of the modified EV-D68.

In certain preferred embodiments, the exogenous nucleic acid includes a target sequence of one or more (e.g., two, three, or four) microRNAs as described above. In certain preferred embodiments, the exogenous nucleic acid comprises a target sequence of miR-133 and/or miR-206. In certain preferred embodiments, the target sequence of the miR-133 is shown in SEQ ID NO: 3. In certain preferred embodiments, the target sequence of the miR-206 is shown in SEQ ID NO: 4.

In certain preferred embodiments, the modified EV-D68 comprises an insertion of at least one exogenous nucleic acid as described above.

In certain preferred embodiments, the genomic sequence of the modified EV-D68 has at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the nucleotide sequence shown in SEQ ID NO: 13. In certain preferred embodiments, the genomic sequence of the modified EV-D68 is a nucleotide sequence as shown in SEQ ID NO: 13.

In certain preferred embodiments, the cDNA sequence of the modified EV-D68 has at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to the nucleotide sequence shown in SEQ ID NO: 8. In certain preferred embodiments, the cDNA sequence of the modified EV-D68 is a nucleotide sequence as shown in SEQ ID NO: 8.

In certain preferred embodiments, the modified EV-D68 is used to treat a tumor in a subject.

In certain preferred embodiments, the tumor includes, but is not limited to, cervical cancer, ovarian cancer, endometrial cancer, lung cancer, liver cancer, kidney cancer, neuroblastoma, glioma, breast cancer, melanoma, prostate cancer, bladder cancer, pancreatic cancer, gastric cancer, colorectal cancer, esophageal cancer, thyroid cancer, laryngeal cancer, osteosarcoma, hematopoietic malignancy (such as lymphoma or leukemia).

In certain preferred embodiments, the tumor is selected from the group consisting of gastric cancer, endometrial cancer, cervical cancer, and thyroid cancer.

In a fifth aspect, the invention provides an isolated nucleic acid molecule comprising a

sequence selected from:

(1) the genomic sequence or cDNA sequence of the modified EV-D68 according to the fourth aspect; and

(2) a complementary sequence of the genomic sequence or cDNA sequence.

In certain preferred embodiments, the isolated nucleic acid molecule consists of the genomic sequence or cDNA sequence of the modified EV-D68 as described above, or the complementary sequence of the genomic sequence or cDNA sequence.

In certain preferred embodiments, the isolated nucleic acid molecule has the genomic sequence of the modified EV-D68 as described above. In certain preferred embodiments, the isolated nucleic acid molecule is RNA. In certain preferred embodiments, the isolated nucleic acid molecule has the nucleotide sequence as shown in any one of SEQ ID NOs: 12-16.

In certain preferred embodiments, the isolated nucleic acid molecule is a vector (e.g. a cloning vector or an expression vector) comprising a genomic sequence or cDNA sequence of EV-D68 or a modified form thereof as described above, or a complementary sequence of the genomic sequence or cDNA sequence. In certain preferred embodiments, the isolated nucleic acid molecule is a vector (e.g., a cloning vector or an expression vector) comprising a cDNA sequence of EV-D68 or a modified form thereof as described above, or a complementary sequence of the cDNA sequence. In certain preferred embodiments, the isolated nucleic acid molecule is a vector (e.g., a cloning vector or an expression vector) comprising a nucleotide sequence as shown in any one of SEQ ID NOs: 1, 8-11 or a complementary sequence thereof.

In certain preferred embodiments, the isolated nucleic acid molecule comprises a complementary sequence of the genomic sequence of the modified EV-D68 as described above. In certain preferred embodiments, the complementary sequence is complementary to a nucleotide sequence selected from:

(1) a nucleotide sequence as shown in any one of SEQ ID NOs: 13-16; and

(2) a nucleotide sequence having a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to a nucleotide sequence as shown in any one of SEQ ID NOs: 13-16.

In certain preferred embodiments, the isolated nucleic acid molecule comprises the complementary sequence of the cDNA sequence of the modified EV-D68 as described above. In certain preferred embodiments, the complementary sequence is complementary to a nucleotide

sequence selected from:

(1) a nucleotide sequence as shown in any one of SEQ ID NOs: 8-11; and

(2) a nucleotide sequence having a sequence identity of at least 70%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to a nucleotide sequence as shown in any one of SEQ ID NOs: 8-11.

In certain preferred embodiments, the isolated nucleic acid molecule has a nucleotide sequence as shown in SEQ ID NO: 13, or the isolated nucleic acid molecule is a vector (e.g., a cloning vector or an expression vector) comprising a nucleotide sequence as shown in SEQ ID NO: 8 or a complementary sequence thereof.

In the present invention, the isolated nucleic acid molecule can be delivered by any means known in the art, for example, a naked nucleic acid molecule (e.g., naked RNA) can be directly injected, or a non-viral delivery system can be used. The non-viral delivery system can be obtained from a variety of materials well known in the art, including, but not limited to, the materials described in detail in "Yin H, et al. Nat Rev Genet. 2014 Aug; 15 (8): 541- 55." and "Riley MK, Vermerris W. Nanomaterials (Basel). 2017 Apr 28; 7(5). Pii: E94.", which are incorporated herein by reference in their entirety, such as liposomes, inorganic nanoparticles (such as gold nanoparticles), polymers (such as PEG), and so on.

In certain preferred embodiments, the isolated nucleic acid molecule is used to treat a tumor in a subject, or to prepare a medicament for treating a tumor in a subject.

In certain preferred embodiments, the tumor includes, but is not limited to, cervical cancer, ovarian cancer, endometrial cancer, lung cancer, liver cancer, kidney cancer, neuroblastoma, glioma, breast cancer, melanoma, prostate cancer, bladder cancer, pancreatic cancer, gastric cancer, colorectal cancer, esophageal cancer, thyroid cancer, laryngeal cancer, osteosarcoma, hematopoietic malignancy (such as lymphoma or leukemia).

In certain preferred embodiments, the subject is a mammal, such as a human.

In another aspect, the present invention also relates to a pharmaceutical composition comprising the modified EV-D68 according to the fourth aspect, or the isolated nucleic acid molecule according to the fifth aspect.

In another aspect, the present invention also relates to use of the modified EV-D68 according

to the fourth aspect, or the isolated nucleic acid molecule according to the fifth aspect, in treating a tumor in a subject, or in the manufacture of a medicament for treating a tumor in a subject.

In another aspect, the invention also relates to a method for treating a tumor, comprising a step of administering to a subject in need thereof an effective amount of the modified EV-D68 as described in the fourth aspect, or the isolated nucleic acid molecule according to the fifth aspect .

Definition of terms

In the present invention, unless otherwise stated, scientific and technical terms used herein have meanings commonly understood by those skilled in the art. In addition, the laboratory procedures of cell culture, biochemistry, cell biology, nucleic acid chemistry and the like used herein are all routine steps widely used in the corresponding fields. Meanwhile, in order to better understand the present invention, definitions and explanations of related terms are provided below.

As used herein, the term "enterovirus D68 (EV-D68)" refers to one kind of Enterovirus D of the genus *Enteroviruses* of *Picornaviridae* family, the genome of which is a single-stranded positive-sense RNA, consisting of a 5' non-coding region (5'UTR), an open reading frame (ORF), a 3' non-coding region (3'UTR), and a poly(A) tail; wherein its ORF encodes a precursor polyprotein, which can be hydrolyzed and cleaved by its protease to produce structural proteins VP1 to VP4 and non-structural proteins 2A, 2B, 2C, 3A, 3B, 3C and 3D. In order to more clearly describe the present invention, the nucleic acid sequences in the EV-D68 genome corresponding to the above proteins are called VP1 gene, VP2 gene, VP3 gene, VP4 gene, 2A gene, 2B gene, 2C gene, 3A gene, 3B gene, 3C gene, and 3D gene, respectively. In the present invention, the expression "enterovirus D68 (EV-D68)" refers to a wild-type EV-D68, which can be isolated from sources in nature and has not been intentionally modified artificially, examples of which include, but are not limited to, prototype strains AY426531 (CA62-1) and AY426488 (CA62-3), and various clinical isolates (for example, the clinical isolate described in Example 1 of the present invention). The genomic sequence or cDNA sequence of the wild-type EV-D68 is well known in the art and can be found in various public databases (for example, GenBank database, accession number KM881710).

As used herein, the term "modified form" of a virus refers to a modified virus obtained by modifying a wild-type virus, which retains the desired activity (e.g., oncolytic activity) of the wild-type virus. In the present invention, a "modified form" of EV-D68 includes, but is not limited to, a modified EV-D68 virus, the genome sequence of which has a substitution, insertion, or deletion of one or more nucleotides as compared to that of the wild-type EV-D68, and at least retains the

oncolytic activity of EV-D68.

As used herein, the term "oncolytic virus" refers to a virus capable of infecting a tumor cell, replicating in the tumor cell, causing the tumor cell death, lysis, or blocking tumor cell growth. Preferably, the virus has minimal toxic effects on a non-tumor cell.

As used herein, the term "tumor-specific" refers to selectively exhibiting a biological function or activity within a tumor cell. For example, in the present invention, when the term "tumor specificity" is used to describe the killing selectivity of a virus, it means that the virus can selectively kill a tumor cell without killing or substantially killing a non-tumor cell, or the virus is more effective in killing a tumor cell than killing a non-tumor cell.

As used herein, the term "oncolytic activity" primarily includes tumor killing activity. When describing the oncolytic activity of a virus, the oncolytic activity of the virus can typically be measured by indicators such as the virus' ability to infect a tumor cell, ability to replicate in a tumor cell, and/or ability to kill a tumor cell. The oncolytic activity of a virus can be measured using any method known in the art. For example, the ability of a virus to infect a tumor cell can be evaluated by measuring the viral dose required to infect a given percentage of tumor cells (for example, 50% of the cells); the ability to replicate in a tumor cell can be evaluated by measuring the growth of the virus in the tumor cell; the ability to kill a tumor cell can be evaluated by monitoring cytopathic effect (CPE) or measuring tumor cell activity.

As used herein, the expression "cDNA sequence of EV-D68" means the DNA form of the viral genomic RNA sequence, which differs from the RNA sequence only in that the ribonucleotides in the RNA sequence are replaced by corresponding deoxyribonucleotides, for example, uracil ribonucleotides (UMP) are replaced by thymine deoxyribonucleotides (dTMP).

As used herein, the term "exogenous nucleic acid" refers to an artificially introduced nucleotide sequence that is foreign to the original sequence. Exogenous nucleic acid includes, but is not limited to, any gene or nucleotide sequence not found in the viral genome. However, in the present invention, it is particularly preferred that the exogenous nucleic acid is composed of at most 1500, such as at most 1200, and at most 1000 nucleotides. In some cases, preferably, the exogenous nucleic acid encodes a protein or polypeptide having antitumor killing activity, such as a cytokine, or an antitumor protein or polypeptide; or, the exogenous nucleic acid comprises a target sequence of microRNA (miRNA). In the present invention, the microRNA is preferably a microRNA having an expression level in a tumor cell significantly lower than that in a normal cell and/or having obvious tissue specificity. Examples of the microRNA include, but are not limited to, miR-122, miR-192, miR-483, etc., which are specifically expressed in liver tissue; miR-1, miR-133a/b, miR-208, etc., which are specifically expressed in heart; miR-192, miR-196a/b, miR-204,

miR-215, etc., which are specifically expressed in kidney tissue; miR-133a/b, miR-206, etc., which are specifically expressed in muscle tissue; miR-124a, miR-125a/b, miR-128a/b, miR-138, etc., which are specifically expressed in brain tissue; and miR-34, miR-122a, miR-26a, which are under-expressed in liver tumor tissue; miR-34, which is l under-expressed in kidney tumor tissue; miR-143, miR-133a/b, which are under-expressed in bladder tumor tissue; miR-Let-7, miR-29, which are under-expressed in lung tumor tissue; and so on (see, for example, Ruiz AJ and Russell S J. MicroRNAs and oncolytic viruses. [J]. Curr Opin Virol, 2015, 13: 40–48; which is incorporated herein by reference in its entirety).

In the present invention, when the modified EV-D68 comprises the target sequence of microRNA described above, it is regulated by the microRNA in a cell/tissue in which the microRNA is highly expressed or specifically expressed, so that replication of the oncolytic virus is attenuated and even its killing activity is lost, while in a tumor cell/tissue in which the microRNA is under-expressed or even not expressed, the oncolytic virus can normally replicate and thus kill the tumor cell.

As used herein, the term "cytokine" has a meaning well known to those skilled in the art. However, in the present invention, when the oncolytic virus of the present invention is used to treat a tumor, it is particularly preferred that the cytokine is a cytokine that can be used for tumor treatment. Examples of "cytokines" include, but are not limited to, interleukins (e.g., IL-2, IL-12, and IL-15), interferons (e.g., IFNα, IFNβ, IFNγ), tumor necrosis factors (e.g., TNFα), and colony-stimulating factors (e.g., GM-CSF), and any combination thereof (see, for example, Ardolino M, Hsu J, Raulet D H. Cytokine treatment in cancer immunotherapy [J]. Oncotarget, 2015, 6 (23): 19346-19347).

As used herein, the term "antitumor protein or polypeptide" refers to a protein or polypeptide having antineoplastic activity, including but not limited to: (1) proteins or polypeptides having toxicity to cells, capable of inhibiting cell proliferation, or inducing apoptosis, examples thereof include, but are not limited to, thymidine kinase TK (TK/GCV), TRAIL, and FasL (see, for example, Candolfi M, King GD, Muhammad AG, et al. Evaluation of proapototic transgenes to use in combination with Flt3L in an immune-stimulatory gene therapy approach for Glioblastoma multiforme (GBM) [J]. FASEB J, 2008, 22: 1077.13); (2) proteins or polypeptides having immunotherapeutic effects, examples thereof include, but are not limited to, single chain antibody (scFv) against cytotoxic T lymphocyte-associated antigen 4 (anti-CTLA-4), against programmed death receptor 1 (anti-PD-1), and against programmed death ligand 1 (anti-PDL-1) (see, for example, Nolan E, Savas P, Policheni AN, et al. Combined immune checkpoint blockade as a therapeutic strategy for BRCA1-mutated breast cancer [J]. Science Trans Med, 2017, 9: eaal 4922;

which is incorporated herein by reference in its entirety); (3) proteins or polypeptides that inhibit tumor angiogenesis, examples thereof include, but are not limited to, single-chain antibody (scFv) against vascular endothelial growth factor (anti-VEGF), VEGF-derived polypeptides (e.g., $_D$(LPR), KSVRGKGKGQKRKRKKSRYK, etc.) and ATN-161 (see, for example, Rosca EV, Koskimaki JE, Rivera CG, et al. Anti-angiogenic peptides for cancer therapeutics [J]. Curr Pharm Biotechnol, 2011, 12 (8): 1101–1116; which is incorporated herein by reference in its entirety).

As used herein, the term "scFv" refers to a single polypeptide chain comprising a heavy chain variable region (VH) and a light chain variable region (VL), wherein the VL and VH are linked by a linker (see, for example, Bird et al., Science 242: 423-426 (1988); Huston et al., Proc. Natl. Acad. Sci. USA 85: 5879-5883 (1988); and Pluckthun, The Pharmacology of Monoclonal Antibodies, No. Volume 113, edited by Roseburg and Moore, Springer-Verlag, New York, pp. 269-315 (1994)). Such scFv molecule may have a general structure: NH_2-VL-linker-VH-COOH or NH_2-VH-linker-VL-COOH.

As used herein, the term "identity" refers to the match degree between two polypeptides or between two nucleic acids. When two sequences for comparison have the same monomer sub-unit of base or amino acid at a certain site (e.g., each of two DNA molecules has an adenine at a certain site, or each of two proteins/polypeptides has a lysine at a certain site), the two molecules are identical at the site. The percent identity between two sequences is a function of the number of identical sites shared by the two sequences over the total number of sites for comparison x 100. For example, if 6 of 10 sites of two sequences are matched, these two sequences have an identity of 60%. For example, DNA sequences: CTGACT and CAGGTT share an identity of 50% (3 of 6 sites are matched). Generally, the comparison of two sequences is conducted in a manner to produce maximum identity. Such alignment can be conducted by for example using a computer program such as Align program (DNAstar, Inc.) which is based on the method of Needleman, et al. (J. Mol. Biol. 48:443-453, 1970). The percentage of identity between two amino acid sequences can also be determined using the algorithm of E. Meyers and W. Miller (Comput. Appl. Biosci., 4:11-17 (1988)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, and with a gap length penalty of 12 and a gap penalty of 4. In addition, the percentage of identity between two amino acid sequences can be determined by the algorithm of Needleman and Wunsch (J. Mol. Biol. 48:444-453 (1970)) which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossum 62 matrix or a PAM250 matrix, and with a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6.

As used herein, the term "vector" refers to a nucleic acid vehicle into which a polynucleotide

can be inserted. When a vector enables expression of a protein encoded by an inserted polynucleotide, the vector is referred to as an expression vector. A vector can be introduced into a host cell by transformation, transduction, or transfection, so that the genetic material elements carried by the vector can be expressed in the host cell. The vector is well known to those skilled in the art and includes, but is not limited to: plasmids; phagemids; cosmids; artificial chromosomes, such as yeast artificial chromosomes (YAC), bacterial artificial chromosomes (BAC) or P1-derived artificial chromosomes (PAC); bacteriophages such as λ-phage or M13 phage and animal viruses. Animal viruses that can be used as vectors include, but are not limited to, retroviruses (including lentiviruses), adenoviruses, adeno-associated viruses, herpesviruses (such as herpes simplex virus), poxviruses, baculoviruses, papillomaviruses, and papovaviruses (such as SV40). A vector may contain a variety of elements that control expression, including, but not limited to, promoter sequences, transcription initiation sequences, enhancer sequences, elements for selection, and reporter genes. In addition, the vector may contain a replication initiation site.

As used herein, the term "internal ribosome entry site (IRES)" refers to a nucleotide sequence located in a messenger RNA (mRNA) sequence that is capable of initiating translation without the need for the 5' cap structure. IRES is usually located in the 5' untranslated region (5'UTR), but may also be located elsewhere in the mRNA.

As used herein, the term "human rhinovirus 2 (HRV2)" refers to a virus of picornaviridae family, the genomic or cDNA sequence of which is well known in the art and can be found in various public databases (e.g., GenBank database, accession number X02316.1).

As used herein, the expression "a nucleic acid molecule comprising a genomic sequence of EV-D68 or a modified form thereof" or "a nucleic acid molecule comprises a genomic sequence of EV-D68 or a modified form thereof" has the meaning commonly understood by those skilled in the art, that is, when the nucleic acid molecule is DNA, the nucleic acid molecule comprises a genomic sequence of EV-D68 or a modified form thereof in form of DNA; when the nucleic acid molecule is RNA, the nucleic acid molecule comprises a genomic sequence of EV-D68 or a modified form thereof.

As used herein, the term "pharmaceutically acceptable carrier and/or excipient" refers to a carrier and/or excipient that is pharmacologically and/or physiologically compatible with the subject and the active ingredient, which is well known in the art (see, for example, Remington's Pharmaceutical Sciences. Edited by Gennaro AR, 19th ed. Pennsylvania: Mack Publishing Company, 1995), and includes, but is not limited to: pH adjusting agents, surfactants, ionic strength enhancers, agents to maintain osmotic pressure, agents to delay absorption, diluents, adjuvants, preservatives, stabilizers, etc. For example, pH adjusting agents include, but are not

limited to, phosphate buffered saline. Surfactants include, but are not limited to, cationic, anionic or non-ionic surfactants, such as Tween-80. Ionic strength enhancers include, but are not limited to, sodium chloride. Agents that maintain osmotic pressure include, but are not limited to, sugar, NaCl, and the like. Agents that delay absorption include, but are not limited to, monostearate and gelatin. Diluents include, but are not limited to, water, aqueous buffers (such as buffered saline), alcohols and polyols (such as glycerol), and the like. Adjuvants include, but are not limited to, aluminum adjuvants (such as aluminum hydroxide), Freund's adjuvants (such as complete Freund's adjuvant), and the like. Preservatives include, but are not limited to, various antibacterial and antifungal agents, such as thimerosal, 2-phenoxyethanol, parabens, trichloro-t-butanol, phenol, sorbic acid, and the like. Stabilizers have the meaning commonly understood by those skilled in the art, which can stabilize the desired activity (such as oncolytic activity) of the active ingredients in the drug, including but not limited to sodium glutamate, gelatin, SPGA, sugars (e.g., sorbitol, mannitol, starch, sucrose, lactose, dextran, or glucose), amino acids (e.g., glutamic acid, glycine), proteins (e.g., dried whey, albumin, or casein) or their degradation products (e.g., lactalbumin hydrolysates).

As used herein, the term "treating" refers to treating or curing a disease (e.g., a tumor), delaying the onset of symptoms of a disease (e.g., a tumor), and/or delaying the development of a disease (e.g., a tumor).

As used herein, the term "effective amount" refers to an amount that can effectively achieve the intended purpose. For example, a therapeutically effective amount can be an amount effective or sufficient to treat or cure a disease (e.g., a tumor), delay the onset of symptoms of a disease (e.g., a tumor), and/or delay the development of a disease (e.g., a tumor). Such an effective amount can be easily determined by a person skilled in the art or a doctor, and can be related to the intended purpose (such as treatment), the general health condition, age, gender, weight of the subject, severity, complications, administration route of the disease to be treated. The determination of such an effective amount is well within the capabilities of those skilled in the art.

As used herein, the term "subject" refers to a mammal, such as a primate mammal, such as a human. In certain embodiments, the subject (e.g., a human) has a tumor, or is at risk for having a tumor.

The beneficial effects of the present invention

Compared with the prior art, the technical solution of the present invention has at least the following beneficial effects:

The inventors of the present application have found for the first time that enterovirus D68 (EV-D68) has broad-spectrum tumor-killing activity. Based on this finding, the present invention further provides an EV-D68-based oncolytic virus, which has a broader-spectrum tumor-killing activity and higher tumor specificity, especially also has a very high killing effect to hematopoietic malignancy (such as lymphoma or leukemia), thus can be used alone for the treatment of tumors, and can also be used as a supplementary method of traditional tumor treatment, or as a treatment in the absence of other treatment methods.

The EV-D68 or a modified form thereof of the present invention has little or no effect on normal cells, and does not induce an immunogenic response against the virus in a subject (for example, a human), and thus can be safely administered to a subject (for example, a human). Therefore, the EV-D68 or a modified form thereof of the present invention has great clinical value.

The embodiments of the present invention will be described in detail below with reference to the drawings and examples, but those skilled in the art will understand that the following drawings and examples are only used to illustrate the present invention, rather than limiting the scope of the present invention. Various objects and advantageous aspects of the present invention will become apparent to those skilled in the art from the following detailed description of drawings and the preferred embodiments.

Description of the Drawings

FIG. 1 shows photomicrographs of the in vitro killing tests of the wild-type EV-D68 on human umbilical vein endothelial cell line HUVEC, human esophageal cancer cell line TE-1, human thyroid cancer cell lines SW-579 and TT in Example 2, wherein MOCK represents cells that are not infected with the virus. The results showed that the EV-D68 had a significant oncolytic effect on human tumor cell lines TE-1, SW-579, and TT after 72 hours of infection at a multiplicity of infection (MOI) of 10, but had no effect on HUVEC of human normal cells.

FIG. 2 shows the photos of crystal violet staining of the in vitro killing tests of the wild-type EV-D68 on human liver cancer cell lines HepG2, SMMC7721, BEL7404, BEL7402, and Huh7, human cervical cancer cell lines Hela and Caski, human lung cancer cell lines NCI-H1299 and A549, human foreskin fibroblast cell line HFF-1, human embryonic kidney cell line HEK-293, and differentiated human liver progenitor cell line HepaRG in Example 2, wherein MOCK represents cells that are not infected with the virus. The results showed that the EV-D68 had significant oncolytic effects on human tumor cell lines HepG2, SMMC7721, BEL7404, BEL7402,

Huh7, Hela, Caski, NCI-H1299 and A549 after 72 hours of infection at MOIs of 10, 1, and 0.1, but had limited effect on HFF-1, HEK-293 and differentiated HepaRG of human normal cells.

FIG. 3 shows an electrophoresis image of four samples of wild-type EV-D68 virus genomic RNA of the same batch obtained by the in vitro transcription method in Example 2.

FIG. 4 shows the killing effect of the wild-type EV-D68 virus genomic RNA on human cervical cancer tumor cell line Hela in Example 2. The results showed that Hela cells showed obvious CPE after 24 hours of transfection with EV-D68 genomic RNA, and were almost all lysed to death by 48 hours.

FIGs. 5A to 5C show the results of in vivo antitumor experiment of the wild-type EV-D68 in Example 3 on human cervical cancer cell line Hela (A), human glioma cell line U118-MG (B), and human lymphoma cell line Raji (C). The results showed that, in the challenge experimental group, 10^6 TCID50 per tumor mass of EV-D68 were injected intratumorally every third day. After 5 treatments in total, the growth of tumors formed by subcutaneous inoculation of Hela, U118-MG, or Raji cells in SCID mice significantly slowed down and arrested, and the tumors were even lysed and disappeared. In contrast, the tumors of the negative group (CTRL) without treatment of oncolytic virus maintained the normal growth, and their tumor volumes are significantly larger than those in the challenge group.

FIG. 6 shows the results of toxicity detection of EV-D68-WT in BALB/c mice in Example 4. FIG. 6A shows the survival rates and health scores of 1-day-old BALB/c mice after challenge with EV-D68 at different doses (10^3, 10^4, 10^5, 10^6, and 10^7 TCID50/mouse) by intraperitoneal injection; FIG. 6B shows the survival rates and health scores of BALB/c mice of different ages (1-day-old, 2-day-old, 3-day-old, 7-day-old and 14-day-old) challenged with a very high dose (10^7 TCID50/mouse) by intraperitoneal injection. The overall toxicity of EV-D68 to BALB/c mice was relatively weak, and only high doses caused the death of 1-day-old to 3-day-old BALB/c mice, but had no effect on 4- or more-day-old BALB/c mice, indicating that EV-D68 had good safety in vivo.

Sequence information

Information of a part of sequences involved in the present invention is provided in Table 1 as below.

Table 1: Sequence description

SEQ ID	Description

NO:	
1	cDNA sequence of wild type EV-D68 (EV-D68-WT)
2	RNA sequence of the internal ribosome entry site of human rhinovirus 2 (HRV2)
3	RNA sequence of the target sequence of miR-133
4	RNA sequence of the target sequence of miR-206
5	RNA sequence of tandem sequence of miR-133 target sequence and miR-206 target sequence
6	DNA sequence of human granulocyte-macrophage colony-stimulating factor (GM-CSF) gene
7	DNA sequence of anti-human programmed death receptor 1 single chain antibody (Anti-PD-1 scFv)
8	cDNA sequence of the modified form of EV-D68 (EV-D68-HRV2)
9	cDNA sequence of the modified form of EV-D68 (EV-D68-miR133&206T)
10	cDNA sequence of the modified form of EV-D68 (EV-D68-GM-CSF)
11	cDNA sequence of the modified form of EV-D68 (EV-D68-Anti-PD1)
12	Genomic sequence of wild-type EV-D68 (EV-D68-WT)
13	Genomic sequence of the modified form of EV-D68 (EV-D68-HRV2)
14	Genomic sequence of the modified form of EV-D68 (EV-D68-miR133 & 206T)
15	Genomic sequence of the modified form of EV-D68 (EV-D68-GM-CSF)
16	Genomic sequence of the modified form of EV-D68 (EV-D68-Anti-PD1)
17	DNA sequence of miR-133 target sequence
18	DNA sequence of miR-206 target sequence
19	DNA sequence of tandem sequence of miR-133 target sequence and miR-206 target sequence
20	DNA sequence of the internal ribosome entry site sequence of human rhinovirus 2 (HRV2)

Specific Models for Carrying Out the Invention

The present invention is now be described with reference to the following examples which are intended to illustrate the present invention (rather than to limit the present invention).

Unless otherwise specified, the molecular biology experimental methods and immunoassays used in the present invention were carried out substantially by referring to the methods described

in J. Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, 1989, and F. M. Ausubel et al., Short Protocols in Molecular Biology, 3rd Edition, John Wiley & Sons, Inc., 1995; restriction enzymes were used under conditions recommended by the product manufacturer. If the specific conditions were not indicated in the examples, the conventional conditions or the conditions recommended by the manufacturer were used. If the reagents or instruments used were not specified by the manufacturer, they were all conventional products that were commercially available. Those skilled in the art will understand that the examples describe the present invention by way of examples, and are not intended to limit the scope of protection claimed by the present invention. All publications and other references mentioned herein are incorporated by reference in their entirety.

Example 1: Obtainment and preparation of EV-D68 and its modified form

1.1 Isolation of enterovirus EV-D68 from patient clinical sample

(1) A throat swab of patient was gained from the Center for Disease Control and Prevention of Xiamen City, China; African green monkey kidney cells (Vero cells; ATCC® Number: CCL-81™) were was kept by the National Institute of Diagnostics and Vaccine Development in Infectious Diseases, Xiamen University, China, and cultured in MEM medium containing 10% fetal bovine serum, as well as glutamine, penicillin and streptomycin.

(2) Sample processing: the throat swab of patient was sufficiently agitated in a sample preservation solution to wash off the virus and virus-containing cells adhering to the swab, and then the sample preservation solution was subjected to a high speed centrifugation at 4000 rpm and 4 °C for 30min;

(3) Inoculation and observation:

A) The Vero cells were plated in a 24-well plate with 1×10^5 cells/well. The growth medium (MEM medium, containing 10% fetal bovine serum, as well as glutamine, penicillin and streptomycin) was aspirated, and 1 mL of maintenance medium (MEM medium, containing 2% fetal calf serum, as well as glutamine, penicillin and streptomycin) was added in each well. Then except the negative control wells, each well was inoculated with 50 μL of the sample supernatant, and cultured in an incubator at 37 °C, 5% CO_2.

B) The cells were observed under a microscope every day for one week, and the occurrence of specific cytopathic effect (CPE) in the inoculated wells was recorded.

C) If the enterovirus-specific cytopathic effect appeared in the cells in the inoculated wells

within 7 days, the cells and supernatant were collected and frozen at -80 °C; if no CPE appeared after 7 days, the cells were subjected to blind passage.

D) If CPE appeared within 6 blind passages, the cells and supernatant were collected and frozen at -80 °C; If CPE did not appear after 6 blind passages, the cells were determined as negative.

(4) Isolation and cloning of viruses:

RT-PCR (Piralla et al., J Clin Microbiol 2015, 53 (5): 1725-1726) and enzyme-linked immunospot method based on specific antibody (Yang et al., Clin Vaccine Immunol 2014, 21 (3): 312 -320; Hou et al., J Virol Methods 2015, 215-216: 56-60) were used to identify the viruses isolated from the clinical sample, and EV-D68 positive cultures were selected and subjected to at least 3 cloning experiments. The virus clones obtained by the limiting dilution method in each experiment were also identified by RT-PCR and ELISPOT, and the EV-D68 positive clones were selected for the next round of cloning. A single EV-D68 strain with strong growth viability was selected as a candidate oncolytic virus strain.

1.2 Rescued strain of enterovirus EV-D68 and its modified form obtained by infectious cloning and reverse genetics technology

This example used wild-type EV-D68 (SEQ ID NO: 1) as an example to show how to obtain EV-D68 and its modified form for the present invention through reverse genetics technology. The specific method was as follows.

(1) Construction of viral infectious clone: the cDNA sequence of wild-type enterovirus EV-D68 (named EV-D68-WT) was shown in SEQ ID NO: 1, and its genomic RNA sequence was SEQ ID NO: 12; or gene insertion or replacement based on the cDNA (SEQ ID NO: 1) of enterovirus EV-D68 was performed, comprising:

Modified form 1: the internal ribosome entry site sequence of wild-type EV-D68 was replaced with the internal ribosome entry site sequence of human rhinovirus 2 (which has a DNA sequence shown in SEQ ID NO: 20) to obtain the cDNA (SEQ ID NO: 8) of the recombinant virus (named as EV-D68-HRV2), which has a genomic RNA sequence shown as SEQ ID NO: 13;

Modified form 2: the tandem sequence (which has a DNA sequence shown in SEQ ID NO: 19) of miR-133 target sequence (which has a DNA sequence shown in SEQ ID NO: 17) and miR-206 target sequence (which has a DNA sequence shown in SEQ ID NO: 18) was inserted between 7293-7294 bp of the 3' untranslated region of the cDNA (SEQ ID NO: 1) of the wild-type EV-D68, to obtain the cDNA (SEQ ID NO : 9) of the recombinant virus (named EV-D68-miR133&206T) , which has a genomic RNA sequence shown as SEQ ID NO: 14;

Modified form 3: the human granulocyte-macrophage colony-stimulating factor (GM-CSF) gene (SEQ ID NO: 6) was inserted between the VP1 gene and 2A gene of the cDNA (SEQ ID NO: 1) of wild-type EV-D68 to obtain the cDNA (SEQ ID NO: 10) of the recombinant virus (named EV-D68-GM-CSF), which has a genomic RNA sequence shown as SEQ ID NO: 15;

Modified form 4: the sequence (SEQ ID NO: 7) encoding the single chain antibody against human programmed death receptor 1 (Anti-PD-1 scFv) was inserted between the VP1 gene and 2A gene of the cDNA (SEQ ID NO: 1) of wild-type EV-D68 to obtain the cDNA (SEQ ID NO: 11) of the recombinant virus (named EV-D68-Anti-PD-1), which has a genomic RNA sequence shown as SEQ ID NO: 16.

Then, the cDNA sequences (SEQ ID NO: 1, 8-11) of the above five oncolytic viruses were sent to the gene synthesis company (Shanghai Biotech Engineering Co., Ltd.) for full gene synthesis, and ligated into the pSVA plasmid (Hou et al. Virus Res 2015, 205: 41-44; Yang et al., Virus Res 2015, 210: 165-168) to obtain the infectious cloning plasmids of enterovirus EV-D68 or modified forms thereof (i.e., EV-D68-WT, EV-D68-HRV2, EV-D68-miR133&206T, EV-D68-GM-CSF and EV-D68-Anti-PD-1).

(2) Plasmid mini-kit and *E coli.* DH5α competent cells were purchased from Beijing Tiangen Biochemical Technology Co., Ltd.; Hela cells (ATCC® Number: CCL-2™) and human rhabdomyosarcoma cells (RD cells; ATCC® Number: CCL-136™) were kept by National Institute of Diagnostics and Vaccine Development in Infectious Diseases, Xiamen University, China, and were cultured with DMEM and MEM media respectively, in which 10% fetal bovine serum as well as glutamine, penicillin and streptomycin were added; transfection reagents Lipofactamine2000 and Opti-MEM were purchased from Thermo Fisher Scientific Company.

(3) The infectious cloning plasmids containing the cDNA sequences of the above five oncolytic viruses were transformed into *E coli* DH5α competent cells, the monoclonal strains were picked out and shaken after the outgrowth of clones, and the plasmids were extracted using the plasmid mini-kit, and then sent to the company (Shanghai Biotech Engineering Co., Ltd.) for sequencing analysis.

(4) The infectious cloning plasmids with correct sequence and the helper plasmid pAR3126 were co-transfected into the cells to rescue virus (Hou et al. Virus Res 2015, 205: 41-44; Yang et al. Virus Res 2015, 210: 165-168). Hela cells were first transfected according to the instructions of the transfection reagent; then observed under a microscope. When CPE appeared in Hela cells, the cells and culture supernatant were harvested, and inoculated with RD cells followed by passaging and culturing, thereby obtaining the candidate strain of oncolytic virus.

Example 2: In vitro antitumor experiment of EV-D68 and modified form thereof

2.1 Viruses and cell lines as used

(1) Viruses: this example used EV-D68-WT (SEQ ID NO: 12), EV-D68-HRV2 (SEQ ID NO: 13), EV-D68-miR133&206T (SEQ ID NO: 14), EV-D68-GM-CSF (SEQ ID NO: 15) and EV-D68-Anti-PD-1 (SEQ ID NO: 16) as provided in Example 1.

(2) Cell lines: human rhabdomyosarcoma cell RD (ATCC® Number: CCL-136™); human cervical cancer cell lines Hela (ATCC® Number: CCL-2™), SiHa (ATCC® Number: HTB-35™), Caski (ATCC® Number: CRL-1550™) and C-33A (ATCC® Number: HTB-31™); human ovarian cancer cell lines SKOV-3/TR (drug-resistant strain of SKOV-3), SKOV-3 (ATCC® Number: HTB-77™) and Caov3 (ATCC® Number: HTB-75™); human endometrial cancer cell lines Hec-1-A (ATCC® Number: HTB-112™), Hec-1-B (ATCC® Number: HTB-113™) and Ishikawa (ECACC No. 99040201); human lung cancer cell lines SPC-A-1 (CCTCC Deposit Number: GDC050), NCI-H1299 (ATCC® Number: CRL-5803™), NCI-H1417 (ATCC® Number: CRL-5869™), NCI-H1703 (ATCC® Number: CRL-5889™), NCI-H1975 (ATCC® Number: CRL-5908™), A549 (ATCC® Number: CCL-185™), NCI-H661 (ATCC® Number: HTB-183™), EBC-1 (Thermo Fisher Scientific, Catalog #: 11875101), and DMS114 (ATCC® Number: CRL-2066™); human liver cancer cell lines MHCC97H (purchased from the Institute of Liver Cancer, Fudan University), C3A (ATCC® Number: CRL-10741™), Hep3B (ATCC® Number: HB-8064™), HepG2 (ATCC® Number: HB-8065™), SMMC7721 (purchased from the Basic Medical Cell Center of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, number: 3111C0001CCC000087), BEL7402 (CCTCC Deposit Number: GDC035), BEL7404 (purchased from the Cell Resource Center, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, number: 3131C0001000700064), Huh7 (CCTCC Deposit Number: GDC134), PLC/PRF/5 (ATCC® Number: CRL-8024™) and SK-Hep-1 (ATCC® Number: HTB-52™); human kidney cancer cell lines A-498 (ATCC® Number: HTB-44™), 786-O (ATCC® Number: CRL-1932™) and Caki-1 (ATCC® Number: HTB-46™); human neuroblastoma cell lines SH-SY5Y (ATCC® Number: CRL-2266™) and SK-N-BE (2) (ATCC® Number: CRL-2271™); human glioma cell lines U87-MG (ATCC® Number: HTB-14™) and U118-MG (ATCC® Number: HTB-15™); human breast cancer cell lines MCF-7 (ATCC® Number: HTB-22™), BcaP37 (CCTCC Deposit Number: GDC206), BT-474 (ATCC® Number: HTB-20™), MDA-MB-231 (ATCC® Number: CRM-HTB-26™) and MDA-MB-453 (ATCC® Number: HTB-131™); human melanoma cell lines A-375 (ATCC® Number: CRL-1619™), SK-MEL-1 (ATCC® Number: HTB-67™) and MeWo (ATCC® Number: HTB-65™);

human prostate cancer cell lines PC-3 (ATCC® Number: CRL-1435™), LNCap (ATCC® Number: CRL-1740™) and DU145 (ATCC® Number: HTB-81™); human bladder cancer cell lines J82 (ATCC® Number: HTB-1™) and 5637 (ATCC® Number: HTB-9™); human pancreatic cancer cell lines Capan-2 (ATCC® Number: HTB-80™), HPAF-2 (ATCC® Number: CRL-1997™), and PANC-1 (ATCC® Number: CRL-1469™); human gastric cancer cell lines AGS (ATCC® Number: CRL-1739™), SGC7901 (CCTCC Deposit Number: GDC150), BGC823 (CCTCC Deposit Number: GDC151), and NCI-N87 (ATCC® Number: CRL-5822™); human colorectal cancer cell lines DLD-1 (ATCC® Number: CCL-221™), SW1116 (ATCC® Number: CCL-233™), SW480 (ATCC® Number: CCL-228™), HCT-116 (ATCC® Number: CCL-247™) and HT-29 (ATCC® Number: HTB-38™); human esophageal cancer cell line TE-1 (purchased from the Cell Resource Center, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, No. 3131C0001000700089); human thyroid cancer SW-579 (ATCC® Number: HTB-107™) and TT (ATCC® Number: CRL-1803™); human laryngeal cancer Hep-2 (ATCC® Number: CCL-23™); osteosarcoma 143B (ATCC® Number: CRL-8303™) and U2OS (ATCC® Number: HTB-96™); human lymphoma and leukemia cell lines K562 (ATCC® Number: CCL-243™), U937 (ATCC® Number: CRL-1593.2™), THP-1 (ATCC® Number: TIB-202™), Raji (ATCC® Number: CCL-86™), Daudi (ATCC® Number: CCL-213™), Jurkat (ATCC® Number: TIB-152™) and MT-4 (obtained from the National Institutes of Health, USA); human normal cell lines include: human embryo lung fibroblast cell line MRC-5 (ATCC® Number: CCL-171™), human embryonic kidney cell line HEK-293 (ATCC® Number: CRL-1573™), human foreskin fibroblast cell line HFF-1 (ATCC® Number: SCRC-1041™), human skin keratinocyte cell line HaCat (CCTCC Deposit Number: GDC106), human prostate stromal cell line WPMY-1 (ATCC® Number: CRL-2854™), human umbilical vein endothelial cell line HUVEC (Thermo Fisher Scientific, Catalog #: C01510C), and differentiated human liver progenitor cell line HepaRG (with characteristics of primary hepatocytes; Thermo Fisher Scientific, Catalog #: HPRGC10). The above cells were kept by National Institute of Diagnostics and Vaccine Development in Infectious Diseases, Xiamen University, China. HepaRG cells were cultured in WME medium (added with 1.5% DMSO), AGS and TT were cultured with F-12K medium, SW-579 was cultured with L-15 medium, SH-SY5Y and SK-N-BE (2) were cultured with DMEM:F12 (1:1) medium, RD, C-33A, EBC-1, J82, SK-Hep-1, SK-MEL-1 and DU145 were cultured with MEM medium, K562, U937, THP-1, Raji, Daudi, Jurkat, MT-4, 5637, 786-O, TE-1, Caski, NCI-H1417, NCI-H1703, NCI-H1975, NCI-H661, SGC7901, BGC823, DLD-1, SW1116, Hep-2, and LNCap were cultured with RPMI-1640 medium, other cells were cultured with DMEM medium. All the mediums mentioned above were supplemented with 10% fetal bovine serum, glutamine and penicillin-streptomycin. All the above cells were cultured under the standard conditions of 37 °C and 5% CO_2.

2.2 Virus culture

RD cells were evenly plated on 10 cm cell culture plates, and the culture conditions included MEM medium containing 10% fetal bovine serum and glutamine, penicillin and streptomycin, 37 °C, 5% CO_2, and saturated humidity. When the cell confluence reached 90% or more, the cell culture medium was replaced with serum-free MEM medium, and each plate was inoculated with 10^7 TCID50 of EV-D68-WT, EV-D68-HRV2, EV-D68-miR133&206T, EV-D68-GM-CSF or EV-D68-Anti-PD-1, the culture environment was changed to 33 °C, 5% CO_2, saturated humidity. After 24 hours, the EV-D68 or its modified form proliferated in RD cells and caused CPE in cells. When more than 90% of the cells turned contracted and rounded, showed increased graininess, and became detached and lysed, the cells and culture supernatants thereof were harvested. After freeze-thawing for three cycles, the culture supernatant was collected and centrifuged to remove cell debris, wherein the centrifuge conditions were 4000 rpm, 10min, 4 °C. Finally, the supernatant was filtered with a 0.22 μm disposable filter (Millipore Company) to remove impurities such as cell debris.

2.3 Determination of virus titer

The RD cells were plated in a 96-well plate with a cell density of 10^4 cells/well. After the cells adhered, the virus solution obtained in Example 2.2 was diluted 10-fold with serum-free MEM medium from the first 10-fold dilution. 50 μl of the dilution of virus was added to the wells with cells. After 7 days, the wells where CPE appeared were monitored and recorded, followed by calculation using Karber method, in which the calculation formula was lg^{TCID50} = L - D (S - 0.5), L: logarithm of the highest dilution, D: difference between the logarithms of dilutions, S: sum of proportions of positive wells. The unit of TCID50 thus calculated was TCID50/50μl, which should be converted to TCID50/ml.

2.4 In vitro antitumor experiment of viruses

Human tumor cells and normal cells were inoculated into 96-well plates at 10^4 cells/well. After the cells adhered, the medium in each well was replaced with the corresponding cell culture medium without serum, and viruses were inoculated at an MOI of 0.1, 1, 10 or 100. Subsequently, CPE of the cells were monitored daily by a microscope.

FIG. 1 shows micrographs of the human umbilical vein endothelial cell line HUVEC, the human esophageal cancer cell line TE-1, and the human thyroid cancer cell line SW-579 and TT, which were not infected with viruses (negative control group, Mock) or which were treated with EV-D68-WT at MOI = 10 for 72 hours. The results showed that after 72 hours of infection at a

multiplicity of infection (MOI) of 10, a significant reduction in the number of the tumor cells, marked shrinking and lysis and the like, were detected in the virus-infected groups; while as compared to the non-tumor cells in the Mock group, the non-tumor cells infected with the viruses showed almost no change in cell morphology. The above results demonstrated that EV-D68 had significant oncolytic effects on human tumor cell lines TE-1, SW-579 and TT, but did not have any effect on non-tumor cells HUVEC.

After 72 hours of virus infection and culture, the cell survival rate was detected using Cell Counting Kit-8 (CCK-8 kit; Shanghai Biyuntian Biotechnology Co., Ltd.) and crystal violet staining method (only for adherent cells), and the specific method was as follows:

(1) Cell survival rate detected by CCK8 method

For adherent cells, the original medium in a 96-well cell culture plate was directly discarded; for suspension cells, the original medium in a 96-well cell culture plate was carefully discarded after centrifugation; and then 100 μl of fresh serum-free medium was added per well. 10 μl of CCK-8 solution was added to each of the wells inoculated with cells, and an equal amount of CCK-8 solution was also added to the blank culture medium as a negative control, followed by incubation at 37 °C in a cell culture incubator for 0.5-3 hours. The absorbance was detected at 450 nm using a microplate reader at 0.5, 1, 2, 3 hours, respectively, and the time point where the absorbance was within a suitable range was selected as a reference for cell survival rate. The CCK-8 test results of EV-D68-WT for each kind of cells were shown in Table 2, where "-" indicated that the cell survival rate after virus treatment was not significantly different from that of the MOCK group; "+" indicated that after virus treatment, the cell number was reduced, the survival rate was still greater than 50% but was significantly different from that of the MOCK group; "++" indicated that the cell survival rate after virus treatment was less than 50%, and was significantly different from that of the MOCK group.

The calculation of cell survival rate was:

$$Cell\_survival\_rate(\%) = \frac{(reading\_of\_test\_group - reading\_of\_negative\_group)}{(reading\_of\_positive\_group - reading\_of\_negative\_group)} \times 100\% .$$

(2) Cell survival rate detected by crystal violet staining method (only for adherent cells)

After the cells were infected with viruses for 3 days, the culture supernatant in the 96-well cell culture plate was discarded, 100 μl of methanol was added to each well, followed by fixation in the dark for 15 min. Crystal violet powder (Shanghai Biotech Biotechnology Co., Ltd.) was weighed, and formulated as 2% (w/v) crystal violet methanol solution, which was stored at 4 °C. An appropriate amount of 2% crystal violet methanol solution was taken and formulated with PBS

solution to prepare 0.2% crystal violet working solution. After fixation for 15 minutes, the methanol fixation solution in the 96-well cell culture plate was discarded, and 100μl of the crystal violet working solution was added to the plate and staining was performed for 30min. After the crystal violet staining solution was discarded, PBS solution was used for washing for 3 to 5 times, until the excess staining solution was washed off, and air-drying was performed. ImmunSpot @ S5 UV Analyzer (Cellular Technology Limited, USA) was used for photographing. FIG. 2 showed the crystal violet staining results of the human liver cancer cell lines HepG2, SMMC7721, BEL7404, BEL7402 and Huh7, the human cervical cancer cell lines Hela and Caski, the human lung cancer cell lines NCI-H1299 and A549, the human foreskin fibroblast cell line HFF-1, the human embryonic kidney cell line HEK-293, and the differentiated human hepatic progenitor cell line HepaRG in the control group (MOCK) and in the experimental groups (infected for 72 hours with EV-D68-WT at MOIs of 0.1, 1, and 10, respectively). As shown in the results, after 72 hours of infection at MOIs of 10, 1, and 0.1, the tumor cells in the experimental groups were significantly reduced as compared to the control group (MOCK) without addition of virus; while the number of non-tumor cells showed no significant change. The above results indicated that the EV-D68-WT had significant oncolytic effects on human tumor cell lines HepG2, SMMC7721, BEL7404, BEL7402, Huh7, Hela, Caski, NCI-H1299 and A549, but had no significant effect on non-tumor cell lines HFF-1, HEK-293 and the differentiated HepaRG.

Table 2: Results of in vitro antitumor experiment of wild-type enterovirus EV-D68

Multiplicity of infection MOI / Cell Line	0.1	1	10	100
RD	++	++	++	++
Hela	++	++	++	++
SiHa	-	-	++	++
Caski	-	+	++	++
C-33A	-	++	++	++
SKOV-3/TR	-	-	-	+
SKOV-3	-	-	++	++
Caov3	+	++	++	++
Hec-1-A	-	-	-	++
Hec-1-B	-	+	++	++
Ishikawa	-	-	++	++
SPC-A-1	-	+	++	++
NCI-H1299	-	++	++	++
NCI-H1417	-	-	-	+
NCI-H1703	-	-	-	+
NCI-H1975	-	++	++	++
A549	+	++	++	++
NCI-H661	-	-	+	++
EBC-1	-	-	+	++
DMS114	++	++	++	++

MHCC97H	+	++	++	++
C3A	++	++	++	++
Hep3B	-	+	+	++
HepG2	-	++	++	++
SMMC7721	+	++	++	++
BEL7402	++	++	++	++
BEL7404	+	++	++	++
Huh7	++	++	++	++
PLC/PRF/5	-	+	++	++
SK-Hep-1	-	-	+	++
A-498	+	++	++	++
786-O	-	-	+	++
Caki-1	++	++	++	++
SH-SY5Y	-	+	++	++
SK-N-BE(2)	-	-	-	+
U87-MG	+	++	++	++
U118-MG	++	++	++	++
MCF-7	-	-	-	+
BcaP37	-	++	++	++
BT-474	-	-	-	+
MDA-MB-231	++	++	++	++
MDA-MB-453	-	-	+	++
A-375	-	+	++	++
SK-MEL-1	+	++	++	++
MeWo	-	+	++	++
PC-3	++	++	++	++
LNCap	-	+	++	++
DU145	++	++	++	++
J82	-	+	++	++
5637	-	-	-	+
Capan-2	-	-	+	++
HPAF-2	-	+	+	++
PANC-1	-	++	++	++
AGS	-	-	+	++
SGC7901	-	-	-	+
BGC823	-	+	+	++
NCI-N87	-	+	++	++
DLD-1	-	-	-	+
SW1116	+	+	++	++
SW480	-	+	++	++
HCT-116	-	-	+	++
HT-29	+	++	++	++
TE-1	-	+	++	++
SW-579	-	-	++	++
TT	-	-	++	++
Hep-2	-	+	++	++
143B	-	-	-	+
U2OS	+	+	++	++
K562	-	+	+	++
U937	-	-	+	++
THP-1	+	++	++	++
Raji	++	++	++	++
Daudi	++	++	++	++
Jurkat	++	++	++	++
MT-4	++	++	++	++

MRC-5	-	-	+	++
HEK-293	-	-	-	-
HFF-1	-	-	+	+
HaCat	-	-	-	-
WPMY-1	-	-	-	-
HUVEC	-	-	-	-
HepaRG	-	-	-	+

Note: "-" indicated that there was no significant difference in cell survival rate between virus treatment group and MOCK group; "+" indicated that after virus treatment, the number of cells was reduced, the survival rate was greater than 50% but was significantly different from that of MOCK group; "++" indicated that the cell survival rate after virus treatment was less than 50%, and was significantly different from that of the MOCK group.

It could be known from Table 2 that the wild-type enterovirus EV-D68 had a killing effect on the tested tumor cells, and therefore had a broad-spectrum anti-tumor activity. In particular, the virus had significant killing effects on liver cancer cell lines, glioma cell lines, prostate cancer cell lines, leukemia and lymphoma cell lines. On the other hand, the virus had little or no toxicity to the non-tumor cell lines tested, except that it was significantly toxic to human embryonic lung fibroblast MRC-5 at higher MOIs.

In addition, in vitro antitumor experiments of EV-D68-HRV2, EV-D68-miR133&206T, EV-D68-GM-CSF and EV-D68-Anti-PD-1 showed that the four modified EV-D68s retained the broad-spectrum killing effect of the wild-type enterovirus EV-D68 on the tested tumor cells, and substantially retained the significant killing effect on the tested tumor cells of human hepatocellular carcinoma cell line, prostate cancer cell line, leukemia and lymphoma cell lines, wherein the CCK-8 test results of oncolytic effect of the four modified EV-D68s on cervical cancer cell line Hela, glioma cell line U118-MG, liver cancer cell line Huh7, prostate cancer cell line PC-3, and lymphoma cell line Raji were shown in Table 3.

Table 3: Results of in vitro antitumor experiment of EV-D68-HRV2, EV-D68-miR133&206T, EV-D68-GM-CSF and EV-D68-Anti-PD-1

Multiplicity of infection MOI / Cell Lines		0.1	1	10	100
EV-D68-HRV2	Hela	+	+	++	++
	U118-MG	+	++	++	++
	Huh7	++	++	++	++
	PC-3	++	++	++	++
	Raji	-	+	+	++
EV-D68-miR133&206T	Hela	++	++	++	++
	U118-MG	++	++	++	++

	Huh7	++	++	++	++
	PC-3	++	++	++	++
	Raji	++	++	++	++
EV-D68-GM-CSF	Hela	++	++	++	++
	U118-MG	++	++	++	++
	Huh7	++	++	++	++
	PC-3	++	++	++	++
	Raji	++	++	++	++
EV-D68-Anti-PD-1	Hela	++	++	++	++
	U118-MG	++	++	++	++
	Huh7	++	++	++	++
	PC-3	++	++	++	++
	Raji	++	++	++	++

Note: "-" indicated that there was no significant difference in cell survival rate between virus treatment group and MOCK group; "+" indicated that after virus treatment, the number of cells was reduced, the survival rate was greater than 50% but was significantly different from that of MOCK group; "++" indicated that the cell survival rate after virus treatment was less than 50%, and was significantly different from that of the MOCK group.

In addition, the inventors unexpectedly found that EV-D68-HRV2 exhibited significantly improved killing activity on some tumors compared to EV-D68-WT, wherein the CCK-8 test results of the oncolytic activity of the human gastric cancer cell line AGS, the human endometrial cancer cell lines HEC-1-A and Ishikawa, the human cervical cancer cell line C-33A, and the human thyroid cancer cell line SW579 were shown in Table 4.

Table 4: Comparison of the results of in vitro oncolytic experiment of EV-D68-WT and EV-D68-HRV2 on some tumor cells

MOI / Cell Line		0.01	0.1	1	10
EV-D68-WT	AGS	-	-	-	+
	HEC-1-A	-	-	-	-
	Ishikawa	-	-	-	++
	C-33A	-	-	++	++
	SW579	-	-	-	++
EV-D68-HRV2	AGS	++	++	++	++
	HEC-1-A	-	+	++	++
	Ishikawa	++	++	++	++
	C-33A	++	++	++	++
	SW579	++	++	++	++

Note: "-" indicated that there was no significant difference in cell survival rate between virus treatment group and MOCK group; "+" indicated that after virus treatment, the number of cells was reduced, the survival rate was greater than 50% but was significantly different from that of MOCK group; "++" indicated that the cell survival rate after virus treatment was less than 50%, and was significantly different from that of the MOCK group.

2.5 Serial passaging of EV-D68 for adaptation

In this example, EV-D68 was serially passaged for adaptation in a certain type of tumor cell to obtain a virus strain with enhanced killing activity to the tumor cell.

The wild-type enterovirus EV-D68 was serially passaged for adaptation in the human cervical cancer cell line SiHa, human ovarian cancer cell line SKOV-3, human liver cancer cell line SK-hep-1, human pancreatic cancer cell line Capan-2, human gastric cancer cell line AGS or human colorectal cancer cell line HCT-116 on which the oncolytic effect of EV-D68 was not very significant, and the specific method was as follows:

One kind of the above tumor cells was evenly plated on a 10 cm cell culture plate, and the culture conditions included a corresponding cell culture media containing 10% fetal bovine serum and glutamine, penicillin and streptomycin, 37 °C, 5% CO_2, and saturated humidity. When the cell confluence reached 90% or more, the cell culture medium was replaced with serum-free cell culture medium, each plate was inoculated with 10^7 TCID50 of EV-D68, the culture environment was changed to 33 °C, 5% CO_2, saturated humidity. When EV-D68 proliferated in tumor cells and caused CPE in the cells (after infection for up to 3 days), the cells and their culture supernatant were harvested. After freeze-thawing for three cycles, centrifugation was performed at 4 °C, 4000 rpm for 10 min. The centrifugation supernatant was taken and added onto new tumor cells with a cell confluence of more than 90% to complete one round of virus passage. The passage was repeated for more than 10 times, and a part of the virus solution was taken for virus titer detection in RD cells in each round of passage, and the specific method referred to Example 2.3. Generally, the virus replication ability would increase with the generation, and when a relatively high infectious titer was reached and the virus replication was stable in the tumor cell, the adapted strain of EV-D68 for the tumor cell was obtained.

Subsequently, by the in vitro antitumor experimental method described in Example 2.4, the human tumor cell SiHa, SKOV-3, SK-hep-1, Capan-2, AGS, or HCT-116 was inoculated to a 96-well plate at 10^4 cells/well. After the cells adhered, the medium in each well was replaced with the corresponding culture medium free of serum, followed by incubation at 37 °C for 30 min, and then the serially passaged EV-D68 virus strains (viral titers of which were detected on RD cells) adapted for each of the above kinds of cells at MOIs of 0.1, 1, 10, and 100 were inoculated. Subsequently, CPE of the cells were monitored daily by a microscope, and the cell survival rate was detected using CCK-8 method 72 hours after the infection and culture of viruses.

The results were shown in Table 5, in which after serial passaging of the wild-type enterovirus

EV-D68 in a certain kind of tumor cells on which EV-D68 had poor oncolytic effect, the killing activity thereof on the tumor cells was significantly enhanced, indicating that the serial passaging method could be used to obtain an EV-D68 adapted strain with enhanced oncolytic effect on the tumor cells.

Table 5: Results of in vitro killing experiment of EV-D68 on a tumor cell after serial passaging for adaptation in the tumor cell

Cell Line	0.1	1	10	100
SiHa	+	++	++	++
SKOV-3	-	++	++	++
SK-hep-1	-	++	++	++
Capan-2	-	+	++	++
AGS	+	+	++	++
HCT-116	-	+	++	++

Note: "-" indicated that there was no significant difference in cell survival rate between virus treatment group and MOCK group; "+" indicated that after virus treatment, the number of cells was reduced, the survival rate was greater than 50% but was significantly different from that of MOCK group; "++" indicated that the cell survival rate after virus treatment was less than 50%, and was significantly different from that of the MOCK group.

2.6 Evaluation of oncolytic effect of genomic RNA of EV-D68

In this example, a large amount of infectious live viruses of EV-D68 could be produced by transfecting the purified genomic RNA of EV-D68 into a certain kind of tumor cells, and thus kill the tumor cells.

The viral genomic RNA was first obtained by in vitro transcription, and this method could be found in, for example, Hadac E M, Kelly E J and Russell S J. Mol Ther, 2011, 19(6): 1041-1047. Specifically, the infectious cloning plasmid of wild-type EV-D68 obtained in Example 1 was linearized, and the linearized plasmid was used as a template for in vitro transcription using MEGAscript™ T7 Transcription Kit (Thermo Fisher Scientific, AM1333) so as to produce a large amount of viral RNA. And the obtained viral RNA was purified using MEGAclear™ Transcription Clean-Up Kit (Thermo Fisher Scientific, AM1908) for next use. The RNA electropherograms of 4 parallel samples were shown in FIG. 3.

Subsequently, according to the method of the in vitro antitumor experiment described in Example 2.4, the human cervical cancer tumor cell line Hela was inoculated to a 24-well plate at 10^5 cells/well. After the cells adhered, the medium in each well was replaced with a corresponding

cell culture medium free of serum, followed by incubation at 37 °C for 30 min. Then Hela cells were transfected with purified virus RNA at 1 μg per well using transfection reagent Lipofectamine® 2000 (Thermo Fisher Scientific, 11668019), and the negative control group was transfected with irrelevant RNA nucleic acid molecules. Subsequently, CPE of the cells were monitored daily by a microscope.

The results showed that CPE began to appear in the Hela cells transfected with genomic RNA of EV-D68 about 8 hours after transfection, and then the cytopathy gradually increased. After 48 hours, the survival rate was measured using the CCK8 method, the Hela cells had almost all died and lysed. And the micrographs of Hela cells at 0, 24 and 48 hours after infection were shown in FIG. 4. The culture supernatant was inoculated into new Hela cells and CPE was quickly produced. The results indicated that the direct administration with the nucleic acid of EV-D68 also had good killing activity and could be used to treat tumors.

Example 3: In vivo antitumor experiments of enterovirus EV-D68 and its modified forms

3.1 Viruses, cell lines and experimental animals

(1) Viruses: the EV-D68-WT (SEQ ID NO: 12), EV-D68-HRV2 (SEQ ID NO: 13), EV-D68-miR133&206T (SEQ ID NO: 14), EV-D68-GM-CSF (SEQ ID NO: 15) and EV-D68-Anti-PD-1 (SEQ ID NO: 16) provided in Example 1 were used in this example. The methods of virus culture and virus titer measurement could be seen in Examples 2.2 and 2.3, respectively.

(2) Cell lines: human cervical cancer cell line Hela (ATCC® Number: CCL-2™), glioma cell line U118-MG (ATCC® Number: HTB-15™), and lymphoma cell line Raji (ATCC® Number: CCL-86™). Except that Raji was cultured with RPMI-1640 medium, the other Hela and U118-MG were all cultured with DMEM medium. These mediums were all supplemented with 10% fetal bovine serum, glutamine and penicillin-streptomycin. All the above cells were cultured under the standard conditions of 37 °C and 5% CO_2.

(3) Experimental animals: female C.B17 SCID mice aged 6-8 weeks were from Shanghai Slark Experimental Animal Co., Ltd.; the mice were raised under SPF conditions, according to the protocol approved by the Experimental Animal Center and Ethics Committee of Xiamen University.

3.2 In vivo antitumor experiments of the virus

The tumor cells used for subcutaneous tumor formation in SCID mice were digested with

0.01% trypsin, and then resuspended into a single-cell suspension using a cell culture medium containing 10% fetal bovine serum. The cell density of the suspension was counted. The cells were precipitated by centrifugation under 1000 g for 3 min, and then the cells were resuspended with an appropriate volume of PBS to reach a concentration of about 10^6-10^7 cells/100 μl PBS. The tumor cells were subcutaneously inoculated in the back of SCID mice at 10^6-10^7 cells/100 μl PBS/site with a syringe. When the tumor cells grew into a tumor mass of about 100 mm^3 under the skin of SCID mice after about 14-21 days, the tumor-bearing SCID mice were randomly divided into experimental groups (administrated with EV-D68-WT, EV-D68-HRV2, EV-D68-miR133&206T, EV-D68-GM-CSF or EV-D68-Anti-PD-1) and negative control group, with 4 mice (n = 4) in each group. Oncolytic virus (EV-D68-WT, EV-D68-HRV2, EV-D68-miR133&206T, EV-D68-GM-CSF or EV-D68-Anti-PD-1) at 10^6 TCID50/100μl serum-free medium/tumor mass or equivalent amount of serum-free medium were intratumorally injected every two days, for a total of 5 treatments. The tumor size was measured with a vernier caliper and recorded every two days, and the method for calculating the tumor size was:

Tumor size (mm^3) = tumor length value × (tumor width value)^2 / 2.

The treatment results of EV-D68-WT for the above three tumors were shown in FIGs. 5A-5C. The results showed that after the challenge of EV-D68-WT, the growth of the three tested tumors of Hela (A), U118-MG (B) and Raji (C) gradually slowed down and arrested, and the tumors were even lysed and disappeared; by contrast, the tumors of the negative group (CTRL) maintained the normal growth, and their tumor sizes were significantly larger than those of the experimental groups.

Table 6 showed the results obtained after a treatment of the Raji tumor model with EV-D68-WT, EV-D68-HRV2, EV-D68-miR133&206T, EV-D68-GM-CSF or EV-D68-Anti-PD-1 for 10 days. The results showed that the tumor volumes were significantly reduced after treatment with EV-D68-WT, EV-D68-HRV2, EV-D68-miR133&206T, EV-D68-GM-CSF, and EV-D68-Anti-PD as compared with the negative control group that was not treated with oncolytic virus, wherein similar reductions in tumor volume were detected after treatment with 4 oncolytic viruses EV-D68-WT, EV-D68-miR133&206T, EV-D68-GM-CSF and EV-D68-Anti-PD-1. The above results indicated that all of EV-D68-WT, EV-D68-HRV2, EV-D68-miR133&206T, EV-D68-GM-CSF and EV-D68-Anti-PD-1 showed remarkable and favorable antitumor activity in vivo.

Table 6: Results of in vivo anti-tumor experiments of EV-D68-WT, EV-D68-HRV2, EV-D68-miR133&206T, EV-D68-GM-CSF and EV-D68-Anti-PD-1 on human lymphoma cell line Raji

Oncolytic virus	In vivo oncolytic effect on Raji after 10 days of treatment

EV-D68-WT	++
EV-D68-HRV2	+
EV-D68-miR133&206T	++
EV-D68-GM-CSF	++
EV-D68-Anti-PD-1	++

Note: "+" indicated that after treatment, the tumor volume reducedand was greater than 50% of the negative control group, but was significantly different from that of the negative control group; "++" indicated that the tumor volume reduced to less than 50% of the negative control group after treatment, and was significantly different from that of the negative control group.

Example 4: Safety evaluation of oncolytic virus

4.1 Viruses and laboratory animals used

(1) Virus: the EV-D68-WT (SEQ ID NO: 12) provided in Example 1 was used in this example. The methods for virus culture and virus titer measurement could refer to Examples 2.2 and 2.3, respectively.

(2) Experimental animals: BALB/c pregnant mice were from Shanghai Slark Experimental Animal Co., Ltd.; according to the protocol approved by the Experimental Animal Center and Ethics Committee of Xiamen University, the mice were raised under clean conditions, and then the 1-day-old, 2-day-old, 3-day-old, 7-day-old and 14-day-old mice produced by the BALB/c pregnant mice were used for in vivo virulence evaluation of EV-D68.

4.2 Evaluation of the safety of the virus in mice

(1) BALB/c suckling mice aged 1 day were selected for challenge with EV-D68-WT by intraperitoneal injection, and the titer doses for challenge were 10^3, 10^4, 10^5, 10^6, or 10^7 TCID50/mouse. Then the survival rates and health scores for the BALB/c mice challenged with different doses were recorded daily, wherein the evaluation criteria of the health score were: score of 5, represents dying or died; score of 4 represents severe limb paralysis; score of 3 represents weakness or mild deformity of limb; score of 2 represents wasting; score of 1 represents lethargy, piloerection, and trembling; and score of 0 represents healthy.

The results were shown in FIG. 6A. Within 20 days after challenge, all mice in the group with extremely high-dose of 10^7 TCID50 became ill and died within 1 week; 80% of the mice in the group with high-dose of 10^6 TCID50 eventually survived and only few mice became ill and died; in addition, no morbidity and death occurred in the mice of the challenge groups with other doses.

(2) The 1-day-old, 2-day-old, 3-day-old, 7-day-old and 14-day-old BALB/c mice were injected with EV-D68-WT at an extremely high dose of 10^7 TCID50/mouse, and then the survival

rates and health scores for the BALB/c with different ages in days were recorded daily, wherein the evaluation criteria of the health score were the same as above.

The results were shown in FIG. 6B. Within 20 days after challenge, the 1-day-old mice all died within 1 week; the 2-day-old mice resisted in a certain extent to EV-D68 toxicity, and eventually had a survival rate of 70%, but with a relatively high incidence of disease and relatively severe symptoms; the 3-day-old mice were already not vulnerable to EV-D68, and eventually had a survival rate of 90%, and with a low incidence of disease and mild symptoms; the 4- or more-day-old mice were fully tolerant to the high doses of EV-D68, and no morbidity and death occurred.

The above results showed that the EV-D68-WT was less toxic to mice, and was only lethal to the 1- to 3-day-old BALB/c mice at an extremely high dose of 10^7 TCID50/mouse, and had no effect on the 4- or more-day-old mice, thereby indicating good safety in vivo.

Although specific embodiments of the present invention have been described in detail, those skilled in the art will understand that according to all the teachings that have been published, various modifications and changes can be made to the detail, and these changes are all within the protection scope of the present invention. The protection scope of the present invention is given by the appended claims and any equivalents thereof.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word “comprise”, and variations such as “comprises” and “comprising”, will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

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The claims defining the invention are as follows:

1. Use of a wild-type Enterovirus D68 (EV-D68) or a modified EV-D68, or an isolated nucleic acid molecule, in the treatment of a tumor in a subject, or in the manufacture of a medicament for treating a tumor in a subject; wherein the nucleic acid molecule comprises a sequence selected from the following:

(1) a genomic sequence or cDNA sequence of the wild-type EV-D68 or the modified EV-D68; and

(2) a complementary sequence of the genomic sequence or cDNA sequence defined in (1);

wherein the wild-type EV-D68 has a genomic sequence defined by SEQ ID NO: 12, and/or has a cDNA sequence defined by SEQ ID NO: 1; and

a genome of the modified EV-D68 has one or more modifications selected from the following as compared to a genome of the wild-type EV-D68:

(1) a substitution of the internal ribosome entry site (IRES) sequence in a 5' untranslated region (5'UTR) with an exogenous IRES sequence; and

(2) an insertion of an exogenous nucleic acid which is selected from a nucleic acid sequence encoding a cytokine, a nucleic acid sequence encoding an antitumor protein or polypeptide, and/or a target sequence of microRNA.

2. The use according to claim 1, wherein the exogenous IRES sequence is an internal ribosome entry site sequence of human rhinovirus 2 (HRV2).

3. The use according to claim 1 or 2, characterized by one or more of the following characteristics:

(1) the cytokine is GM-CSF;

(2) the antitumor protein or polypeptide is a scFv against PD-1 or PD-L1; and

(3) the microRNA is selected from miR-133 and/or miR-206.

4. The use according to any one of claims 1 to 3, wherein the modified EV-D68 has one of the following characteristics:

(1) the genomic sequence of the modified EV-D68 is selected from the nucleotide sequences

as shown in any one of SEQ ID NOs: 13-16; and

(2) the cDNA sequence of the modified EV-D68 is selected from the nucleotide sequences as shown in any one of SEQ ID NOs: 8-11.

5. The use according to any one of claims 1 to 4, characterized by one of the following characteristics:

(1) the isolated nucleic acid molecule consists of a genomic sequence of the wild-type EV-D68 or the modified EV-D68; and

(2) the isolated nucleic acid molecule is a vector comprising a cDNA sequence of the wild-type EV-D68 or the modified EV-D68, or a complementary sequence of the cDNA sequence.

6. The use according to claim 5, wherein the isolated nucleic acid molecule has a nucleotide sequence defined by any one of SEQ ID NOs: 12-16, or is a vector comprising a nucleotide sequence defined by any one of SEQ ID NOs: 1, 8-11 or a complementary sequence thereof.

7. The use according to any one of Claims 1 to 6, wherein the wild-type EV-D68 or the modified EV-D68, or the isolated nucleic acid molecule, is used in combination with an additional pharmaceutically active agent having antitumor activity.

8. The use according to claim 7, wherein the additional pharmaceutically active agent is selected from an additional oncolytic virus, chemotherapeutic agent or immunotherapeutic agent.

9. The use according to claim 8, wherein the additional pharmaceutically active agent is characterized by one or more of the following:

(1) the additional oncolytic virus is selected from herpesvirus, adenovirus, parvovirus, reovirus, Newcastle disease virus, vesicular stomatitis virus, measles virus or any combination thereof;

(2) the chemotherapeutic agent is selected from 5-fluorouracil, mitomycin, methotrexate, hydroxyurea, cyclophosphamide, dacarbazine, mitoxantrone, anthracyclines, etoposide, platinum compounds, taxanes, or any combination thereof; and

(3) the immunotherapeutic agent is selected from immune checkpoint inhibitors, tumor-specific targeting antibodies or any combination thereof.

10. The use according to any one of claims 1 to 9, wherein the tumor is selected from cervical cancer, ovarian cancer, endometrial cancer, lung cancer, liver cancer, kidney cancer, neuroblastoma, glioma, breast cancer, melanoma, prostate cancer, bladder cancer, pancreatic cancer, gastric cancer, colorectal cancer, esophageal cancer, thyroid cancer, laryngeal cancer, osteosarcoma, hematopoietic malignancy.

11. The use according to any one of claims 1 to 10, wherein the subject is a human.

12. A method for treating a tumor, comprising the step of administering to a subject in need thereof an effective amount of a wild-type EV-D68 or a modified EV-D68, or an effective amount of an isolated nucleic acid molecule; wherein the isolated nucleic acid molecule comprises a sequence selected from:

(1) a genomic sequence or cDNA sequence of the wild-type EV-D68 or the modified EV-D68; and

(2) a complementary sequence of the genomic sequence or cDNA sequence defined in (1);

wherein the wild-type EV-D68 has a genomic sequence defined by SEQ ID NO: 12, and/or has a cDNA sequence defined by SEQ ID NO: 1; and

a genome of the modified EV-D68 has one or more modifications selected from the following as compared to a genome of the wild-type EV-D68:

(1) a substitution of the internal ribosome entry site (IRES) sequence in a 5' untranslated region (5'UTR) with an exogenous IRES sequence; and

(2) an insertion of an exogenous nucleic acid which is selected from a nucleic acid sequence encoding a cytokine, a nucleic acid sequence encoding an antitumor protein or polypeptide, and/or a target sequence of microRNA.

13. The method according to claim 12, wherein the tumor is selected from cervical cancer, ovarian cancer, endometrial cancer, lung cancer, liver cancer, kidney cancer, neuroblastoma, glioma, breast cancer, melanoma, prostate cancer, bladder cancer, pancreatic cancer, gastric cancer, colorectal cancer, esophageal cancer, thyroid cancer, laryngeal cancer, osteosarcoma,

hematopoietic malignancy.

14. The method according to claim 12, wherein the subject is a human;

15. The method according to any one of claims 12-14, wherein the wild-type EV-D68 or modified EV-D68, or the isolated nucleic acid molecule, is as defined in any one of Claims 2 to 11.

16. A modified EV-D68, a genome of which has one or more modifications selected from the following as compared to a genome of a wild-type EV-D68:

(1) a substitution of the internal ribosome entry site (IRES) sequence in a 5' untranslated region (5'UTR) with an exogenous IRES sequence;

(2) an insertion of an exogenous nucleic acid which is selected from a nucleic acid sequence encoding a cytokine, a nucleic acid sequence encoding an antitumor protein or polypeptide, and/or a target sequence of microRNA;

wherein the wild-type EV-D68 has a genomic sequence defined by SEQ ID NO: 12, and/or has a cDNA sequence defined by SEQ ID NO: 1.

17. The modified EV-D68 according to claim 16, wherein the exogenous IRES sequence is an internal ribosome entry site sequence of a human rhinovirus 2 (HRV2).

18. The modified EV-D68 according to claim 16 or 17, characterized by one or more of the following characteristics:

(1) the cytokine is GM-CSF;

(2) the antitumor protein or polypeptide is a scFv against PD-1 or PD-L1; and

(3) the microRNA is selected from miR-133 and/or miR-206.

19. The modified EV-D68 according to any one of Claims 16 to 18, wherein the modified EV-D68 has at least one of the following characteristics:

(1) the genomic sequence of the modified EV-D68 is selected from the nucleotide sequences as shown in any one of SEQ ID NOs: 13-16; and

(2) the cDNA sequence of the modified EV-D68 is selected from the nucleotide sequences as shown in any one of SEQ ID NOs: 8-11.

20. An isolated nucleic acid molecule, which comprises a sequence selected from the group consisting of:

(1) a genomic sequence or cDNA sequence of the modified EV-D68 according to any one of Claims 16 to 19; and

(2) a complementary sequence of the genomic sequence or cDNA sequence defined in (1).

21. The isolated nucleic acid molecule according to claim 20, characterized by one of the following characteristics:

(1) the isolated nucleic acid molecule consists of a genomic sequence of the modified EV-D68; or

(2) the isolated nucleic acid molecule is a vector comprising a cDNA sequence of the modified EV-D68, or a complementary sequence of the cDNA sequence.

22. The isolated nucleic acid molecule according to claim 21, wherein the isolated nucleic acid molecule has a nucleotide sequence defined by any one of SEQ ID NOs: 12-16, or is a vector comprising a nucleotide sequence defined by any one of SEQ ID NOs: 1, 8-11 or a complementary sequence thereof.

Drawings

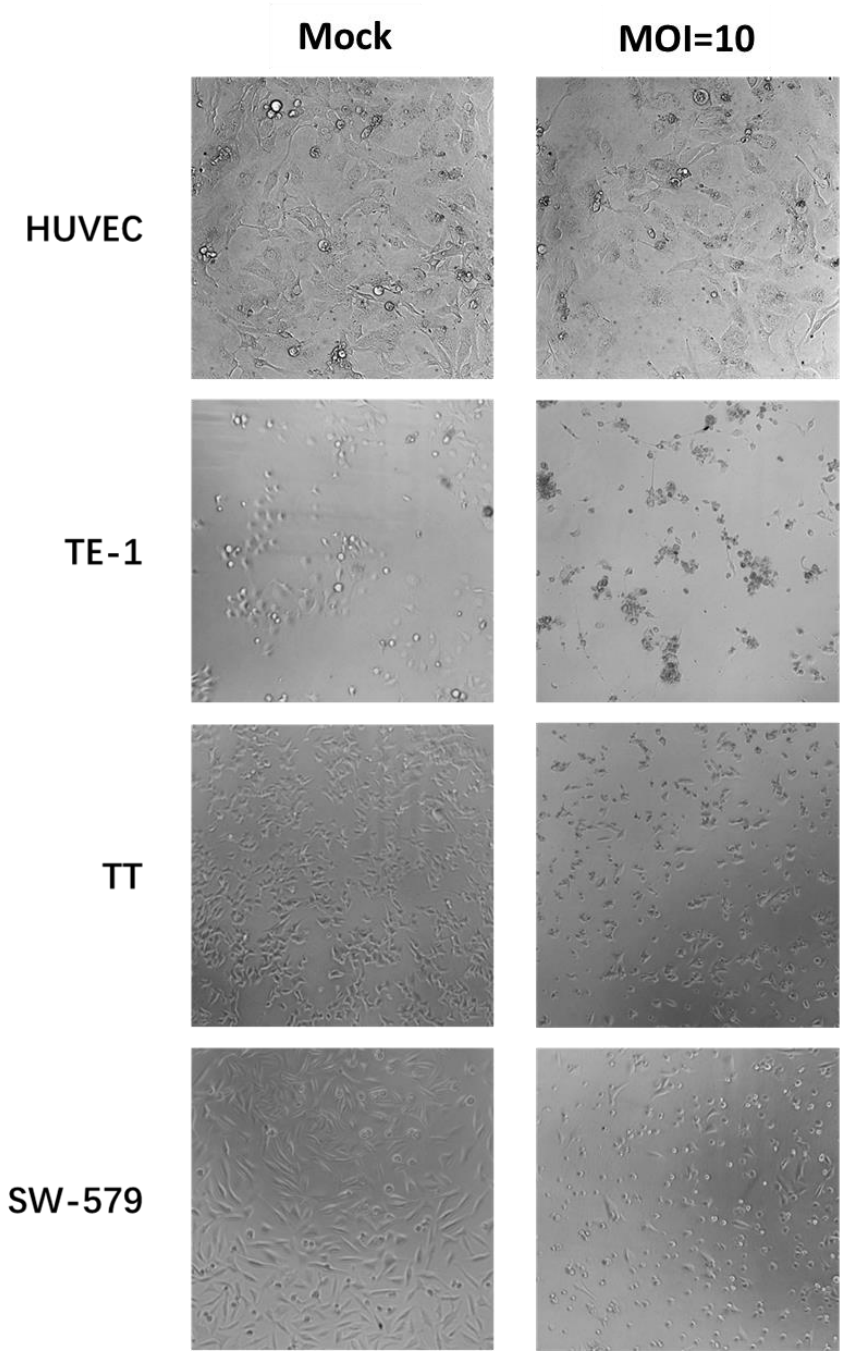
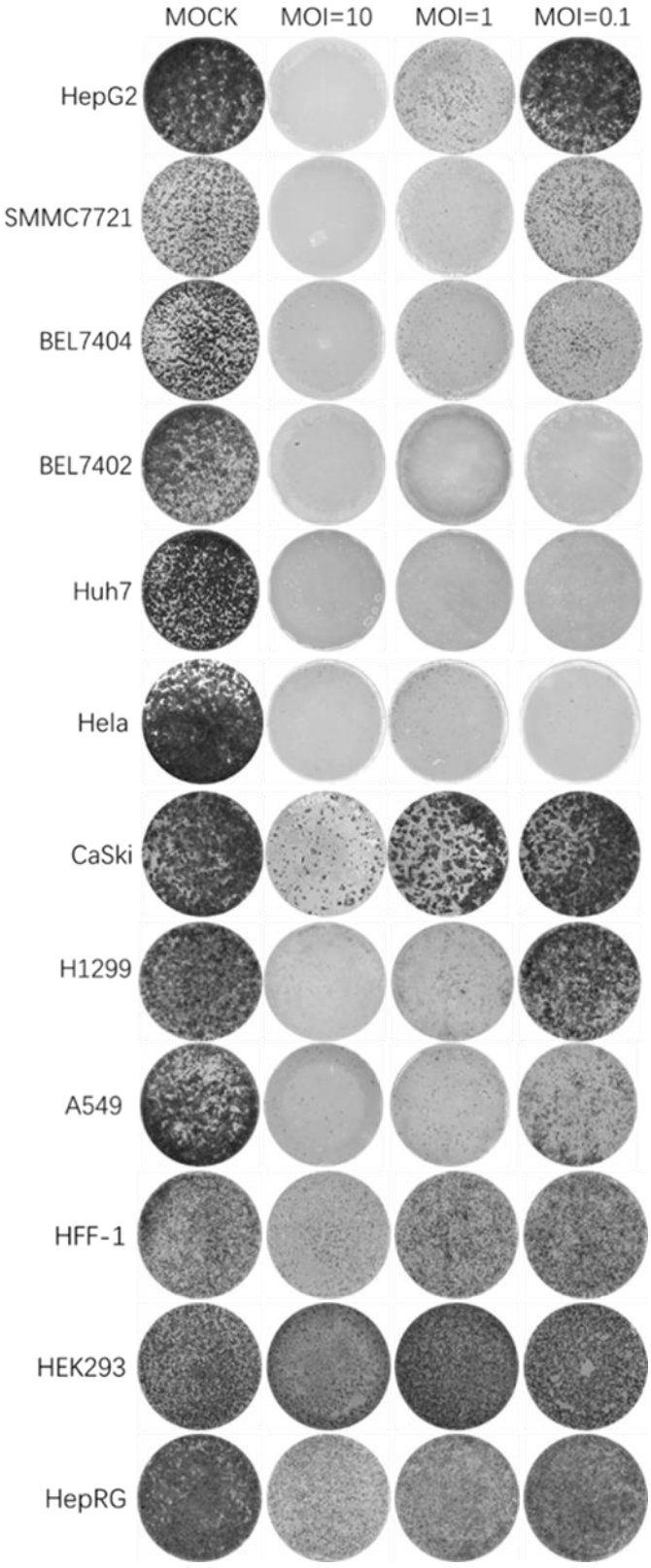


FIG. 1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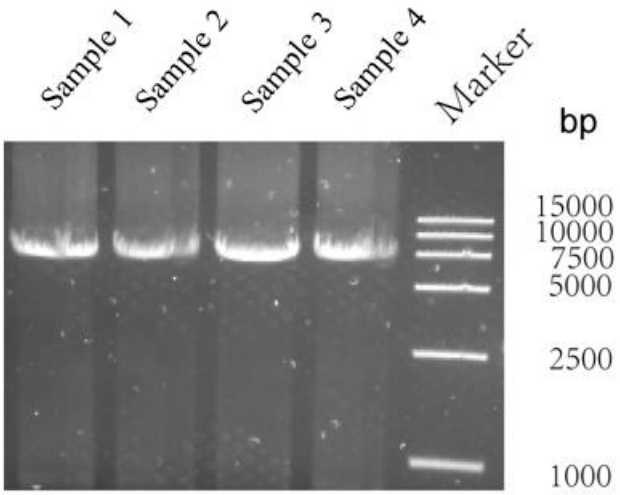
HEK293
HepRG

FIG. 2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Sample 3
Sample 4
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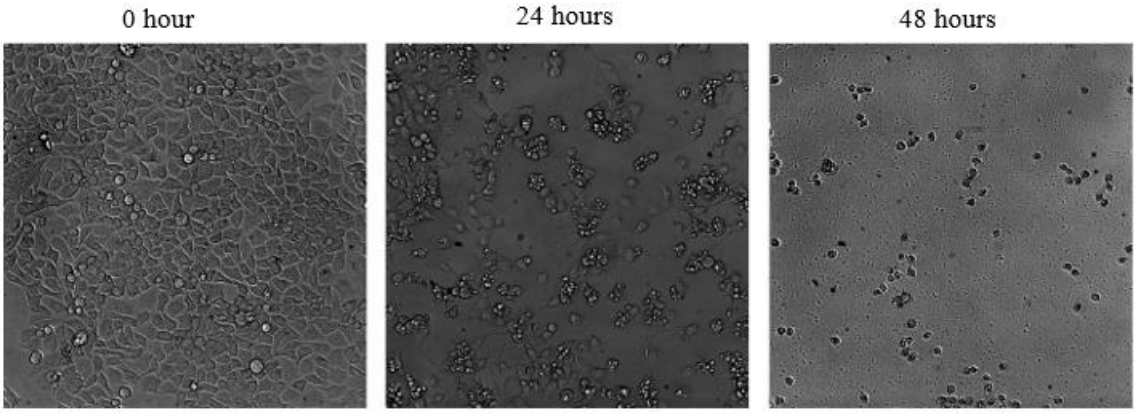
5000
2500
1000

FIG. 3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48 hours

FIG. 4

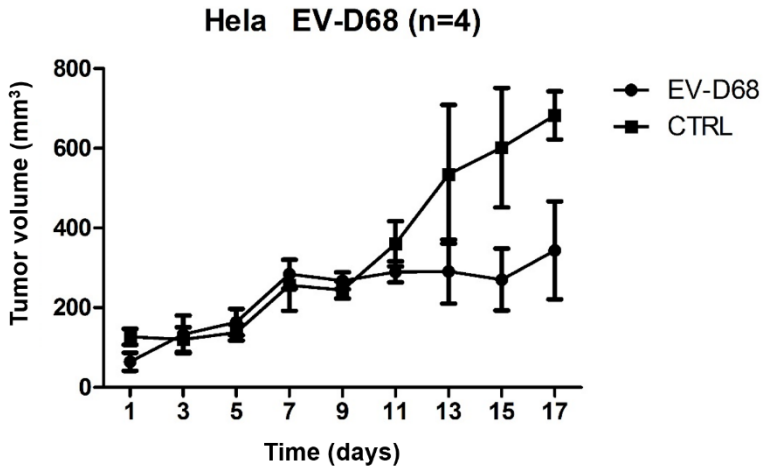


FIG. 5A

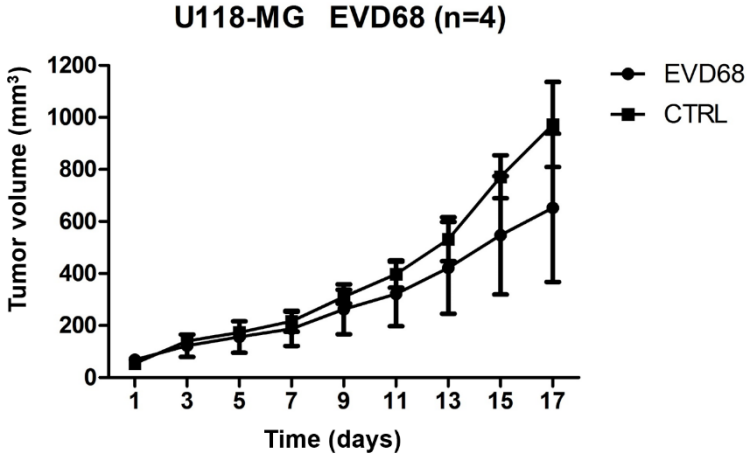


FIG. 5B

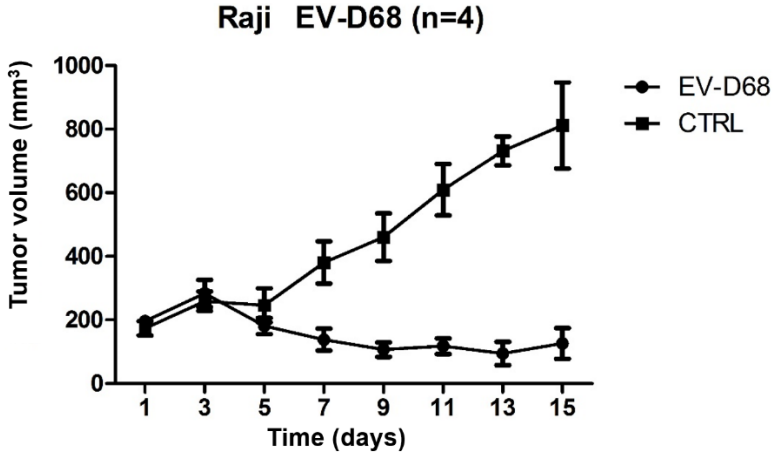
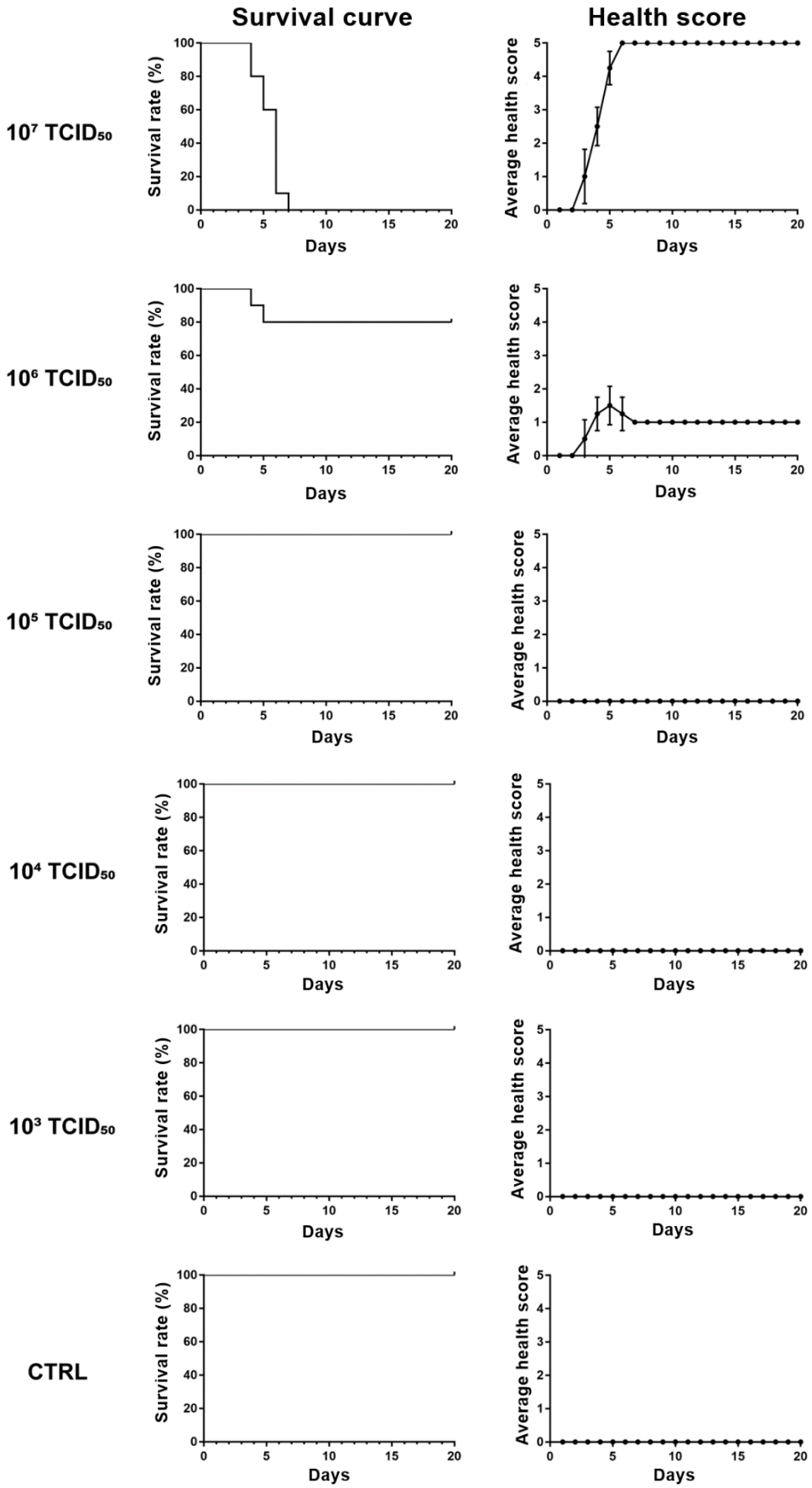


FIG. 5C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10⁶ TCID₅₀
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10³ TCID₅₀
CTRL
Survival rate (%)
Average health score
Days

FIG. 6A

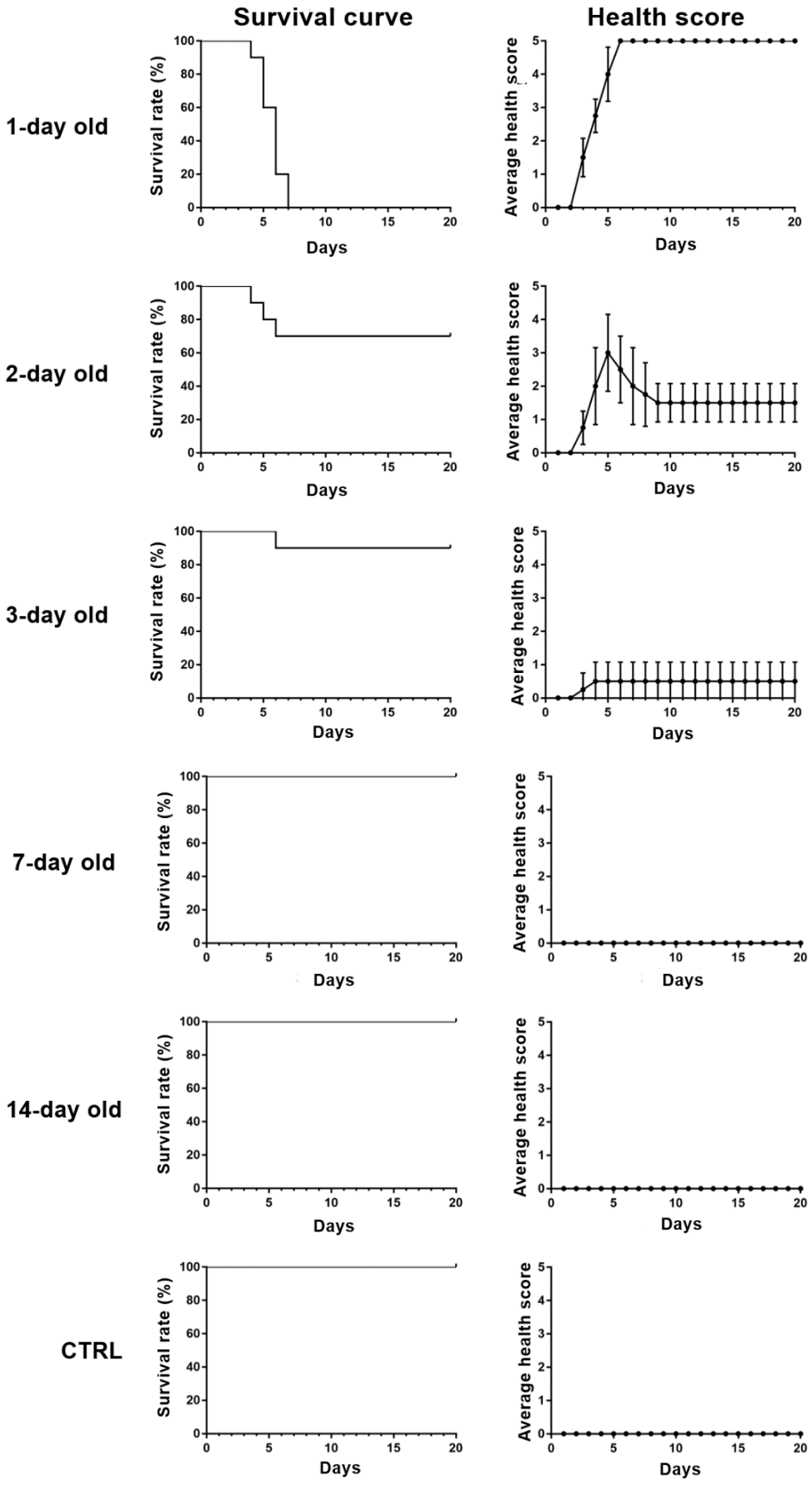
Survival curve
Health score
1-day old
2-day old
3-day old
7-day old
14-day old
CTRL
Survival rate (%)
Average health score
Days

FIG. 6B

序列表

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养生堂有限公司

<120> 一种用于治疗肿瘤的病毒

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