DEVELOPMENT OF A RECOMBINANT ONCOLYTIC POLIOVIRUS TYPE 3 STRAIN WITH ALTERED CELL TROPISM

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Diffuse gliomas are incurable, prevalent, and aggressive central nervous system tumors. Therefore, the development of selective oncolytic viral strains for malignant neoplasms is highly relevant. This study aimed to create an oncolytic virus based on a vaccine strain of poliovirus type 3 with natural antitumor activity. To achieve this goal, we replaced the internal ribosome entry site (IRES) of poliovirus with the corresponding fragment of human rhinovirus 30. The resulting recombinant oncolytic strain RVP3 retained the serotype of poliovirus type 3, as confirmed by virus neutralization micro-test with specific antiserum. In addition, the oncolytic efficacy of RVP3 was assessed *in vitro* on a broad panel of cell cultures. According to the results, RVP3 has changed its tropism, losing the ability to replicate in conditionally normal cell lines of embryonic astrocytes and embryonic fibroblasts while retaining the ability to replicate in tumor cells.

Keywords: oncolytic viral therapy, glioma, vaccine strain of poliovirus

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Compliance with ethical standards: the study was conducted per the requirements of the World Medical Association Declaration of Helsinki 2000 and its subsequent revisions; in compliance with the principles of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

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РАЗРАБОТКА РЕКОМБИНАНТНОГО ОНКОЛИТИЧЕСКОГО ШТАММА ПОЛИОВИРУСА 3-ГО ТИПА С ИЗМЕНЕННЫМ КЛЕТОЧНЫМ ТРОПИЗМОМ

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Диффузная глиома является неизлечимым заболеванием, наиболее распространенным и агрессивным типом опухолей ЦНС. Разработка высокоонкоселективных вирусных штаммов для терапии злокачественных новообразований является актуальной задачей. Целью работы было создание онколитического вируса на базе вакцинного штамма полиовируса 3-го типа, обладающего природной противоопухолевой активностью, путем замены IRES полиовируса соответствующим участком из риновируса человека 30 типа. В результате был успешно получен рекомбинантный онколитический штамм RVP3, сохранивший серотип полиовируса 3-го типа, что было подтверждено микрореакцией нейтрализации специфической антисывороткой. Онколитическая эффективность RVP3 была оценена *in vitro* на широкой панели клеточных культур. RVP3 изменил тропизм, потеряв способность эффективно реплицироваться в условно нормальных клеточных линиях эмбриональных астроцитов и эмбриональных фибробластов, сохранив способность эффективно реплицироваться в опухолевых клетках.

Ключевые слова: онколитическая виротерапия, глиома, вакцинный штамм полиовируса

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Соблюдение этических стандартов: исследование проведено в соответствии с требованиями Хельсинкской декларации Всемирной медицинской ассоциации (2000 г.) и последующих ее пересмотров; с соблюдением принципов Европейской конвенции по защите позвоночных животных, используемых в экспериментальных исследованиях.

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Table. Oligonucleotide sequences used for RVP3 DNA amplification

№	ID	Sequence	Melting temperature, °C
1	HRV30 IRES for	tttatactccctcccttagaagttttacataaagaccaataggt	56
2	HRV30 IRES rev	taagttaaggagtaaaacgcaattgctcattacgact	56
3	P3vect for	ttttactccttaacttattgaaattgtttgaagac	57
4	P3vect rev	gggagggagtataaaacaggcgta	57

Diffuse glioma (glioblastoma) is the most prevalent and aggressive type of primary central nervous system malignancies [1]. Relapses occur in almost 100% of the cases. Despite all standard treatment approaches, the average survival is 12–18 months post-diagnosis, including surgical resection, radiation, chemo- and targeted therapies [2]. Alternative treatment approaches include viral treatment based on non-pathogenic oncolytic strains. Such strains may show natural tropism to tumor cells; besides, the antitumor properties can be deliberately enhanced by modifying viral genomes. The development of productive viral infection in a tumor activates innate and adaptive antitumor response [3–5], facilitating the elimination of tumor cells.

A recombinant oncolytic strain PVS-RIPO was developed to treat glioblastoma [6]. Intratumor administration of PVS-RIPO combined with chemotherapy and radiotherapy afforded prolonged remissions (over three years) in 21% of the patients [7].

PVS-RIPO is an attenuated non-pathogenic oncolytic virus created based on poliovirus type 1 Sabin vaccine strain, where the internal ribosome entry site (IRES) is replaced with the corresponding sequence of human rhinovirus type 2 (HRV2). The antitumor efficacy of PVS-RIPO is due primarily to the natural tropism of polioviruses to tumor cells, notably in tumors of the nervous system. Polioviruses enter cells by binding the PVR/CD155 cellular receptor, which is significantly overexpressed in solid tumors and in the tumor microenvironment [7–9]. However, polioviruses may exhibit neurotoxicity when administered systemically or intratumorally that is related with their ability to infect normal neurons. Tropism of polioviruses is not only determined by cell surface receptors and the structure of the viral IRES, which mediates the interactions of viral RNA with some tissue-specific cytoplasmic factors that modulate translation initiation efficiency [10, 11]. For instance, cellular factor DRBP76 can specifically bind the 3'-region of IRES in the rhinovirus HRV2 RNA, thereby inhibiting its translation in cells of neuronal origin [12]. The high tropism of polioviruses to neurons can be partially explained by the low binding affinity between DRBP76 and IRES. The oncolytic PVS-RIPO contains chimeric 5′ UTR combining the cloverleaf of polio- with IRES of rhinovirus, limiting the infection to cells originating from glia and some other tissues; most importantly, it spares neurons [13]. Apart from brain tumors, PVS-RIPO is undergoing clinical trials for unresectable melanomas [14], and many other immunotherapy-sensitive tumors that may also respond to the viral therapy. In the context of the increasing incidence of malignant neoplasms, the development of oncolytic viral strains becomes particularly urgent. The use of heterologous IRESes allows deliberate customization of viral tropism depending on the individual characteristics. At the same time, the expanded arsenal of available oncolytic strains enables a personalized selection of antitumor medications. Here we report a new recombinant oncolytic virus derived from the poliovirus type 3 Sabin vaccine strain by replacing a large segment of polioviral IRES with the corresponding sequence of human rhinovirus 30.

METHODS

Development of recombinant viral strain RVP3

The recombinant viral strain RVP3 was created based on the poliovirus type 3 Sabin vaccine strain (PV3S). A pUC18 plasmid construct containing PV3S genome sequence under the control of T7 promoter was developed in the Laboratory of Cell Proliferation at Engelhardt Institute of Molecular Biology. The nucleotide sequence of the rhinovirus A30 (HRV30) 5'-noncoding region was accessed from the GenBank database (Human rhinovirus 30 strain ATCC VR-1140, FJ445179.1). The HRV30 genome fragment (nucleotides 108–521) was custom synthesized by IDT DNA (USA). The plasmid encompassing the final recombinant strain genome was obtained by ligase-free cloning of two fragments generated by PCR with primers listed in Table following a published protocol [15]. A scheme of the product is given in Fig. 1.

To obtain the infectious genomic RNA, the plasmid construct was transfected into HEK293T cells with a plasmid expressing codon-optimized T7 polymerase as described elsewhere [16]. At 36–48 h post-transfection, the medium was harvested and used for infection of fresh HEK293T cells to account for cytopathic foci and plaque formation.

Production of viral particles

The PV3S and RVP3 enteroviruses were produced in the RD and HEK293T cells. The cells were plated 24 h before the

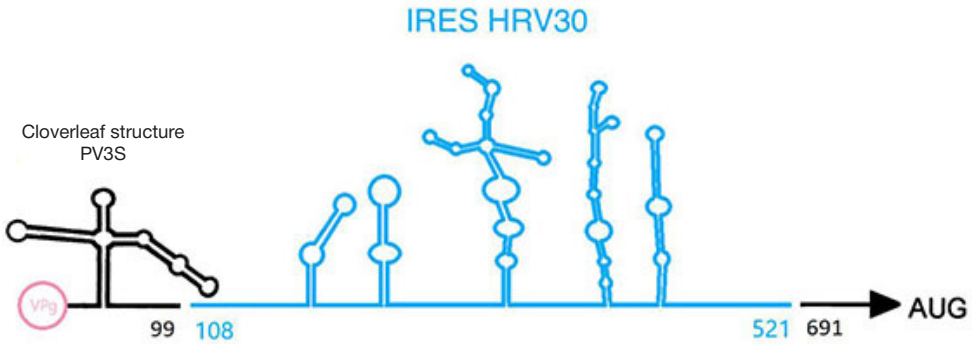

Fig. 1. A scheme of 5′ UTR in RVP3. The chimeric region combines sequences of rhinovirus and poliovirus type 3

infection at 70% confluence and MOI = 0.1. The cytopathic effect developed at 24 h post-transfection. The harvested medium was clarified from cell debris by centrifugation at +4 °C, 2500 rpm for 15 min. The virus-containing supernatant was stored in 0.5 mL aliquots at –80 °C.

Production of anti-PV3 sheep antiserum and virus neutralization micro tests

The study used a six-month-old lamb of the Dorper breed. The animal was obtained from the "Capri" breeding farm (Kaluga region, Russia) and housed under standard farming conditions with regular anthelmintic prophylaxis; the diet included hay, compound feed, and vitamin supplements.

For immunization, 1 mL of virus-containing supernatant (10^9 infectious units) was mixed with an equal volume of complete Freund's adjuvant (DIFCO; USA). The mixture was homogenized to a stable suspension and administered to the animal intradermally (0.1 mL on both sides in the hind thigh area) and intramuscularly (0.4 mL on both sides in the thigh muscles). Further injections were carried out similarly, but the virus was mixed with incomplete Freund's adjuvant (DIFCO; USA) 4 times at 7-day intervals. During the experiment, the lamb was housed separately from the herd. Seven days after the fifth injection, the blood was collected in several 9 mL vacuum tubes with coagulant — 40 mL from the jugular vein. The animal was not subject to slaughter and joined the herd. The collected blood was left for 4 h until the clot formation; the serum was clarified by centrifugation at 5000 rpm for 15 min. The serum was stored in aliquots at –70 °C.

Serotype of the new strain was verified by infectivity neutralization micro test using specific sheep antiserum to poliovirus type 3 in accordance with a published protocol [17]. The virus-containing supernatant was incubated for 2 hours with antiserum in 23 consecutive dilutions (1:10,000, 1:7,500, 1:5,000, 1:4,000, 1:3500, 1:3000, 1:2500, 1:2000, 1:1500, 1:1000, 1:750, 1:500, 1:200, 1:100, 1:75, 1:50, 1:30, 1:20, 1:15, 1:10, 1:7, 1:5, and 1:1). Apart from PV3S and RVP3, we tested other non-pathogenic oncolytic strains: Coxsackie B5 (CVB5, GenBank: MG642820.1), Coxsackie A7 (CVA7, GenBank: JQ041367.1) and prototype strain echovirus 12 Travis (ECHO12T, GenBank: X79047.1).

Cell line sensitivity and viral replication efficiency assays

To assess the sensitivity of cell lines to viral infections, the cells were plated in 96-well plates to 40% confluence (4×10^3 to 4×10^3 cells per well, depending on the culture). The next day, the cells were infected with the virus in serum-free DMEM at a wide multiplicity range (MOI = 0.001–100). After 1 h adsorption of the virus, the medium was replaced with DMEM supplemented with 1% fetal bovine serum (FBS). The viability was assessed by MTT test at 72 h. TCID50 values were calculated for ease of interpretation by the Reed–Muench method [18]. The titrations were carried out in four technical parallels and three independent biological replicates.

To assess the replication efficiency, the cells were plated in 12-well plates to 50% confluence and infected by incubation with the virus (MOI = 0.1) in DMEM at 37 °C for 1 h. Then, the cells were washed from the virus and placed in a fresh medium with 0.5% FBS. The supernatants were harvested 48 h after infection. Viral replication capacity was determined by titration on HEK293T cells infected with serially diluted supernatants (Reed–Muench method).

RESULTS

RVP3 reconstitution and production in cell cultures

At 48 h after cotransfection of HEK293T cells with RVP3 genome-containing construct and codon-optimized T7 polymerase-expressing plasmid, accumulation of infectious particles in the supernatant was determined by measuring the cytopathic effect upon re-infection of fresh HEK293T monolayers. The virus was subjected to over 15 adaptation passages before its preparative production in HEK293T and RD cell lines. The titers were determined by Reed–Muench method; the results of the replication efficiency assay for RVP3 and PV3S are shown in Fig. 2. HEK293T cell line, which showed higher replication efficiency, was chosen for subsequent virus production.

RVP3 retains the serotype of poliovirus type 3

In virus neutralization micro tests, effective inactivation of PV3S was achieved at 1:3000–1:2500 dilutions of anti-PV3 sheep antiserum, whereas cross-inactivation of enteroviruses of another serotype (Coxsackie A7 and B5, and echovirus 12) was effective in 1:10–1:7 dilutions only, which confirmed high specificity of the serum. Effective inactivation of RVP3 was achieved at 1:2000–1:1500 dilutions of anti-PV3 sheep antiserum, indicating the retention of poliovirus type 3 serotype by RVP3 (Fig. 3).

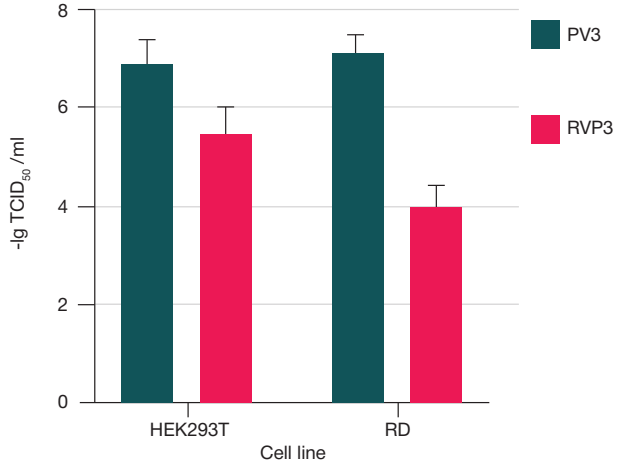

Fig. 2. Comparison of viral titers for enterovirus-sensitive cell cultures RD and HEK293T

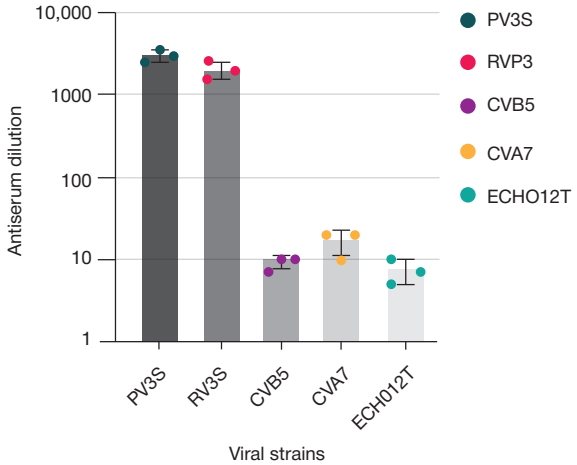

Fig. 3. Results of anti-poliovirus type 3 neutralization microtests for different viral strains

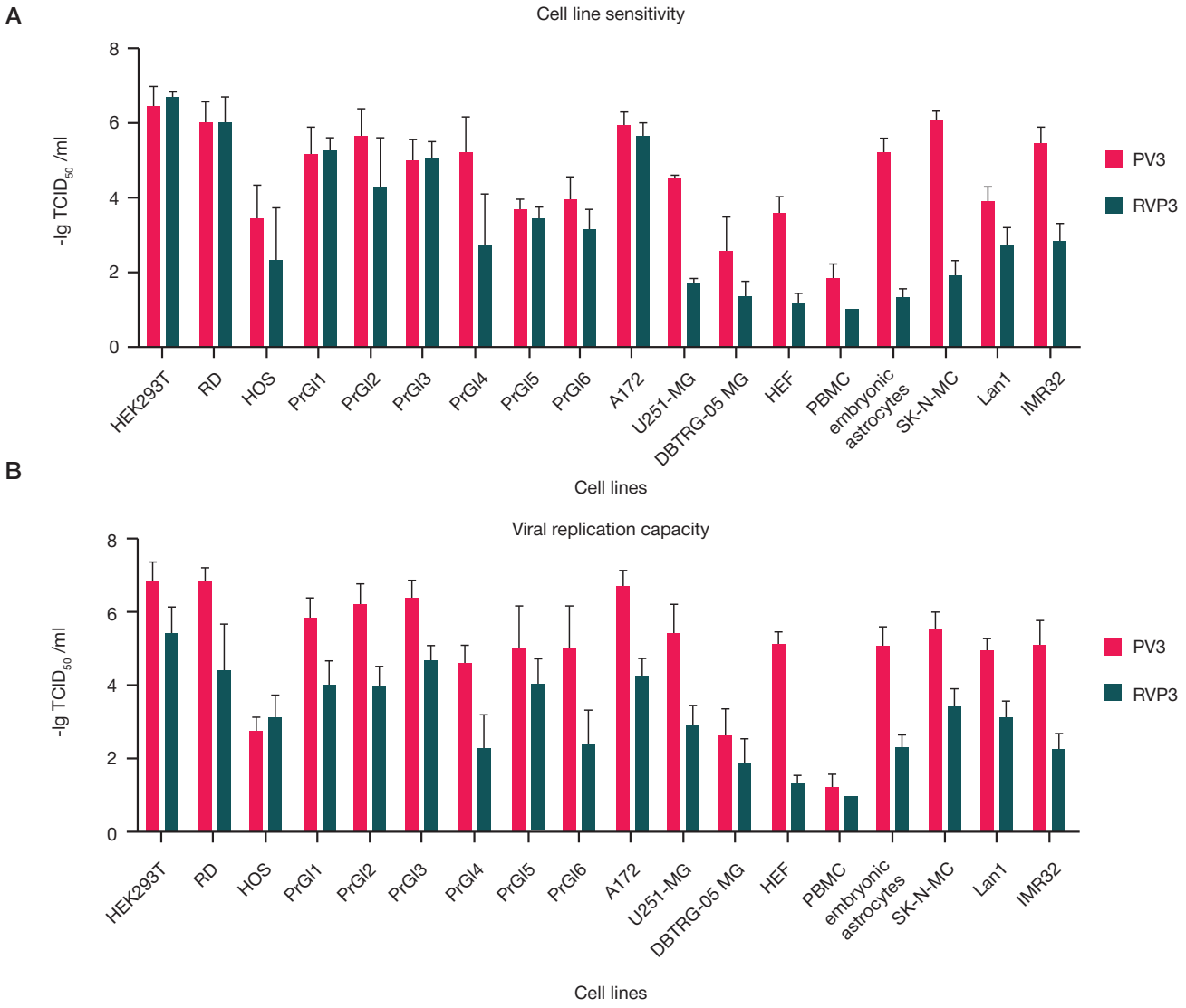

Fig. 4. *In vitro* functional tests for RVP3 and PV3S. **A**. Cell line sensitivity to infection. **B**. Viral replication capacity

Comparison of *in vitro* cytolytic activity for RVP3 and PV3S

Comparative assessment of cytolytic effects for the new RVP3 and the original PV3S viral strains involved the scope of tumor and conditionally normal cell lines; the results are shown in Fig. 4. The tumor cell lines included three model glioblastoma cultures DBTRG-05 MG, A-172, and U-251 MG (ATCC; USA); six low-passage glioblastoma cell lines PrGI1, PrGI2, PrGI3, PrGI4, PrGI5, and PrGI6 developed and characterized in Laboratory of Cell Proliferation at Engelhardt Institute of Molecular Biology [19]; three model neuroblastoma cell lines SK-N-MC, Lan1, and IMR-32 purchased from "Collection of vertebrate cell cultures" at the Institute of Cytology RAS (Russia); and two tumor cell lines RD and HOS from the collection of Laboratory of Cell Proliferation at Engelhardt Institute of Molecular Biology. The conditionally normal cells included human embryonic fibroblasts (HEF) and normal embryonic astrocytes from the Laboratory of Immunochemistry at Serbsky Research Center for Psychiatry and Narcology (Russia); and peripheral blood mononuclear cells (PBMC) from two patients.

Both viruses demonstrated high oncolytic activity against the studied panel of cultures. The RVP3 replication efficiency was significantly lower, with a profound decrease in HEF and embryonic astrocytes.

DISCUSSION

The development of selective oncolytic viral strains showing specific enhancement of replication efficiency in malignant neoplasms with negligible damage to normal cells is pivotal for the future of oncolytic viral strategies.

The differential tropism of enteroviruses toward cell type is determined by differential expression of specific cell surface proteins used as entry receptors and viral 5' UTR structure, notably its internal ribosome entry site (IRES). The efficiency of viral RNA translation largely depends on its binding with certain cellular regulatory factors, the availability of which varies in tissue- and tumor-specific manner. Accordingly, the same viral strain may show high antitumor efficacy in one patient. Still, it cannot replicate and kill tumor cells in another patient because of the difference in expression profiles of receptors or translation factors. Considering the considerable diversity of tumor cell types, the oncolytic viral strategies must involve a personalized selection of suitable therapeutic options from a large arsenal of viral strains with different cell tropisms. IRES modification provides an additional source of viral strain diversity and a tool for studying the effects of individual structural elements on the target cell specificity and replication efficiency of therapeutic viruses.

We constructed a recombinant variant based on the poliovirus type 3 Sabin (PV3S) vaccine strain using this concept. Of the three

available vaccine strains of polioviruses, PV3S showed the most effective lytic properties against primary glioblastoma cell lines. The IRES of PV3S differs by only one nucleotide substitution from the IRES of wild pathogenic PV3 Leon strain, from which it has been derived by lengthy passaging in monkey cells [20]. For this reason, PV3S is more susceptible to reversions causing vaccination-associated poliomyelitis than PV1S [21]. Replacement of the 5' regulatory region in PV3S with the corresponding sequence of rhinovirus prevents the possibility of reversions and enhances the safety of a candidate oncolytic construct.

Thanks to the global anti-polio vaccination campaign involving the trivalent live poliovirus vaccine, almost the entire population of the planet have lifelong immunity to polio. For PV3S, neutralization with vaccine-derived antibodies in vaccinated individuals is less effective compared with the other two variants of polioviruses [22], which may endow it with higher anticancer efficacy.

Functional tests using a panel of tumor and conditionally normal cell cultures (Fig. 4) revealed significantly altered cell tropism of RVP3 compared with PV3S. Despite the similar rates of RVP3 and PV3S replication in HOS osteosarcoma cells, in other cultures replication of the recombinant virus was 2–4 orders less productive. The relative replication efficiency changed accordingly: in cultures with top rates of PV3S replication (e.g., PrGl6 and U251MG), RVP3 replication was medium or low. We also observed no substantial differences for neuroblastoma cell lines. Despite the reduced replication efficiency of RVP3 in SK-N-MC, Lan1, and IMR-32 cells compared with PV3S, the infection still occurred and killed the cells. We can assume that neuroblastoma cells may have specific IRES-interacting protein expression signatures different from tumors and normal central nervous system neurons.

Moreover, the novel RVP3 strain revealed enhanced selectivity toward malignant cells: its replication efficiency in normal astrocytes and embryonic fibroblasts was, respectively, three and four orders of magnitude lower compared with PV3S, whereas its ability to replicate in peripheral blood mononuclear leukocytes were minimal.

The global experience of over a billion administrations of the live oral polio vaccines, entering the circulation via Peyer's patches and crossing the blood-brain barrier, confirms their safety in terms of neurological symptoms or emergence of the virus in the central nervous system. It should be noted that human cells propagated *in vitro* are usually more susceptible to viral infections than cells and tissues of the body. Continual passaging in artificial media significantly reorganize cell metabolism [23]. Therefore, *in vitro* cell sensitivity cannot be considered ultimate evidence of viral pathogenicity. However, the results of *in vitro* tests must not be discounted altogether: viruses with low *in vitro* replication efficiency are less likely to be pathogenic. For this reason, cell cultures provide essential test targets for the candidate therapeutic viral strains, which should be highly active in a tumor, but have minimal effect in normal tissues.

Further investigation of possibilities for targeting enteroviral replication within the human body by modifying IRES sequences will increase the availability of safe therapeutic viral strains for personalized anticancer therapy.

CONCLUSIONS

The recombinant oncolytic viral strain RVP3 was developed by replacement of the internal ribosome entry site (IRES) of poliovirus with the corresponding sequence of human rhinovirus 30. The developed strain retains the original serotype of poliovirus type 3 and can be successfully replicated in model cell cultures. The oncolytic activity of RVP3 was validated *in vitro* on a broad panel of cell cultures. The strain shows altered tropism, with a profoundly decreased efficiency to replicate in conditionally normal cell lines of embryonic astrocytes and embryonic fibroblasts. The enhanced target scope of the new recombinant virus with regard to tumor cell lines makes it an excellent oncolytic alternative to the prototype vaccine strain of poliovirus.

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