UDC 615.37

Original article

ADVANCING THE PRODUCTION OF VIRAL VECTORS FOR CAR-T CELL THERAPY IN KAZAKHSTAN

Syzdykova L.R*, Keyer V.V., Arsienko R. Y., Inirbay B.K., Shustov A.V.

National center for biotechnology, 13/5 Korgalzhyn Road, Astana, 010000, Kazakhstan
**syzdykova@biocenter.kz*

ABSTRACT

Therapy of hematological tumors with chimeric antigen receptor-expressing T-cells (CAR-T) is a recent technology which holds promise to become the most significant achievement in oncohematology over the past 20 years. One aspect of the clinically applied CAR-T technology is that the process of production of CAR+ cells requires viral vectors which are used to deliver the CAR gene in T lymphocytes. At present the production of CAR-T therapy depends on the availability of packaged lentiviral or retroviral vectors. There is a worldwide deficiency in a production capacity to produce CAR vectors, and clinical-use vectors are not sold as off-the-shelf products.

At the National center for biotechnology the CAR receptor was assembled and this CAR targets the CD19 antigen which is a marker of tumor cells of the B-cell origin, lentiviral vectors were constructed. Studies on the production of packaged vectors were conducted at which three types of transfection reagents were compared for efficiency, in terms of the produced functional titers. A method was developed to measure functional titers using flow cytometry. High titers of the packaged vectors were obtained.

As the CAR-T is effective for treatment of patients after failure of traditional therapy, with relapse or refractory disease, all works on transfer of the CAR-T technology to Kazakhstan are of life importance to the patients with blood cancers in the country. The results underscore a necessity to organize a full production process to produce CAR vectors for clinical use and therapeutic cells for the CAR-T therapy at the NCB.

Key words: viral vectors, CAR-T therapy, transfection, lentiviruses, polyethylenimine, hematological tumors.

INTRODUCTION

An important achievement in the genetic engineering is that genetic modification of cultured primary human cells has moved beyond purely scientific research and gained a practical utilization in clinic for the treatment of patients. These are gene therapy which is based on making controlled modifications to the genome of patient's own cells, and novel technologies such as chimeric antigen receptor T-cells (CAR-T) which is actually a treatment with genetically modified (GM) lymphocytes. The CAR-T has been approved by a variety of regulatory authorities in major biopharmaceutical markets such as the US, EU and South-East Asia. In this industry, viral vectors are currently the most commonly used tools for delivering genes either in vivo for gene therapy, or ex vivo for GM-cells therapy. Among the clinical viral vectors those based on adeno-associated virus (AAV), lentiviruses (LV) and retroviruses (RV) are of particular importance [1].

The technology of T-cells modified with chimeric antigen receptor, also known as CAR-T, is a prominent example of adoptive cellular immunotherapy which already has a great importance in oncohematology. The CAR-T in its clinical applications relies completely on the use of LV and RV [2].

Traditional treatments for cancer, such as surgery, chemotherapy, and radiation therapy, probably reached the limit in inherent therapeutic efficiency. For treatment of particular tumors, therapeutic monoclonal antibodies which target tumor-specific antigens, or immune checkpoint inhibitors, may be used. However, the CAR-T technology has emerged as a breakthrough in the treatment of hematological tumors: leukemia, lymphoma and myeloma [3].

The CAR-T technology being an adoptive cell immunotherapy, is essentially a means to direct patient's own immunity against tumor cells by injecting the patient with GM T-lymphocytes capable of attacking cellular targets which carry tumor-specific or tumor-associated antigens on their surface. CAR-T therapy is implemented as follows: 1. leukocytes (WBC) are collected from peripheral blood from a patient; 2. CD3+ cells are isolated from the WBC. Upon that, CD3+ cells are cultured for some time in ex vivo conditions to activate proliferation. 3. CD3+ are genetically modified so that the cells start producing a chimeric antigen receptor (CAR), a genetically engineered protein which makes T cells to recognize tumor target cells. 4 the resulting CAR+ cells are expanded in ex vivo cultures and injected into the patient. A CAR+ cells dose per injection is in the range of 10^6-10^8 cells per 1 kg of patient weight. Then, in the patient's body, CAR+ cells persist, proliferate and carry out effector functions. For example, GM cytotoxic T-lymphocytes (CAR+ CTL) eliminate tumor cells, resulting in a therapeutic effect. Treatment of blood tumors using the CAR-T has a clinical success in the majority of patients, and many (>50%) patients have the complete elimination of tumor cells from the bone marrow and peripheral blood, and achieve a long-term (>5 years) remission. Currently there are sufficient data from clinical trials and from routine use of approved CAR-T therapy to show the ability of CAR-T to rescue terminal oncohaematology patients after a failure of traditional treatments.

An important aspect of the technology is that to produce clinical-grade CAR+ cells, the cultured CD3+ cells are infected with packaged lentiviral or retroviral vectors (LV/RV). Such use of LV or RV in the field is because the vectors not only deliver the CAR gene to lymphocytes, but also integrate the CAR transgene into the host genome providing a stable expression.

A CAR receptor has an extracellular domain for immuno-

logical recognition of the target antigen, a transmembrane domain and intracellular signaling domains, namely the CD3z (common T-cell co-receptor molecule) signaling domain, and one or more co-stimulatory domains (such as 4-1BB, etc.). The CARs are classified into several "generations" having varying molecular designs [4].

As was already mentioned, the CAR-T therapy demonstrates remarkable clinical efficiency. For example, the CAR-T with a receptor specific to human antigen CD19 is used to treat B-cell malignancies and shows a complete response (CR) of 93% in acute lymphoblastic leukemia [5-6], 54% in non-Hodgkin's lymphoma [7-9] and 67% in mantle cell lymphoma [10]. One revolutionizing feature of the CAR-T technology is that CAR-T is effective in patients who have failed conventional treatment and present after the last line of convenbtional therapy with a relapse or refractory disease. Because the CAR-T is capable of providing a complete remission in such cases, which otherwise have no more therapeutic options, the CAR-T has become a game-changer therapeutic technology.

Increasing the availability of viral vectors (LV and RV) is a prerequisite for the transfer of the CAR-T technology in Kazakhstan, as well as in other developing countries. In our parts of the world there is still no production of LV and RV for medical use, in amounts needed. Also, prices for such vectors in the world market are exorbitantly high. In fact, the whole world is experiencing a shortage of the production capacity for packaged LV and RV (for clinical use). Moreover, current practice in the field is that viral vectors are not offered in open markets as ready-to-purchase products. Rather, an interested user must find a contract management organization (CMO) which will produce on-demand; almost all providers of CAR-T services have their own vector production facilities but do not offer the vector as a separate product. The authors of this work, having encountered the similar problem, are convinced that the shortage of a clinical-grade vector is among the most important obstacles which slow down the acquisition of the CAR-T technology to Kazakhstan. As the shortage of vectors is actually worldwide problem, it is constantly reverberated in the industry's media, e.g. the Executive Vice President of the Pharmaceutical Division of Johnson & Johnson, Jennifer Taubert summarized this as follows: «Lentivirus remains the rate-limiting factor in the development of all types of cell therapies» [11].

In preparation to conducting a transfer of the CAR-T technology to Kazakhstan, efforts were made to start producing LV at the National center for biotechnology (Astana). This work describes a process of engineering and producing the packaged LV for CAR-T, optimization of packaging conditions and a method to determine LV titers.

MATERIALS AND METHODS

Cell Lines

HEK293FT cells were kindly provided by Prof. E.R. Bulatov from Kazan Federal University (Kazan, Russia). HEK293FT cells have an integrated gene encoding the SV40 large T-antigen and stably express this protein, which was shown to increase titers of packaged LVs. The cells were cultured in DMEM (Gibco, USA) supplemented with 10% fetal serum (FBS, Gibco, USA), 1% penicillin/streptomycin, 1% non-essential amino acids and 1% L-glutamine (all components from ThermoFisher, USA).

CD19 CAR Receptor and CAR-GFP Fusion Protein

The gene encoding CD19 CAR receptor (Figure 1) was constructed by the authors of this work using de novo synthe-

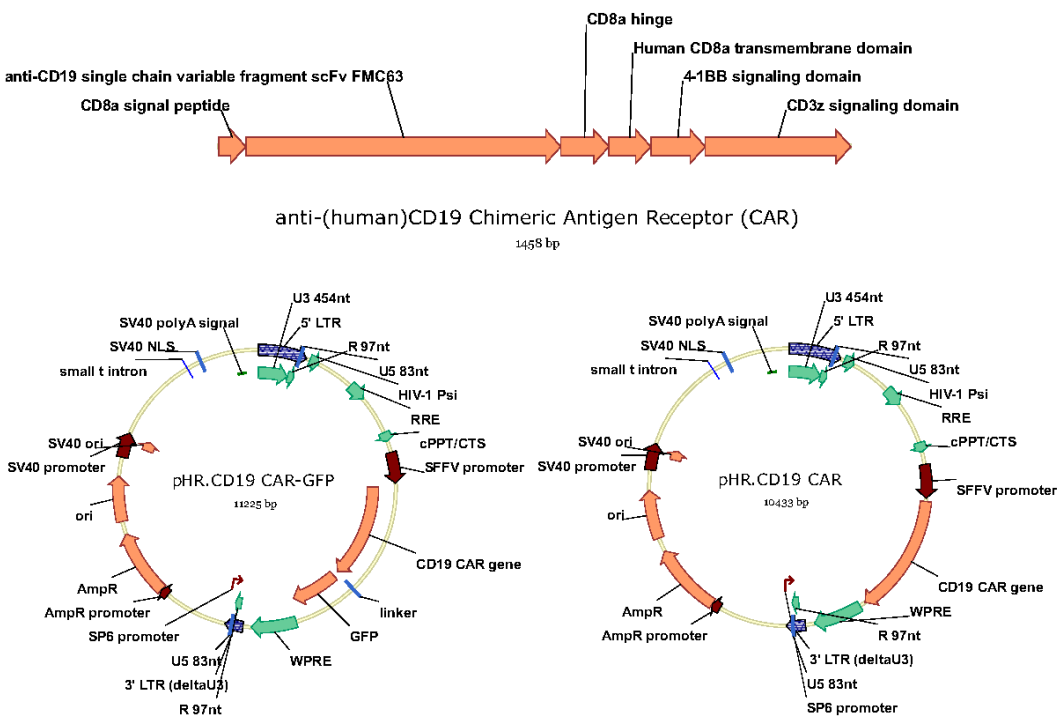


Figure 1. Top panel, a schematic for the chimeric antigen receptor CD19 CAR which recognizes the CD19 antigen present on the majority of cells in the B-cell lineage. Bottom panel, genetic maps for the plasmids pHR.CD19_CAR-GFP and pHR.CD19_CAR. Both plasmids carry lentiviral vectors (LVs) with a transgene (CAR or CAR-GFP) placed under control of an internal promoter, the spleen focus-forming virus (SFFV) LTR promoter.

sis of DNA fragments. The synthetic gene CD19 CAR codes for a 486 a.a.-long protein, which is composed from the following sequences and domains: signal peptide of the human CD8a (21 a.a.); a fragment of the single-chain monoclonal antibody FMC63 (242 a.a.) which serves as an extracellular domain of the CAR receptor; CD8a hinge domain (37 a.a.); CD8a transmembrane domain (32 a.a.); intracellular signaling domain from 4-1BB (42 a.a.); CD3z signaling domain (112 a.a.). All amino-acid sequences, except scFv FMC63, are from human proteins.

For one of the planned vectors, an additional gene was produced which codes for a fusion protein of the CD19 CAR receptor with a fluorescent marker, green fluorescent protein (GFP). This fusion protein is composed from (from N- to C-terminus) CD19 CAR, a non-cleavable glycine-rich linker (24 a.a.) and a GFP protein (240 a.a.).

Construction of Lentiviral vectors

The plasmid pLenti-C-Myc-DDK-IRES-Puro (OriGene Cat. PS100069) was used as a starting construct to produce the planned LVs. Heterologous inserts between HIV-1 terminal repeats (LTRs) were removed from the parental construct, except for sequences important for lentivirus replication. The 5'LTR region, self-inactivating (SIN) 3'-LTR, and signals important for the replication and packaging (packaging signal (Psi), central polypurine tract/central termination sequence cPPT/CTS), as well as post-transcriptional regulatory element (WPRE), were retained and are present in the LVs created in this work. The promoter of spleen focus-forming virus (SFFV) was cloned between cPPT/CTS and WPRE, and the CD19 CAR or CAR-GFP genes were inserted under the control of the SFFV promoter. The resulting plasmids were named pHR.CD19_CAR and pHR.CD19_CAR-GFP. Genetic maps for the plasmids are presented in Figure 1.

All plasmids for transfections were produced by isolation from cultures of transformed XL1 Blue cells. Plasmid DNA was isolated using the alkaline lysis method and purified by banding in cesium chloride gradients.

Lentivirus Vector Packaging

HEK293FT cell were cultured in T-175 flasks and transfected with mixtures of plasmids consisting of a transfer vector, packaging helper (psPAX2, Addgene Cat. 12260) and envelop plasmid VSV-G (pMD2.G, Addgene Cat. 12259). During optimization experiments, transfections were carried out using different transfection reagents, i.e. two types of polyethylenimine (PEI) or cationic liposomes. Branched PEI (Mw~25 kDa, Sigma Cat. 408727), or linear PEI MAX Mw~ 40 kDa (Polysciences Cat. 24765) or cationic liposomes Lipofectamine 2000 (ThermoScientific Cat. 11668500) were used as transfection reagents. For typical transfection, HEK293FT cells were seeded in T-175 flasks to achieve confluency 40-60% on the day of transfection.

Transfection with PEI was done as follows: a mixture of plasmids was prepared, consisting of the transfer vector pHR.CD19_CAR or pHR.CD19_CAR-GFP; psPAX and pMD2G in a mass ratio of 3:3:1. A PEI solution in water (1 mg/ml) was added to the plasmids mixture in a mass ratio 3:1 (PEI/DNA). The resulting mixture was incubated at room temperature for 15-20 minutes. Medium was removed from flasks with HEK293FT cells, and the monolayers were washed with PBS. The DNA:PEI complex was slowly added to the cells and this solution was distributed to cover the cells. Serum-free medium was added to the final volume 20 ml and the cultures were incubated for 4 h in a CO2-incubator. After 4 h, the medium containing the transfection mixture was replaced with complete growth medium.

Transfection with Lipofectamine was done using the protocol recommended by the manufacturer. A mixture of plasmids was prepared. Lipofectamine 2000 reagent was added to the plasmid mixture in a 3:1 ratio (3 µl of Lipofectamine 2000 for every 1 µg of DNA). OptiMEM serum-free medium (ThermoScientific Cat. 31985062) was added to the transfection mixture and the mixture was applied to cell monolayers. The volume was adjusted with OptiMEM medium to 20 ml in the flask. The cultures were incubated overnight (18-20 h). The next day, the medium was replaced with complete growth medium. At 24, 48, and 72 h after transfection, medium containing packaged vector particles was collected and used for determining the LV titers.

Determining the Functional Titers Using Flow Cytometry

Titers of the packaged GFP-expressing vector were determined by infecting cells in 6-well plates with different dilutions of the LV particles, followed by measuring a fraction (%) of GFP+ cells by flow cytometry. Functional LV titers, e.g. numbers of transducing particles per 1 ml, were then computed from known numbers of seeded cells and GFP+ percents.

10-fold dilutions of the LV were prepared in complete growth medium supplemented with 8 µg/ml polybrene (Sigma Cat. H9268). HEK293FT cells were seeded into 6-well plates in amount of 2 □ 10^5 cells per well. After 4 h, medium was removed from the wells and 2 ml-aliquots of LV dilutions were added into the wells. The plates were incubated for 24 h. Then, medium was replaced with complete growth medium (DMEM with 10% FBS) without polybrene. GFP fluorescence was monitored for several days by examining the plates under a fluorescence microscope. At 72 h after transduction, cells from the transduced cultures were detached by trypsin/EDTA treatment and pelleted by centrifugation (1000 rpm for 5 min). Cells were washed twice with 1x PBS (resuspended and pelleted again). Final cell pellet was resuspended in 1 ml of cytometry buffer (FACS buffer: PBS + 0.5% BSA + 0.1% NaN3).

To measure the fraction (%) of GFP+ cells, MACS Quant 10 flow cytometer (Miltenyi Biotec, Germany) was used. GFP fluorescence was detected in channel B1 488/(530/30) nm. Forward Scatter Channel (FSC) and Side Scatter (SSC) Channel were used to set up gates to filter out debris. To set up the GFP+ gate for the cytometer, control GFP-producing and fluorescent cells were used; the latter have been constructed by infceting HEK293FT cells with a GFP-producing alphavirus (VEEV/GFP-SEAP virus, which infects all cells in culture and makes all cells GFP+). A sample of naïve HEK293FT cells was used to set up a gate for GFP-negative cells.

After fractions of GFP+ cells in wells (transduced with the LV) were measured, titers were calculated. Only cultures in which the fraction (GFP+) was less than 5% were used to compute titers. In our experiments, this commonly

corresponds to the LV-dilution 1/1000. Functional titer was calculated as, Titer (transducing units in 1 ml, TU/ml) = ((%GFP+)/100) x 2 x 10^5 (number of seeded cells) x D (LV dilution) x 5 (factor to correct for the volume of inoculum 200 µl).

RESULTS

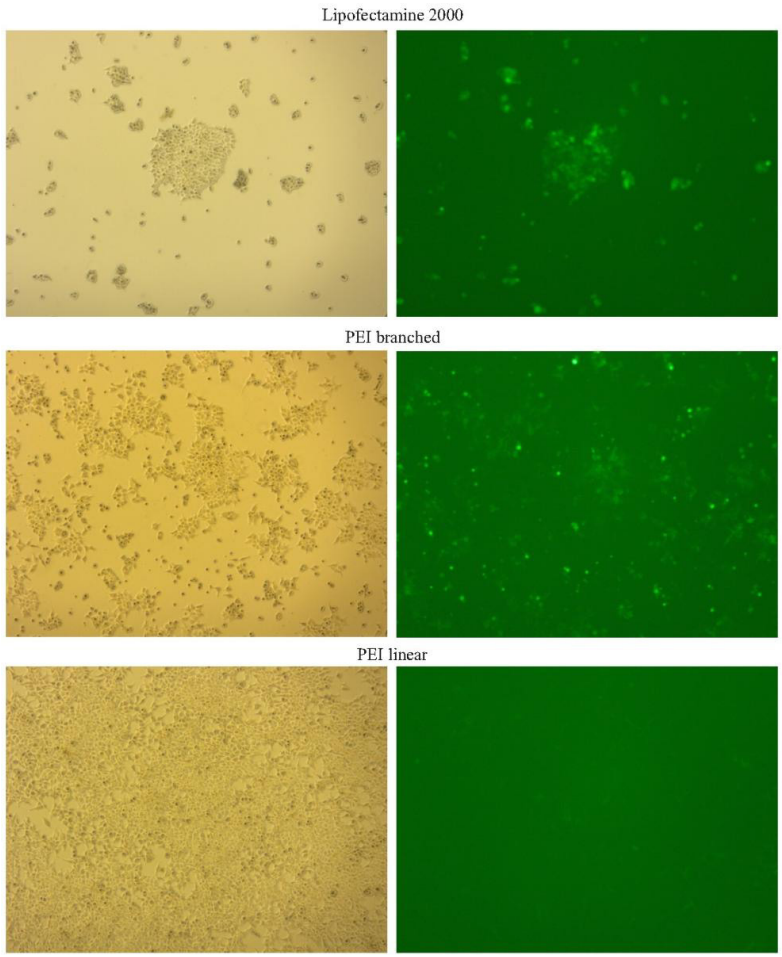
Construction of CAR, Transgenes and Lentiviral Vectors

A gene encoding receptor CAR which has an extracellular domain targeting human CD19 antigen (CD19 CAR) was synthesized in constructive PCR. The molecular design of the CAR gene was done with accordance to the principles defined in the work [12]. This CAR is of the second generation, i.e. its cytoplasmic part has two activation domains, one from CD3z and the other from costimulatory molecule 4-1BB. The CAR contains sequences derived human proteins, except for the extracellular domain which is a humanized murine single-chain antibody. This CAR was designed to modify human T-lymphocytes and change their phenotype to CAR+ cells. In potential, the resulting CAR+ cells will gain a use in therapy against B-cell line (CD19+) hematological tumors.

Two constructs were engineered: pHR.CD19_CAR and pHR.CD19_CAR-GFP (Figure 1). Shown plasmids carry the lentiviral vectors LV/CD19_CAR and LV/CD19_CAR-GFP, respectively. Transgenes in the LVs are under the control of the LTR promoter of spleen focus-forming virus (SFFV); this promoter is highly active in hematological cells and also has acceptable activity in HEK293FT cells. The LV/CD19_CAR is a transfer vector with CD19 CAR and no other transgene-coded expressed proteins. This vector has been intended for the use in future CAR-T therapies. A different vector LV/CD19_CAR-GFP has a transgene of CD19 CAR genetically fused to GFP. The presence of GFP provides production conveniences: the GFP fluorescence in packaging cultures makes easy controlling the transduction efficiency; also the transduction of GFP during titering enables simple titering using flow cytometry. This means, when naive cells are transduced, a fraction of GFP+ cells is proportional to the functional titer, at least when the multiplicity of infection (MOI) is sufficiently low. The GFP-producing vector was designed to be used in experiments for optimization of packaging conditions.

Lentivector Packaging

Experiments were carried out to optimize packaging protocols and maximize the packaged LV titers. A second-generation packaging system (pHR.CD19_CAR-GFP + psPAX2 + pMD2.G) was used. Three transfection reagents were compared. These are branched PEI (Sigma), linear PEI (Polysciences), and cationic liposomes (Lipofectamine 2000 from Thermo Scientific). Photographs of cultures after transfection with the use of the transfection reagents are shown in



Panels (top to bottom): cultures transfected with the use of Lipofectamine 2000, branched PEI, and linear PEI. Same microscopic fields are shown: bright field at left panels; GFP fluorescence at right panels. Objective magnification 5X.

Figure 2. Photographs of packaging cultures.

Figure 2. Transfection with pHR.CD19_CAR-GFP results in the appearance of GFP+ cells. Fractions of GFP+ cells at 48 h post-transfection (hpt) appeared to be high (>90% by microscopy) in cultures with Lipofectamine 2000 and branched PEI (Figure 2). In a culture with linear PEI, there were few GFP+ cells at 48 hpt. Cell viability as determined by microscopy also varied, as fewer live cells remained in a culture with Lipofectamine 2000; more live cells were present in PEI cultures. It follows, that Lipofectamine 2000 is more toxic to cells compared to branched PEI. Medium from the packing cultures was collected at 24, 48 and 72 hpt; complete replacement of the medium was done. The collected samples were used to determine functional titers of packaged LV, because the titer is the main parameter when choosing the optimal packaging protocol.

Flow Cytometry for Titering

In this work, a method was developed and used to determine functional titers of packaged LVs. The method applies to LVs which transduce a gene for fluorescent marker and utilizes flow cytometry. Fixed numbers of cells were seeded and infected with dilutions of the GFP-transducing LV. Upon entry into cell, LV RNA-containing capsid provides genomic integration of the transgene. Then, transcription of the transgene and translation of the mRNA lead to accumulation of GFP in the cytoplasm. Thus transduced cells may be counted using fluorescence microscopy or flow cytometry. The cells upon infection with LVs are given sufficient time to develop fluorescence (72 h), after which the cultures are examined using a flow cytometer. In this way, fractions of GFP+ cells are measured. Virus titers are computed from the collected data.

Profiles of fluorescence intensity in channel B1 (the channel to detect GFP) for cultures in titering experiment are graphed in Figure 3. These pertain to cultures infected with LV/CD19_CAR-GFP at 72 hpi. The results show histograms of the intensity of GFP fluorescence which developed in cultures infected with different dilutions (from 1/10 to 1/10^6) of three samples. These samples are media collected at 24 hpt, 48 hpt and 72 hpt from the same packaging culture. The histograms at Figure 3 correspond well to what was observed by visual microscopy of the same wells. Expectedly, fractions of GFP+ cells decrease with the dilution. Also, amounts of GFP+ cells in the titration experiment are higher and similar for samples collected at 24 hpt and 48 hpt, and ~2-fold lower in the last sample (72 hpt).

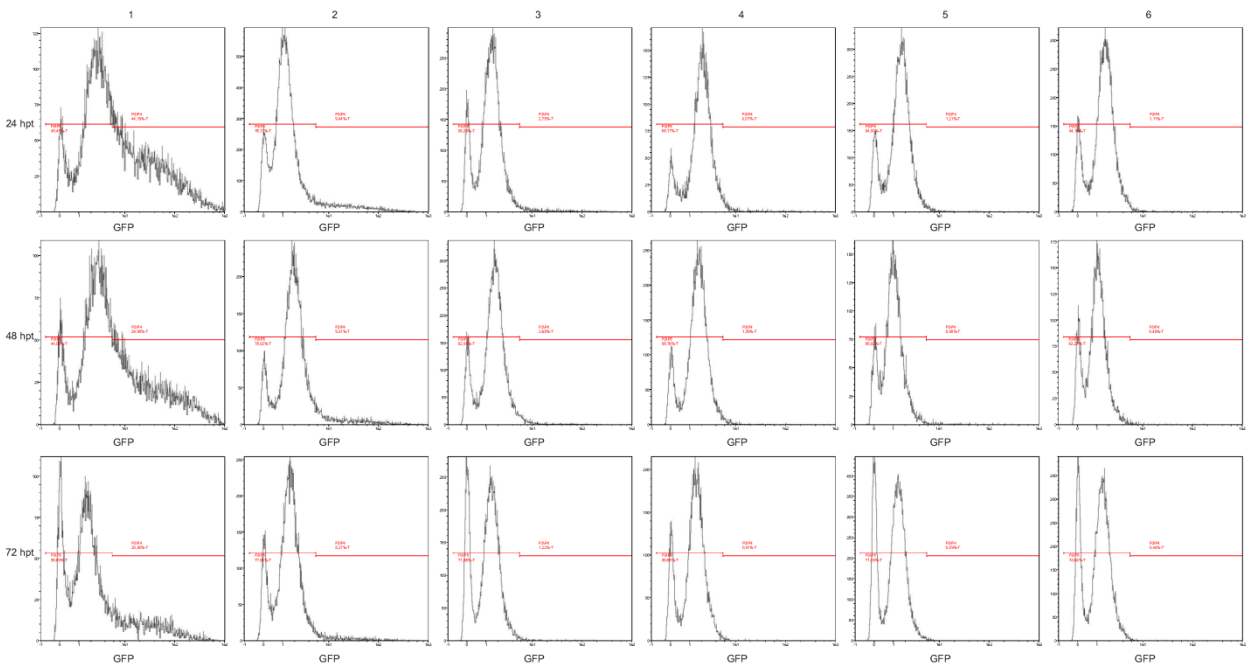
The flow cytometry enables accurately measuring fractions of transduced (GFP+) cells as shown in Figures 3 and 4. The plot at Figure 4 depicts how the fraction of the transduced cells changes with increasing different dilution of transducing particles. Of three curves in Figure 4 each pertain to one sample, namely LV-particles-containing media collected form a packaging culture at 24 hpt, 48 hpt 72 hpt.

Three different samples of LV particles (labeled: 24 hpt, 48 hpt, 72 hpt) were used to produce dilutions (1/10 - 1/10^6). Cell cultures in wells (in 6-well plates) were infected with the dilutions. Then fractions (%) of transduced cells were measured and plotted against the dilution number.

DISCUSSION

This work contains results of testing of a set of transfecting reagents in experiments for lentiviral vectors (LV) packaging and describes a method to measure LV functional titers. This research was conducted as a part of works which have being conducted in the National Center of Biotechnology to transfer the CAR-T technology to Kazakhstan.

To successfully localize the CAR-T therapy and make it economically sustainable it is necessary to organize produc-



Cultures in wells of 6-well plates were infected with dilutions of the GFP-transducing LV. Panels (left to right) represent different wells in the same 6-well plate (i.e. different dilutions of one sample). Rows (top to bottom) represent different samples: 24 hpt (top), 48 hpt (middle) and 72 hpt (bottom). Histograms show the fluorescence intensity by X-axis, cell counts by Y-axis.

Figure 3. Results of flow cytometry of cultures infected with serial dilutions of GFP-transducing LV particles: histograms of the intensity of GFP fluorescence in infected cultures.

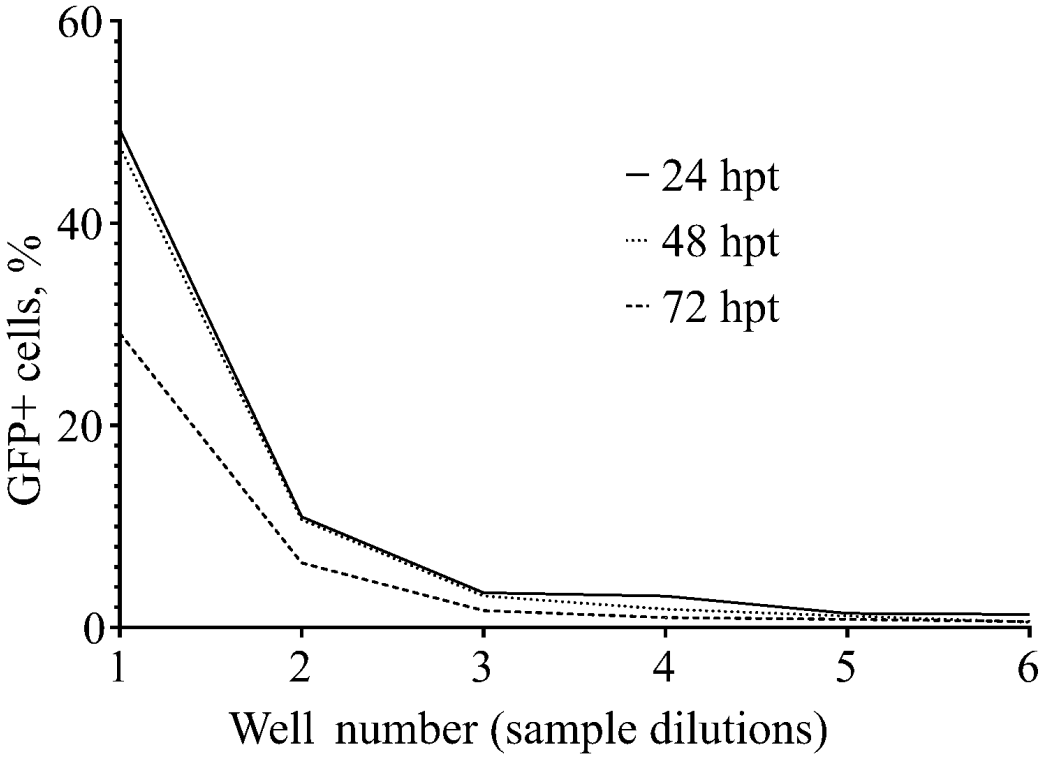


Figure 4. Fractions of GFP+ cells in cultures infected with different increasing dilutions of packaged LVs.

Functional titers which are expressed as amount of transducing units per ml (TU/ml) were determined from percents of transduced cells at dilution 1/1000 (which corresponds to less than 5% GFP+ cells). The functional titers were: 34.4 million TU/ml (24 hpt), 31.3 million TU/ml (48 hpt) and 16.8 million TU/ml (72 hpt). Thus, titers are at a level of 10^7 TU/ml which in general correspond to the titers (before concentration) published in the industry of LV manufacturing.

tion of the LVs locally, and ensure that the LVs have properties of suitability for clinical usage. Organizing the local LV production is much importance because of high prices of clinical-grade LVs and many obstacles in importation of LVs to Kazakhstan. In fact there is a worldwide deficiency of LVs for medical use. Moreover, packaged viral vectors are not sold like commodities and not available on world markets as off-the-shelf products. Large companies organize and maintain their own production of vectors which are commonly used only in-house. For example, in relation to CAR-T, such companies as Novartis or Kite Pharma sell CAR-T therapeutic services, but do not make their vectors commercially available. For those companies or clinics which do not have own vector production, the one available option is to contact a Contract Manufacturing Organization (CMO). There are CMOs in the market which can produce a clinical-grade packaged viral vector provided that they are supplied with a vector-containing plasmid (constructing this plasmid is a customer's responsibility). However, prices remain high, as a standard price tag is USD 50,000 per 1 LV lot (amount sufficient to treat 1 patient). This level of prices makes impossible treatment of patients in Kazakhstan with the use of imported vectors and maintain economical feasibility of the local CAR-T production. This underscores a worldwide shortage of viral vectors which are used for ex vivo or in vivo therapy. Actually, there is a worldwide shortage of production capacity for the industrial production of packaged LVs and RVs.

At present, the CAR-T technology which is actually applied to treat patients, uses only one means of creating CAR+ cells, this is viral transduction with LVs or RVs [13]. Vectors of these two types provide the reverse transcription and genomic integration of transgene. As a result of genomic integration, the transgene is not lost during cell division, leading to stable expression of the transgene [14]. This is important in CAR-T therapy, because the CAR+ cells are injected into the patient's body in limited numbers, but upon injection the CAR+ cells undergo multiple divisions so that CAR+ population may expand 10000 times.

To date, five CAR-T therapies are registered (approved for the use apart from clinical trials), of which two therapies (Yeskarta and Tecartus) are made using RVs, and three others (Kymriah, Breyanzi, Abecama) are produced using LVs.

All LVs having permits for the use in producing therapeutical cells, were developed from the genome of the human immunodeficiency virus type 1 (HIV-1) [15]. At present such vectors are of the 2nd or 3rd generation. The classification by generations applies to systems for packaging. For example, the 1st generation uses vectors which differ from the HIV-1 genome only in that the virion glycoprotein gp160 is deleted in the LV. To package these LVs it is sufficient to supply only the envelope glycoprotein in trans. Later generations have all protein-coding sequences removed from the LV. Thus, all proteins required for the replication, packaging and genomic integration must be provided in trans during packaging. In the 2nd generation packaging systems, at least three plasmids are present in a transfection mixture. Commonly these are a plasmid with a cloned LV with a transgene, named «transfer vector»; a plasmid to provide GAG-POL, Rev and Tat, called «packaging helper»; and a plasmid encoding an envelope protein, accordingly dubbed «envelope plasmid» [16]. In the 3rd generation, the genes for GAG-POL and Rev are separated into different plasmids, and also Tat is excluded from

the packaging system by placing an LV under the control of a Tat-independent promoter.

With regard to the real clinical use, vectors for the production of the registered CAR-T therapies are of the 2nd generation. When considering a selection of a packaging system, using more different plasmids in a mixture (during transfection) increases safety of the final product, but reduces titers and overall yields of vectors.

The most commonly used production method to produce packaged LVs, is transient expression of all components of packaging system in the same cell achieved by plasmid transfection [17]. In the transfected cell, those plasmids are transported into the cell nucleus because of the work of intracellular transport machinery, circulating transcription factors and transporters such as karyopherins. When in the nucleus, strong promoters in a transfer vector and helpers are recognized by RNA polymerase II. Subsequently, an LV RNA is transcribed, and mRNAs for helper proteins are transcribed. In particular cases such RNAs may undergo splicing, ultimately these RNAs are transported to the cytoplasm. In the cytoplasm, the LV RNA participates in the process of packaging into a nucleocapsid, which is a central structural element within virion. By the translation, polyproteins GAG/GAG-POL, envelope protein and accessory proteins, transactivators such as Rev, if needed, are synthesized on ribosomes. Proteolytic processing of GAG/GAG-POL results in the formation of functional proteins (capsid protein, nucleocapsid protein, matrix protein) and viral enzymes (protease, RNA-dependent DNA polymerase and integrase). The packaging process starts when the viral structural proteins recognize the packaging signal in LV RNA and form an RNA-protein complex (immature capsid), which also includes protease, integrase and revertase. Immature capsid interacts with cytoplasmic tails of envelope protein on the plasma membrane. At sites of this interaction, new virions are formed.

Alternative vector-packaging approaches are known. Of these, stable packaging cell lines (PCLs) are being developed. PCL is a cell line which constitutively produces one or more components of a packaging system. Packaging with a PCL allows using fewer numbers of plasmids in transfection mixtures, which in turn increases efficiency of transfection. PCL have been produced for RVs, for example, such PCLs are Phoenix-ECO and Phoenix-AMPHO. However, for LVs no industrially applicable PCLs have been developed so far. This is because particular viral proteins required in the LV packaging process are toxic when produced intracellularly. Cellular toxicity is well described for HIV-1 protease PR and envelope glycoprotein VSV-G (the glycoprotein most commonly used for LV packaging).

The transient transfection for LV packaging is carried out using chemical transfection facilitators which are needed to enable cell entry of plasmid DNA. The majority of published protocols use cationic liposomes or polyethylenimine (PEI) as transfection reagents. However, older transfection methods, namely co-precipitation of plasmid DNA with calcium phosphate (CaPO4), are actually effective and remain of practical importance. When optimizing a method of transfection, the key result is a high functional titer of the packaged LV. Functional titer measures amounts of «full-biologically-active» particles which infect and transduce target cells and is expressed in transducing units (TU) in 1 milliliter. Functional titer is different from physical titer, which measures numbers of all particles or merely a concentration of virus antigen. Also, apart from the titer, other considerations may appear important such as costs of materials, availability of equipment, possibility to scale up the production.

A variety of approaches have been described to determine LV titers. ELISA to measure virion's p24 antigen is routinely used to determine physical titer. It is possible to use quantitative RT-PCR to measure RNA in the particles. However, determining functional titer is a more laborious procedure because it requires testing by infecting live cells. After sufficient time is given to the cells to undergo molecular processes of genomic integration (of transgene), transcription, translation, etc., the cells expressing the transgene can be counted [18].

In this work, the authors utilized a 2nd generation packaging system and tested three transfection reagents (Lipofectamine 2000, branched PEI, linear PEI) in comparing for the LV-packaging efficiency. The highest titers were obtained with Lipofectamine 2000 and branched PEI. However, high costs of the commercial compound Lipofectamine prevent the routine use at a large scale, e.g. for industrial vector production. Branched PEI is an inexpensive reagent and can be used in the industrial-scale processes.

Flow cytometry was used in experiments for titration. Fractions of transduced GFP-positive cells were measured by the cytometry and appeared to be in good correspondence with what was observed by visual microscopy. Advantages of flow cytometry include comparably low labor load and high throughput. Thus the flow cytometry seems to be a preferred method for measuring functional titers [19].

Producing packaged vectors for the therapeutic use remains an expensive process. The costs of the vector were reported to be 10-30% of the price of the CAR-T therapy. To supply domestic needs for the CAR-T therapy in Kazakhstan, it is necessary to organize local production of packaged LVs, starting from a testing yearly production rate of 20-40 lots (approx. 10^10 TU).

CONCLUSION

At present the CAR-T therapy looks like the most significant achievement in treatment of blood cancers over the past 20 years. It is the first therapy with genetically modified cells which gained mass usage in the clinic. Obviously, it is important for Kazakhstan's science and technology to keep pace with such prominent achievements by doing a transfer of the CAR-T technology and industrial vectors production in the country. Results are presented to illustrate steps undertaken by the NCB's scientists to ultimately set up production processes for the vector and CAR-T cells.

FUNDING

This research has been funded by the Science Committee of the Ministry of Science and Higher Education of the Republic of Kazakhstan (Grant No. AP19678901).

LITERATURE

1. Quinonez R., Sutton R.E. Lentiviral Vectors for Gene Delivery into Cells // *DNA and Cell Biol-*

ogy. – 2004. – Vol. 21, № 12. – P. 937-951. https://doi.org/10.1089/104454902762053873

2. Labanieh L., Majzner R.G., Mackall C.L. Programming CAR-T cells to kill cancer // Nature Biomedical Engineering. – 2018. – Vol. 2. – P. 377-391. https://doi.org/10.1038/s41551-018-0235-9

3. Perica K., Varela J.C., Oelke M., Schneck J. Adoptive T Cell Immunotherapy For Cancer // Cancer Immunotherapy. – 2015. – Vol. 6, № 1. – P. e0004. https://doi.org/10.5041%2FRMMJ.10179

4. Rafic S., Hackett C.S., Brentjens R.J. Engineering strategies to overcome the current roadblocks in CAR T cell therapy // Nature Reviews Clinical Oncology. – 2020. – Vol. 17. – P. 147-167. https://doi.org/10.1038%2Fs41571-019-0297-y

5. Maude S.L., Laetsch T.W., Buechner J., Rives S., Boyer M., Bittencourt H., Bader P., Verneris M.R., Stefanski H.E., Myers G.D., Qayed M., De Moerloose B., Hiramatsu H., Schlis K., Davis K.L., Martin P.L., Nemecek E.R. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia // N. Engl. J. Med. - 2018. - Vol. 378, No. 5. - P. 439-448. https://doi.org/10.1056/nejmoa1709866

6. Shah B.D., Ghobadi A., Oluwole O.O., Logan A.C., Boissel N., Cassaday R.D., Leguay T., Bishop M.R., Topp M.S., Tzachanis D., Arellano M.L., Lin Y., Baer, M.R., Schiller G.J., Park J.H., Subklewe M., Abedi M. KTE-X19 for relapsed or refractory adult B-cell acute lymphoblastic leukaemia: phase 2 results of the single-arm, open-label, multicentre ZUMA-3 study // Lancet. - 2021. - Vol. 398. - P.491-502. https://doi.org/10.1016/s0140-6736(21)01222-8

7. Abramson J.S., Palomba M.L., Gordon L.I., Lunning M.A., Wang M., Arnason J., Mehta A., Purev E., Maloney D.G., Andreadis C., Sehgal A., Solomon S.R., Ghosh N., Albertson T.M., Garcia J., Kostic A., Mallaney M., Ogasawara K. Lisocabtagene maraleucel for patients with relapsed or refractory large B-cell lymphomas (TRANSCEND NHL 001): a multicentre seamless design study // Lancet. - 2020. - Vol. 396. - P. 839-852. https://doi.org/10.1016/s0140-6736(20)31366-0

8. Neelapu S.S., Locke F.L., Bartlett N.L., Lekakis L.J., Miklos D.B., Jacobson C.A., Braunschweig I., Oluwole O.O., Siddiqi T., Lin Y., Timmerman J.M., Stiff P.J., Friedberg J.W., Flinn I.W., Goy A., Hill B.T., Smith M.R. Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma // N. Engl. J. Med. – 2017. – Vol. 377, - 2017. - Vol. 377, № 26. – P. 2531-2544. https://doi.org/10.1056/nejmoa1707447

9. Schuster S.J., Svoboda J., Chong E.A., Nasta S.D., Mato A.R., Anak Ö., Brogdon J.L., Pruteanu-Malinici I., Bhoj V., Landsburg D., Wasik M., Levine B.L., Lacey S.F., Melenhorst J.J., Porter D.L., June C.H. Chimeric antigen receptor T cells in refractory B-cell lymphomas // N. Engl. J. Med. – 2017. – Vol. 377, № 26. – P. 2545-2554. https://doi.org/10.1056/nejmoa1708566

10. Wang M., Munoz J., Goy A., Locke F.L., Jacobson C.A., Hill B.T., Timmerman J.M., Holmes H., Jaglowski S., Flinn I.W., McSweeney P.A., Miklos D.B., Pagel J.M., Kersten M.J., Milpied N. KTE-X19 CAR T-cell therapy in relapsed or refractory mantle-cell lymphoma // N. Engl. J. Med. – 2020. – Vol. 382, № 14. – P. 1331-1342. https://doi.org/10.1056/nejmoa1914347

11. Stanton D. Commercial CAR-Ts: Ensuring lentivirus supply key for J&J and BMS // *BioProcess International*. – 2022. https://bioprocessintl.com/bioprocess-insider/global-markets/commercial-car-ts-ensuring-lentivirus-supply-key-for-jj-and-bms/

12. Sadelain M., Brentjens R., Rivière I. The basic principles of chimeric antigen receptor design // Cancer Discov. – 2013. – Vol. 3, № 4. – P. 388-398. https://doi.org/10.1158/2159-8290.CD-12-0548

13. Rodrigues A., Alves P. M., Coroadinha A. Production of retroviral and lentiviral gene therapy vectors: challenges in the manufacturing of lipid enveloped virus // *Viral Gene Therapy*. – 2011. - https://doi.org/10.5772/18615

14. Miller A.D. Retroviral vectors: from cancer viruses to therapeutic tools // Human Gene Tharapy. – 2014. – Vol. 25, № 12. – P. 989-994. https://doi.org/10.1089/hum.2014.2542

15. Irving M., Lanitis E., Migliorini D., Ivics Z., Guedan S. Choosing the right tool for genetic engineering: clinical lessons from chimeric antigen receptor-T cells // Human Gene Therapy. – 2021. – Vol. 32, № 19. – P. 1044-1058. https://doi.org/10.1089/hum.2021.173

16. Ding P., Kharytonchyk S., Kuo N., et.al. 5'-Cap sequestration is an essential determinant of HIV-1 genome packaging // PNAS. – 2021. – Vol. 118, № 37. – P. e2112475118. https://doi.org/10.1073/pnas.2112475118

17. Merten O-W., Hebben M., Boolenta C. Production of lentiviral vectors // Mol. Ther. Methods Clin Dev. – 2016. – Vol. 3. – P. 16017. https://doi.org/10.1038%2Fmtm.2016.17

18. Allahverdi A., Eskandari F. et.al. Lentiviral vectors titration using real-time PCR // Archives of Advances in Biosciences. – 2015. – Vol. 6, № 1. – P. 80-84. https://doi.org/10.22037/jps.v6i1.8324

19. Tang Y., Garson K., Li L., Vanderhuden B.C. Optimization of lentiviral vector production using polyethylenimine-mediated transfection // Oncology letters. - 2014. – Vol. 9, № 1. – P. 55-62. https://doi.org/10.3892/ol.2014.2684

REFERENCES

1. Quinonez R., Sutton R.E. Lentiviral Vectors for Gene Delivery into Cells // *DNA and Cell Biology*. – 2004. – Vol. 21, № 12. – P. 937-951. https://doi.org/10.1089/104454902762053873

2. Labanieh L., Majzner R.G., Mackall C.L. Programming CAR-T cells to kill cancer // Nature Biomedical Engineering. – 2018. – Vol. 2. – P. 377-391. https://doi.org/10.1038/s41551-018-0235-9

3. Perica K., Varela J.C., Oelke M., Schneck J. Adoptive T Cell Immunotherapy For Cancer // Cancer Immunotherapy. – 2015. – Vol. 6, № 1. – P. e0004. https://doi.org/10.5041%2FRMMJ.10179

4. Rafic S., Hackett C.S., Brentjens R.J. Engineering strategies to overcome the current roadblocks in CAR T cell therapy // Nature Reviews Clinical Oncology. – 2020. – Vol. 17. – P. 147-167. https://doi.org/10.1038%2Fs41571-019-0297-y

5. Maude S.L., Laetsch T.W., Buechner J., Rives S., Boyer M., Bittencourt H., Bader P., Verneris M.R., Stefanski H.E., Myers G.D., Qayed M., De Moerloose B., Hiramatsu H.,

Schlis K., Davis K.L., Martin P.L., Nemecek E.R. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia // N. Engl. J. Med. - 2018. - Vol. 378, No. 5. - P. 439-448. https://doi.org/10.1056/nejmoa1709866

6. Shah B.D., Ghobadi A., Oluwole O.O., Logan A.C., Boissel N., Cassaday R.D., Leguay T., Bishop M.R., Topp M.S., Tzachanis D., Arellano M.L., Lin Y., Baer, M.R., Schiller G.J., Park J.H., Subklewe M., Abedi M. KTE-X19 for relapsed or refractory adult B-cell acute lymphoblastic leukaemia: phase 2 results of the single-arm, open-label, multicentre ZUMA-3 study // Lancet. - 2021. - Vol. 398. - P.491-502. https://doi.org/10.1016/s0140-6736(21)01222-8

7. Abramson J.S., Palomba M.L., Gordon L.I., Lunning M.A., Wang M., Arnason J., Mehta A., Purev E., Maloney D.G., Andreadis C., Sehgal A., Solomon S.R., Ghosh N., Albertson T.M., Garcia J., Kostic A., Mallaney M., Ogasawara K. Lisocabtagene maraleucel for patients with relapsed or refractory large B-cell lymphomas (TRANSCEND NHL 001): a multicentre seamless design study // Lancet. - 2020. - Vol. 396. - P. 839-852. https://doi.org/10.1016/s0140-6736(20)31366-0

8. Neelapu S.S., Locke F.L., Bartlett N.L., Lekakis L.J., Miklos D.B., Jacobson C.A., Braunschweig I., Oluwole O.O., Siddiqi T., Lin Y., Timmerman J.M., Stiff P.J., Friedberg J.W., Flinn I.W., Goy A., Hill B.T., Smith M.R. Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma // N. Engl. J. Med. – 2017. – Vol. 377, - 2017. - Vol. 377, № 26. – P. 2531-2544. https://doi.org/10.1056/nejmoa1707447

9. Schuster S.J., Svoboda J., Chong E.A., Nasta S.D., Mato A.R., Anak Ö., Brogdon J.L., Pruteanu-Malinici I., Bhoj V., Landsburg D., Wasik M., Levine B.L., Lacey S.F., Melenhorst J.J., Porter D.L., June C.H. Chimeric antigen receptor T cells in refractory B-cell lymphomas // N. Engl. J. Med. – 2017. – Vol. 377, № 26. – P. 2545-2554. https://doi.org/10.1056/nejmoa1708566

10. Wang M., Munoz J., Goy A., Locke F.L., Jacobson C.A., Hill B.T., Timmerman J.M., Holmes H., Jaglowski S., Flinn I.W., McSweeney P.A., Miklos D.B., Pagel J.M., Kersten M.J., Milpied N. KTE-X19 CAR T-cell therapy in relapsed or refractory mantle-cell lymphoma // N. Engl. J. Med. – 2020. – Vol. 382, № 14. – P. 1331-1342. https://doi.org/10.1056/nejmoa1914347

11. Stanton D. Commercial CAR-Ts: Ensuring lentivirus supply key for J&J and BMS // *BioProcess International*. – 2022. https://bioprocessintl.com/bioprocess-insider/global-markets/commercial-car-ts-ensuring-lentivirus-supply-key-for-jj-and-bms/

12. Sadelain M., Brentjens R., Rivière I. The basic principles of chimeric antigen receptor design // Cancer Discov. – 2013. – Vol. 3, № 4. – P. 388-398. https://doi.org/10.1158/2159-8290.CD-12-0548

13. Rodrigues A., Alves P. M., Coroadinha A. Production of retroviral and lentiviral gene therapy vectors: challenges in the manufacturing of lipid enveloped virus // *Viral Gene Therapy*. – 2011. - https://doi.org/10.5772/18615

14. Miller A.D. Retroviral vectors: from cancer viruses to therapeutic tools // Human Gene Tharapy. – 2014. – Vol. 25, № 12. – P. 989-994. https://doi.org/10.1089/hum.2014.2542

15. Irving M., Lanitis E., Migliorini D., Ivics Z., Guedan S. Choosing the right tool for genetic engineering: clinical lessons from chimeric antigen receptor-T cells // Human Gene Therapy. – 2021. – Vol. 32, № 19. – P. 1044-1058. https://doi.org/10.1089/hum.2021.173

16. Ding P., Kharytonchyk S., Kuo N., et.al. 5'-Cap sequestration is an essential determinant of HIV-1 genome packaging // PNAS. – 2021. – Vol. 118, № 37. – P. e2112475118. https://doi.org/10.1073/pnas.2112475118

17. Merten O-W., Hebben M., Boolenta C. Production of lentiviral vectors // Mol. Ther. Methods Clin Dev. – 2016. – Vol. 3. – P. 16017. https://doi.org/10.1038%2Fmtm.2016.17

18. Allahverdi A., Eskandari F. et.al. Lentiviral vectors titration using real-time PCR // Archives of Advances in Biosciences. – 2015. – Vol. 6, № 1. – P. 80-84. https://doi.org/10.22037/jps.v6i1.8324

19. Tang Y., Garson K., Li L., Vanderhuden B.C. Optimization of lentiviral vector production using polyethylenimine-mediated transfection // Oncology letters. - 2014. – Vol. 9, № 1. – P. 55-62. https://doi.org/10.3892/ol.2014.2684

РАЗВИТИЕ ПРОИЗВОДСТВА ВИРУСНЫХ ВЕКТОРОВ ДЛЯ CAR-T – КЛЕТОЧНОЙ ТЕРАПИИ В КАЗАХСТАНЕ

Сыздыкова Л.Р.*, Кеер В.В., Арсиенко Р.Ю., Инирбай Б.К., Шустов А.В.

Национальный центр биотехнологии, Кургальжинское шоссе, 13/5, Астана, 010000, Казахстан
**syzdykova@biocenter.kz*

АННОТАЦИЯ

Терапия гематологических опухолей с помощью Т-клеток, экспрессирующих химерные антигенные рецепторы (CAR-T), является новейшей технологией, которая обещает стать самым значительным достижением в онкогематологии за последние 20 лет. Одним из аспектов клинически применяемой технологии CAR-T является то, что для процесса производства клеток CAR+ требуются вирусные векторы, которые используются для доставки гена CAR в Т-лимфоциты. В настоящее время производство CAR-T-терапии зависит от наличия упакованных лентивирусных или ретровирусных векторов. Во всем мире существует дефицит производственных мощностей по производству векторов CAR, а векторы для клинического использования не продаются как готовые продукты.

В Национальном центре биотехнологии был собран CAR-рецептор, нацеленный на антиген CD19, который является маркером опухолевых клеток В-клеточного происхождения, сконструированы лентивирусные векторы. Были проведены исследования по получению упакованных векторов, в ходе которых три типа реагентов для трансфекции сравнивались по эффективности с точки зрения вырабатываемых функциональных титров. Разработан метод измерения функциональных титров с помощью проточной цитометрии. Были получены высокие титры упакованных векторов.

Поскольку CAR-T эффективен для лечения пациентов после неэффективности традиционной терапии, с рецидивом или рефрактерным заболеванием, все работы по переносу технологии CAR-T в Казахстан имеют жизненно важное значение для больных раком крови в стране. Полученные результаты подчеркивают необходимость организации в НЦБ полноценного производственного процесса по производству CAR-векторов для клинического использования и терапевтических клеток для CAR-T-терапии.

Ключевые слова: вирусные векторы, CAR-T терапия, трансфекция, лентивирусы, полиэтиленимин, гематологические опухоли.

ҚАЗАҚСТАНДА CAR-T ЖАСУШАЛЫҚ ТЕРАПИЯ ҮШІН ВИРУСТЫҚ ВЕКТОРЛАРДЫ ӨНДІРУДІ ІЛГЕРІЛЕТУ

Сыздыкова Л.Р.*, Кеер В.В., Арсиенко Р.Ю., Инирбай Б.К., Шустов А.В.

Ұлттық биотехнология орталығы Қорғалжын тас жолы, 13/5, Астана қ., 010000, Қазақстан
**syzdykova@biocenter.kz*

ТҮЙІН

Химериялық антигенді рецепторларды экспрессиялайтын Т-клеткаларды (CAR-T) қолданатын гематологиялық ісіктерді емдеу соңғы 20 жылдағы гематологиялық онкологиядағы ең маңызды жетістіктерге жетуге уәде беретін жаңа технология болып табылады. Клиникалық қолданылатын CAR-T технологиясының бір аспектісі CAR+ жасушаларын өндіру процесі CAR генін Т жасушаларына жеткізу үшін пайдаланылатын вирустық векторларды қажет етеді. Қазіргі уақытта CAR-T терапиясының өндірісі пакеттелген лентивирустық немесе ретровирустық векторлардың болуына байланысты. Бүкіл әлемде CAR векторлары үшін өндірістік қуат тапшылығы бар және клиникалық қолдануға арналған векторлар дайын өнім ретінде сатылмайды.

Ұлттық биотехнология орталығында В-клетка тектес ісік жасушаларының маркері болып табылатын CD19 антигеніне бағытталған CAR рецепторы жиналып, лентивирустық векторлар құрастырылды. Буып-түйілген вектор өндірісі бойынша зерттеулер жүргізілді, онда трансфекциялық реагенттердің үш түрі олардың генерацияланған функционалдық титрлері бойынша тиімділігі бойынша салыстырылды. Ағындық цитометрияның көмегімен функционалдық титрлерді өлшеу әдісі әзірленді. Қапталған векторлардың жоғары титрлері алынды.

CAR-T дәстүрлі терапия сәтсіз болған және қайталанатын немесе рефрактерлі ауруы бар науқастарды емдеуде тиімді болғандықтан, CAR-T технологиясын Қазақстанға енгізу бойынша барлық күш-жігер елдегі қан қатерлі ісігімен ауыратын науқастар үшін өте маңызды. Алынған нәтижелер клиникалық қолдану үшін CAR векторларын және CAR-T терапиясы үшін терапевтік жасушаларды өндірудің Ұлттық орталығында толыққанды өндіріс процесін ұйымдастыру қажеттілігін көрсетеді.

Түйінді сөздер: вирустық векторлар, CAR-T терапиясы, трансфекция, лентивирустар, полиэтиленимин, гематологиялық ісіктер.