Vaccine Research
ISSN: 2423-4923
eISSN: 2383-2819

Establishment of a Carcinoembryonic Antigen Stable Cell Line for Cancer Immunotherapy Study

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A R T I C L E I N F O

Research Article

VacRes, 2022
Vol. 9, No.1, 1- 7
Received: August 03, 2022
Accepted: September 03, 2022
Pasteur Institute of Iran

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KEYWORDS: Cancer; Immunotherapy; CEA; lentivector; RT-PCR

A B S T R A C T

Introduction: Cancer immunotherapy is one of the effective treatment methods that provide a better quality of life with limited side effects for patients. Carcinoembryonic Antigen (CEA) can be an appropriate target for cancer immunotherapy. **Methods:** A lentivector expressing CEA antigen, pCDH-CEA, was constructed by cloning CEA cDNA downstream of the CMV promoter. The constructed plasmid was co-transfected with helper plasmids, into Lenti-X 293 T cells. The lentivector-containing supernatant was collected. Titers of the CEA- lentivector were estimated using the RT-PCR method. The CT26 cells were then infected by CEA- lentivector. Puromycin as a selective antibiotic was added to the culture for 2 weeks to select CEA-positive cells. The ability to produce tumors in BALB/c mice was investigated. **Results:** The results showed that CEA expressing lentivector plasmids and the two other helper plasmids could be transfected into Lenti-X 293T cells efficiently and packaged successfully as a pseudo-lentivector. The detection of CEA mRNA and protein expression in the 6th and 14th passages of CT26-CEA cells was confirmed in the engineered stable cell line. Tumor formation was confirmed in cell inoculated mice. **Conclusion:** CT26-CEA cell line with stable expression of CEA can be used as a suitable tumor model to facilitate research on colorectal cancer in vitro and in mice models; therefore, it could be served as a valuable tool for cancer immunotherapy.

INTRODUCTION

Common treatments for colorectal cancer (CRC) such as chemotherapy, radiotherapy, and surgery have many side-effects due to lack of specificity and toxicity in the growing and dividing cells. Therefore, alternative therapies with fewer side-effects and more effectiveness for the patients should be used. Cancer immunotherapy can be used as one of the most effective alternative treatments, compared to common standard methods. In cancer immunotherapy, the patient's own immune system is manipulated against the cancer cells. Patients who respond well to cancer immunotherapy show better prognosis and better quality of life [1]. Tumor antigens associated with human cancers can be used as targets for specific immunotherapy. One of the antigens associated with CRC is carcinoembryonic antigen (CEA) which was identified in 1965 as one of the important tumor markers for diagnosis of CRC [2-4]. Expression of tissue carcinoembryonic antigen (t-CEA) and serum carcinoembryonic antigen (s-CEA) of CEA are considered as appropriate tumor markers in the evaluation of CRC in the world [2]. The rate of CEA tumor marker increases in stages of the disease and reduces by surgery and removal of the tumor [5].

Based on previous studies, CEA protein blocks cell differentiation and promotes cancer [6, 7]; hence, CEA antigen can be evaluated as an appropriate choice for diagnosis and monitoring of the disease during and after the treatments [2]. Since the CEA CAM prototype is highly expressed on the plasma membrane of a wide range of tumor epithelial cells, it can be an ideal target for treatment through cellular immunotherapy, radio immunotherapy, antibody therapy, and cancer vaccines [8]. Lentivirus vectors (LVs) are derived from human immunodeficiency viruses (HIV) and have been widely used for scientific research in recent decades [9]. Using LVs to transfer genes into dividing and non-dividing cells is highly effective in scientific research; therefore, accuracy in producing and determining virus titer is very important [10]. In addition to

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[DOI: 10.52547/vacres.8.2.55]

gene transfer, LVs are appropriate tools for the stable expression of genes in target cells. This potential ability of LVs can be used as targeted therapies with high efficiency and cost-effectiveness [11].

In recent years, considering the positive steps that have been taken around the world to treat CRC, research on preclinical *in vivo* models are found to be essential for new therapeutic approaches with minimal side-effects. In this regard, animal models have been used for research to treat CRC for more than 80 years [12].

Human CRC models have long been considered by researchers to advance the optimal treatments. Successful modeling that have been performed by scientists include predicting therapeutic responses using patient-derived organs, predicting therapeutic response and outcome through molecular classification of tumors, investigating the metastasis process using mouse models and using transplanted tumor models [13]. In this study, a CEA expressing lentivector was constructed and used for transduction on CT26 cell line (mice colon carcinoma cell line) and was made stable CT26/CEA for inducing tumor in BALB/c mice model for immunotherapy challenges to study CRC.

MATERIALS AND METHODS

Ethical Statement

Ethical approval for treatment of the mice was granted by Tarbiat Modares University (ethical code: IR.MODARES.REC.1398.024).

Cloning of Human CEACAM5 Gene into pCDH-CMV Vector

To add appropriate restriction sites to whole CEA fragment (2857 bp), a PCR reaction was performed using forward primer: GCTCTAGAAGCTTGGTACCATGGAGTC and reverse primer: CGGAATTCTTATATCAGAGCAACCCCAACC on CEA containing plasmid (Sino biological,China). The PCR reaction included 10 P/mol of each primers and master mix (SMOBIO, Taiwan). After initial denaturation at 95 °C for 10 min, thermal program (95 °C for 10 s, 64°C for 30 s and 68°C for 120 s) was applied for 35 cycles plus a final extension at 68°C for 10 min. The PCR product was run on 1% agarose gel and the CEA fragment was purified with gel extraction method (GeneAll, Korea). Gel purified CEA was cloned into a TA vector made by Dr. Khan Ahmad (Isfahan University of Medical Sciences) and the ligation product (TA-CEA) was verified by restriction enzyme analysis. The TA-CEA was digested with XbaI and EcoRI enzymes and was run on 1% gel. CEA fragment was purified from the gel (GeneAll, Korea) and was subcloned into XbaI and EcoRI sites of pCDH under a cytomegalovirus (CMV) promoter. The pCDH-CEA construct was verified by restriction enzyme analysis and bidirectional sequencing and then transformed into *E. coli* TOP 10 strain competent cell. To verify CEA protein production, pCDH-CEA was transfected to HEK293A and the supernatant was analysis after 48 h by CEA-ECL analysis (Elecsys CEA, cobas, Roche).

Production of CEA Recombinant Lentivirus

Lenti-X 293T cells (5×10^5 Cell/Well; Iranian biological Resource Center, Iran) were cultured in 6-well plate in complete DMEM (containing 10% FBS) for 24 h. The medium was replaced with fresh medium 4 h before the transfection. The transfection was performed using 2.6 µg of pCDH-CEA, 3.5 µg pSPAX2 and 13.9 µg pMD2G for each well according to the manual of Turbofect kit (Thermo Scientific, USA). The Lenti-X 293T cells were kept in incubator with 37 °C for 72 h. The transfection medium was replaced with fresh medium at 24 h after the transfection. The efficiency of the transfection was assessed by GFP expression. The lentivector-containing supernatant was collected every 12 h for a 72-h-period, filtered through a 0.45-µm pore-size polyethersulfone membrane and concentrated to 70-fold by ultracentrifugation (50,000 x g, for 120 min at 16 °C). The pellet was re-suspended in the complete medium and incubated at 4°C on 250 RPM shaker overnight. Subsequently, aliquots of the supernatant were kept at -80°C.

Real –Time PCR – Based Assay for CEA LVs Titration

For evaluation of the lentiviral titer, the Lenti-X 293T cells were seeded in 24-well plate (1×10^5 Cell/Well) at 24 h before transduction and then were incubated overnight. Transduction was then conducted by 100 µl of concentrated lentiviruses. Transduction was performed in the presence of Polybrene (5 µg/ml, Solarbio, China) and continued for a 72-h incubation period. After the incubation time, the cells were harvested using trypsin-EDTA, and RNA was extracted from LENTI-X 293T cells using QIAamp® Viral RNA kit (QIAGEN, Germany) and stored at −20 °C, according to manufacturer's protocol. AltoStar®HIV RT-PCR Kit 1.5 (Altona, Germany) was used to determine the titer of the lentil virus produced, according to the kit instructions. The PCR reaction included 2.5, 10 µl of Mastermix A and B respectively, 1.25 µl Internal control (IC) and 11.25 µl Template, Standards add to each reaction. After the enzyme activation (50 ˚C for 20 min) and initial denaturation (95 °C for 2 min), thermal program (95 °C for 15 s, 55 °C for 45 s and 72 °C for 15 s) was applied for 44 cycles.

Analysis of CEA-Lenti Vectors by Transmission Electron Microscopy (TEM)

The recombinant lentivirus (500 µl) was sent before and after concentration to Partow Rayan Rastak Imaging Center (Tehran, Iran) in cold chain conditions for microscopic examination (Philips EM208s-100kv, Netherland).

Antibiotic Sensitivity Assay of CT26 Cells

A serial concentration of Puromycin (Santa Cruz, USA) from 1 to 10 µg/ml was added to 24-well plates containing 1×10^6 CT26 Cells/Well; each concentration was seeded in duplicate followed by a 10-day incubation at 37 °C with 5% CO_2. The optimal and lethal concentrations of Puromycine were determined in which the lowest concentration of antibiotic that has the highest rate of cell death between 7 and 10 days of transduction was selected as optimal dose does.

Transduction of Lentivirus to CT26 Cells

In accordance with the standard instructions for transduction of recombinant lentiviruses considering the requirements for transduction of colorectal cell lines including CT26, some modifications were performed to increase the optimal transduction efficiency of CT26 cell line. Briefly, the CT26 cells (4×10^5 Cell/Well; Cell Bank of Pasteur Institute of Iran) were cultured in 24-well plates, one day prior to the transduction. On transduction day, the cells were infected by 10 multiplicity of infection (MOI) of CEA lentivector in combination with polybrene (5 µg/ml, Solarbio, China) and incubated for 48 h. One well was left untreated as non-

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transduced control cells. After incubation time, the medium was replaced every 2 days with the complete medium containing MEM Non – Essential Amino Acid Solution (Sigma Aldrich, Germany). When cells transduction rate was observed to be 30 to 40% by evaluation the GFP expression rate, the cells were harvested using trypsin-EDTA and cultured in 6-well plates. As in previous steps, the work process continued until the percentage of transduction increases. The medium was then replaced every 2 days with the complete medium containing 4 µg/ml puromycin. The replacing of the medium was continued to kill whole untreated control cells. Subsequently, the stable cell line was frozen and stored in liquid nitrogen for future use.

Expression of CEA in CT26/CEA Stable Cell Line

The expression of CEA mRNA and protein was evaluated in the CT26/CEA stable cell line by Real Time RT- PCR and Enzyme Linked Florescent Assay (ELFA) methods. CT26/CEA cells were sub-cultured for 6 and 14 times for RT-PCR Real Time and ELFA, respectively. For RT- PCR Real Time analysis, 10^5 cells were collected after each passage. Total RNA was extracted from each passage by Gene All Rib spin vRD DNA/RNA Extraction Kit (Gene All, Korea) and RT-PCR Real Time was performed by CEACAM5 primers (Forward CEA: AAT GGG ATA CCG CAG CAA CA and Reverse CEA: ATC AGA GCA ACC CCA ACC AG). The PCR reaction included 10 pmol of each primer, 10µl master mix (Real Q Plus 2x Master Mix Green; Biotech Rabbit, Germany) and 2-µl reverse transcription enzyme (Biotech Rabbit, Germany). After the reverse-transcription at 50˚C for 30 min and initial denaturation at 95 °C for 5 min, thermal program (95 °C for 5s, 62 °C for 30s and 68 °C for 120s) was applied for 40 cycles by Mic Real-Time PCR System (Bio Molecular Systems, Australia). For positive and negative control, pCMV3-CEACAM5-GFPSpark plasmid (Sino Biological, China) and CT26 cells were used, respectively.

For protein expression analysis by ELFA, after 14 times passage, the CEA CT26 cells were trypsinized, centrifuged and 3 times washed by PBS (200 x g, for 5 min at 4 °C). The pellet was re-suspended in the PBS and freeze-thawed in liquid nitrogen for 5 times. After preparing the cells, protein expression was measured by ELFA method (BIOMERIEUX VIDAS CEA, USA).

Optimal Number of Cells for Tumor Formation in BALB/c Mice

The amount of 3×10^6 [14], CT26/CEA stable cells and CT26 cells suspended in 500 µl of PBS were inoculated subcutaneously in right flanks of groups of 3 BALB/c mice (Inbred mice, purchased from Royan Research Institute, Tehran, Iran). The mice were monitored from appearing of tumor between 10 to 15 days after the inoculation.

Optical Imaging Analysis of CEA /CT26 Mouse Tumor

The in vivo imaging was performed using KODAK imaging system (system FX Pro, Kodak, USA) at the Preclinical Core Facility (TPCF) based at Tehran University of Medical Sciences, with florescent mode and 1 min exposure time. The excitation and emission filters were set to 470 nm and 535 nm, respectively. The light emitted from the mice were detected by the KODAK camera system, integrated, digitized, and displayed. To record fluorescent signaling, the black–white and color images were overlaid together. Then, the pseudo color format was overlapped on the light normal image.

RESULTS

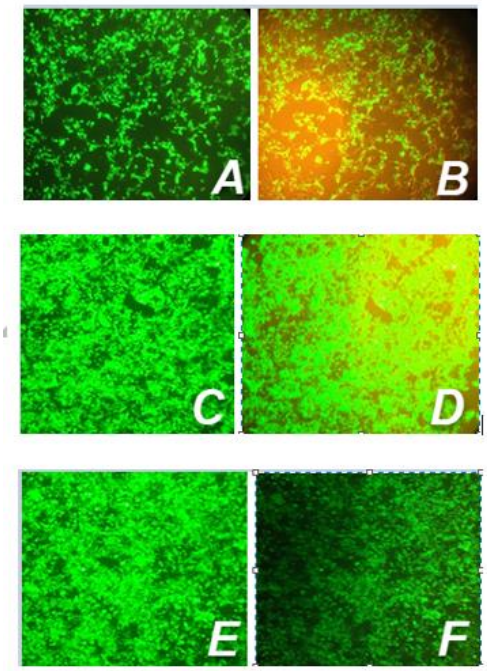
PCR Amplification and Construction of the Recombinant Plasmid

The CEA PCR product was analyzed on 1% agarose gel and the expected 2857-bp fragment was observed. Gel purified CEA PCR product was cloned into TA vector and restriction enzyme analysis verified insertion of CEA in TA-vector. The TA-CEA was digested with *Xba*I and *Eco*RI enzymes. The purified CEA fragment was ligated to *Xba*I and *Eco*RI digested pCDH. The ligation was verified by restriction enzyme analysis and bidirectional sequencing. The CEA-ECL analysis (Elecsys CEA) confirmed the presence of CEA protein in the supernatant of pCDH-CEA transfected HEK293A cells after 48 h (66.1 ng/ml) compared to the controls (CEA positive control: 376 ng/ml, CEA negative control: <0.200ng/ml)

Construction and Confirmation of The recombinant Lentivirus

The pCDH-CEA vector with pSPAX2 and pMD2G (as helper plasmids) were transfected in Lenti-X 293T cells. To check the efficiency of the transfection, the expression of GFP marker by florescent microscopy was examined which was more than 90% (Fig.1).

Fig. 1. LentiX-293T cells 24, 48 and 72 h after the transfection.



A) Transfected LentiX-293T cells in florescent inverted microscope after 24h. **B)** The same field after light and florescent illumination, simultaneously after 24h. **C)** Transfected LentiX-293T cells in florescent inverted microscope after 48h. **D)** The same field after light and florescent illumination, simultaneously after 48 h. **E)** Transfected LentiX-293T cells in florescent inverted microscope after 72h. **F)** The same field after light and florescent illumination, simultaneously after 72h.

Titration of Lentivirus

Titration of the virus was performed with real-time RT-PCR. The number of lentiviral vector copies was calculated by Absolute Quantification with Rotor Gene Software based on standard curves and estimation of absolute DNA titers was

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achieved by comparing crossing point values derived from DNA samples to those obtained from a standard curve of known concentrations of plasmid lentiviral DNA. Based on the software calculations of the device, the titer of lentil virus in CT 8: 949.771 IU / µl, which was calculated to determine the virus titer as a copy in ml and using Qiagen extraction kit according to the following formulas:

Copies/µl: IU/µl × 0.5 = 949.771 × 0.5 = 474.88
Copies /ml = Copies/µl × Elution Buffer volume (µl) / Sample volume (ml) = 474.88 × 60 / 0.14 = 203520

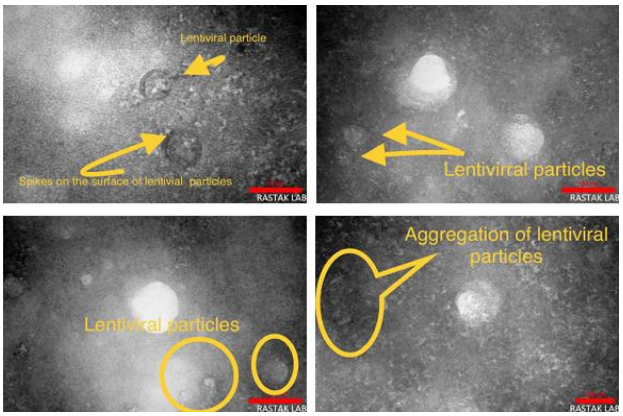

Fig. 2. Diagnosis of the viral particle by TEM. Different conditions are investigated by a TEM

TEM Imaging

Negative staining and TEM imaging characterized the purity, stability, and size distribution of the lentiviral particles. Viral particles were clearly visible in different forms (Fig. 2).

Lethal Evaluation of Different Dilutions of Puromycin

To evaluate the limiting dilution of Puromycin, the CT26 cell lines was cultured in a range of 1-10 µg/ml Puromycin concentrations.The lowest antibiotic concentration that killed 100% of CT26 cells for up to 7 days of Puromycin was 4µg/ml (as optimal dose; Fig. 3).

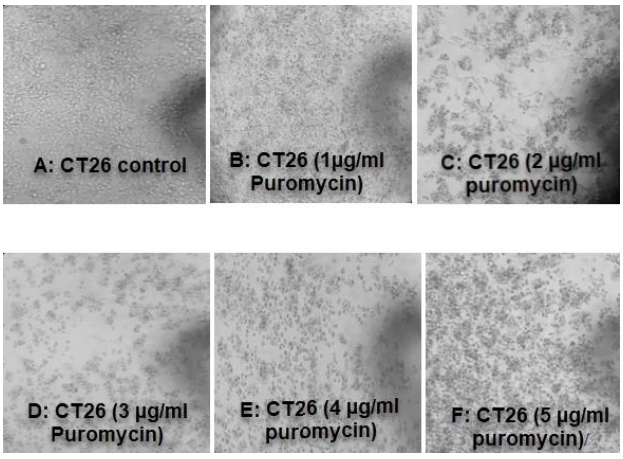

Fig. 3. The CT26 cells after 7-days Puromycin treatment. A) CT26 control with 100 % cell viability. **B to D)** According to Puromycin concentrations curve, the concentrations (1,2 and 3 µg/ml are not appropriate for optimal mortality rate for CT26 cells line. **E)** CT26 with 4µg/ml Puromycin, optimal dose. **F)** CT26 with 5 µg/ml Puromycin, lethal dose. **G)** Viability percentage of CT26 cells during the increase of Puromycin concentration.

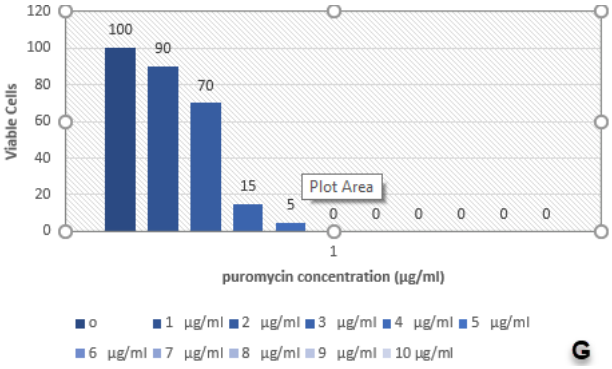

Fig. 3. G) Viability percentage of CT26 cells during the increase of Puromycin concentration.

Transduction of CT26 Cells

Transduced CT26 cells was evaluated with GFP expression as shown in Fig. 4.

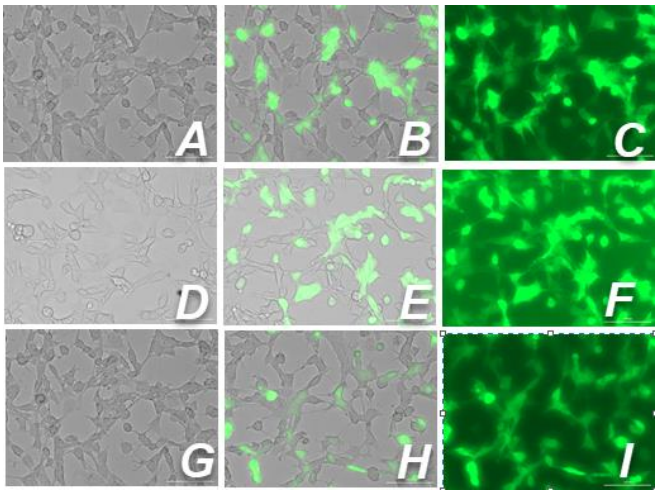

Fig. 4. GFP positive cell identifies by CYTATION imaging reader in multi fields of 6- well plate, the three selected fields are imaged with microscopic, visible and fluorescent light formats. **A, D, G)** Microscopic image without visible and fluorescent light. **B, E, H)** Microscopic image with visible light. **C, F, I)** Microscopic image with fluorescent light.

Expression of CEA in CT26/CEA Stable Cell Line

Total RNA was extracted from the 6th passage of CEA/CT26 stable cell line and RT-PCR was performed using CEACAM5 primers to analyze the stable expression of CEA (Fig. 5).

To evaluate CEA protein expression after passage 14, trypsinized cells are measured by ELFA method (Table1 and Fig.6).

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Table 1. The amount of CEA protein expression by ELFA method. CEA protein expression in stable 26 CT cells was reported to be 3.4 ng/ml compared to CT 26 cells (as control cells).

Sample ID	Test BKG	Test RFV	Result	Unit	Interspersion	Reference Interval
ST1	270	2684	8740	ng/ml	Standard	0.5-3.0
ST2	270	2581	84.04	ng/ml	Standard	0.5-3.0
C	271	1010	32.89	ng/ml	Positive Control	0.5-3.0
CT26	270	72	1.66	ng/ml	Negative	0.5-3.0
CT26/CEA	177	143	3.4	ng/ml	Positive	0.5-3.0

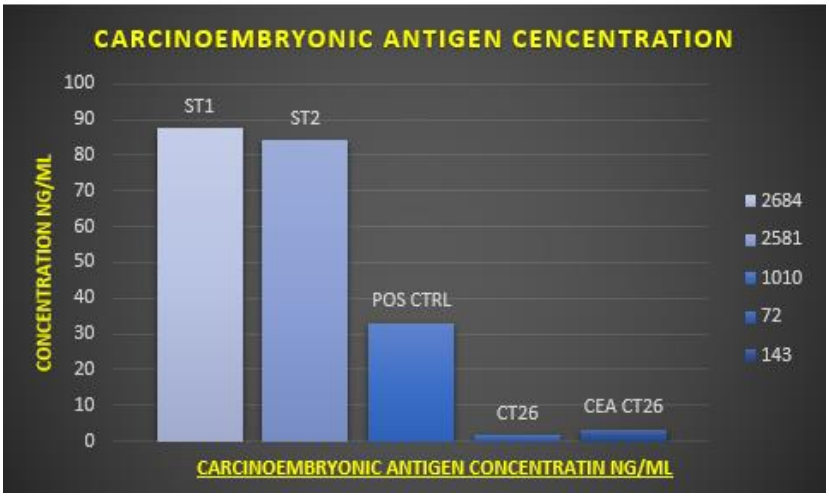

Fig. 6. Comparison of CEA protein levels between the standards, the positive control, the stable cells and the normal cells.

In vivo Imaging of CT26/CEA Tumors in Mouse Model

Comparison and evaluation of GFP expression in CT26/CEA tumor-bearing mice and CT26 tumor-bearing mice as a control group were performed by optical imaging, 16 days after the cells inoculation (Fig.7). The excitation and emission wavelengths received from the CT26-CEA cell were set up at 470 to 535 nm respectively. Imaging results with set-up wavelengths indicated that CT26-CEA tumor-bearing mice showed a different signal compared to CT26-tumor-bearing mice in the tumor area.

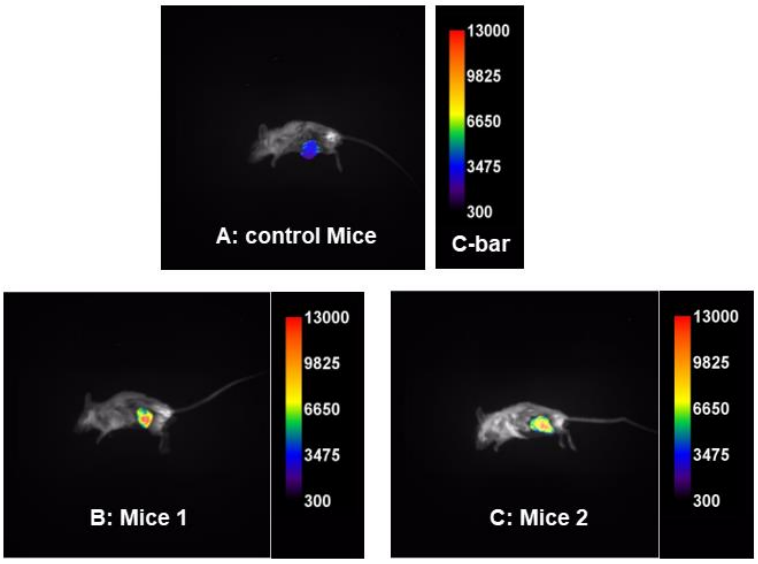

Fig. 7. Optical X-Ray imaging report. **A)** The control mouse inoculated with CT26 cells. **B and C)** Mouse 1 and 2 inoculated with CT26/CEA indicates accumulation of GFP in the tumor lesion.

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In recent years, tumor markers have been used as important factors in identification of malignant cells in the early stages of cancer, as well as in immunotherapy of cancer. Moreover, tumor-related antigens (TTAs), expressed specifically or non-specifically by the tumor cells, can be used as an ideal candidate for vaccine production to start and re-stimulate the immune system [15]. In gastrointestinal-related tumors, the level of CEA antigen increases as a sensitive biomarker in tissue and serum [16]. Previous studies have shown that CEA antigen play a significant role in cancer cell adhesion, innate immunity, attachment of cancer cells to metastatic sites, and support for disease progression in CRC. Therefore, due to the critical characteristics of the CEA antigen in CRC [17], it can be an essential target for treatment through new methods of immunotherapy.

For tumor therapy studies using tumor antigens, availability of a tumor cell line expressing the tumor antigen is a prerequisite. In cancer studies, animal models can be used to examine the tumor microenvironment and metastatic processes. In this regard, mouse models are very important in cancer research; therefore, transgenic mouse models and stable cell lines have been developed to study the growth and behavior of different types of the tumors [18]. Among the viral vectors, lentiviruses have features such as gene transfer to dividing or nondividing cells, carrying large genetic payloads and stable long-term transgene expression which can be used optimally for targeted therapies and production of in vitro stable cell lines [19].

Stable expression of human CEA gene as a CRC tumor marker on mouse colorectal CT26 cell line can be used as a suitable platform for research on the effect and role of CEA on CRC in the normal environment of in vitro and in vivo. In fact, CT26 cell line without the presence of CEA antigen will not have positive results for cancer immunotherapy in animal model. Overall, the in vivo and in vitro perspective of mice models in CRC has been significantly studied and researched. Although there are different methods (physical, chemical, viral vectors, etc.) for the transfer of nucleic acids into the cells and most of which are effective, a number of cell lines including the colorectal cell lines are difficult to transfect and transduct [20, 21]. In this study, pCMV3-CEACAM5-GFPSpark was directly transfected to CT26 cells by lipofectamin 3000, Demriec, and Polyethylenimine. However, due to the nature of CT26 cells, the percentage of transfection was insufficient for establishment of CT26 cells. Therefore, to facilitate the transfection, transduction and persistent stable expression, the CEA gene was cloned into pCDH vector and after production of lentiviruses, the transduction of lentivirus to CT26 cells was performed by modifications in standard protocol to increase the transduction efficiency of CT26 cell line. We used in vivo imaging as one of the most accurate and reliable tools to evaluate the tumor-bearing mice which expressed GFP to evaluate the cell behavior [22]. Moreover, we used X-Ray-Optic for measurement and comparison of GFP expression and the control group (i.e., inoculated by CT26 cell line with no GFP) as well as CT26-CEA inoculated mice. Based on the imaging results, evolution of GFP expression in vivo was successful and confirmed the proper function of the cells in tumor formation.

In conclusion, CT26-CEA cell line with stable expression of CEA transgenic mouse model may serve as a model of choice for trials involving CEA-targeted therapies. This model may also lead to better understanding of various cancers associated with these human-specific CEA family members and, eventually may lead to development of more successful therapies.

ACKNOWLEDGMENT

We gratefully acknowledge Department of Arboviruses and Viral Hemorrhagic Fevers (National Reference Laboratory) of Pasteur Institute of Iran. This study as a part of a Ph.D. program was financially supported from the Research Deputy of Tarbiat Modares University, Faculty of Medical Sciences.

CONFLICT OF INTEREST

The authors declare they have no conflict of interests.

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[DOI: 10.52547/vacres.8.2.55]