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Original Article

Evaluation of lentiviral vector-based green fluorescent protein expression in human gastric cancer cell line evaluation of expression lentivirus vector-based GFP in AGS

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Abstract

Background and aims: Human immunodeficiency virus type 1 (HIV-1) based-lentivirus vector is one of the most promising viral vectors for gene delivery in different cell lines including gastric cell lines. Therefore, the aim of this study was to produce a lentivirus vector for transduction and expression of green fluorescent protein (GFP) in human gastric cancer cell line, AGS.

Materials and Methods: In this piece of work, *Escherichia coli HB101* was transformed with plasmids psPAX2, pTD, and pMD2.G, following the purification of which their DNA was extracted along with their quantity and quality evaluated to be used in the next experiments. Subsequently, to produce the vector, the packaging cells were transfected with the plasmids and the vector containing supernatant was collected and purified using ultracentrifuge. ELISA was used to confirm the construction of the vector. Fluorescent microscopy and flow cytometry were used to check the expression of GFP in the cell line and to calculate the percentage of GFP expression, respectively.

Results: In this study, the results of ELISA confirmed the **c**onstruction of the plasmid used in this study. AGS cells were infected with viruses produced to detect the viral activity and GFP expression was evaluated by fluorescence microscopy and flow cytometry after 72 hours. Based on the results of flow cytometry, GFP was expressed in over 90% of transduced AGS cells.

Conclusion: The results of this study showed that lentiviral vector is a highly efficient vector for expression of GFP gene in AGS cell line.

Keywords: Lentivirus-based vector, Transfection, Transduction, GFP, AGS

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Introduction

Different gene delivery methods including microinjection, liposomes, and viral vectors are used in gene therapy. Viral vectors and in particular lentivirus-based vectors are the most efficient ones for expression of the transgene in different cell lines (1), which are capable of infecting a wide range of dividing and non-dividing cells. This vector can enter the desired gene into the host genome and thus cause long-term expression of the transgene (2). Several studies have shown that lentivirus is one of the tools for genetic manipulation in stem cells (3). Today, the use of lentiviruses in basic research and clinical trials of gene therapy and regenerative technology is considered. Studies have shown that transgenes transmitted by the lentivirus can be stably expressed in hematopoietic progenitor cells and endothelial cells derived from embryonic stem cells (4). A lentivirus is an appropriate tool for expressing external genes in most mammalian cells. The packaging of genetic vector bearing lentiviral constructs allows for the efficient transfer of these expression structures and their subsequent expression in different cells. This vector can be used for various gene therapy and gene modification therapies. In addition, lentiviral viruses are widely used in basic medical sciences for gene transfer with the goal of expressing protein and RNA, for example, shRNA in gene silencing. On the other hand, the use of lentiviral viruses for vaccine production is under investigation (5).

The lentiviral vector consists of 3 plasmids. The transfer vector contains the cis-acting sequences needed to generate RNA and pack the virus. The packaging system consists of two separate plasmids, (psPAX2 and pMD2.G) with transacting Gag-Pol and Rev factors for psPAX2 and the envelope virus (VSVG) for pMD2.G. Gag-Pol encodes structural proteins, integrase, reverse transcriptase. The Rev protein with the RRE sequence in the transmitted plasmid increases the viral titer and the unprocessed transfer of RNA into the nucleus. To produce lentivirus, the plasmid is transfected into HEK-293T cells (genetically engineered cells from human embryos). The virus produced by exocytosis enters the cellular supernatant, is accumulated

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in large quantities, and then is used (6).

Gastric cancer is one of the important factors in mortality worldwide, which is more prevalent in developing countries than in industrial societies (7). Gastric adenocarcinomas include 90% of gastric cancer cases and are among the deadliest cancers in Iran (8). According to statistics from the Cancer Research Center, gastric cancer is the most common cancer among Iranian men as well as the third most common one among Iranian women after breast and colon cancer (9). Histologically, two distinct types of gastric adenocarcinoma have been known: intestinal form, which produces pseudo-tuberous structures, and a diffuse form that has a discohesive nature. The intestinal form typically occurs in older people, which causes premature damage and spreads to the liver through the blood vessels. The diffuse form of gastroduodenal adenocarcinomas occurs in any age group. Primary damage is not detected and extends to most adjacent tissues. Most gastroduodenal adenocarcinomas are identified in the advanced stages; therefore, the mortality rate is high (10).

Cancer often occurs when the mechanisms controlling the growth and proliferation of the cells are disrupted. It can be argued that the cause of all or most of the cancers is a defect in cellular regulation (11). Translating cell mRNAs and controlling them play a major role in cell growth, proliferation, and differentiation (12,13). An AGS cell line isolated from the stomach adenocarcinoma of a 54-year-old Caucasian woman was one of these types of cells (14). In this study, the efficacy of lentiviral vector containing the green fluorescent gene for expression in AGS cell line of human stomach cancer was evaluated.

Materials and Methods

Cells and cell culture

Human gastric cancer cell line AGS and Human Embryonic Kidney 293T cells were purchased from the Royan Institute of Tehran. Dulbecco's modified Eagle's medium (DMEM) with 10% FBS and 1% penicillin-streptomycin (Gibco, Life Technologies, USA) was used to culture the cells (15). Cells were cultured in a T25 flask with DMEM medium containing 10% FBS and 1% penicillin-streptomycin under standard incubation conditions (37°C, 5% CO2, and 98% moisture). The cells were passaged once per week using Trypsin and kept in logarithmic phase (16).

Transformation and plasmid purification

The vector carrying the GFP gene and the two auxiliary vectors for cloning separately into *E. coli HB101* were made and plasmids were extracted according to the protocol. Three vectors prepared for the production of the virus were introduced into a packaging cell line simultaneously with calcium phosphate transfection into HEK-293T cells (isolated from human embryos). In this study, two packaging plasmids called pMD2.G and psPAX2 were used.

Production of lentiviral vector

HEK-293T cells are cells isolated from human embryos, which are used as a cell line for packaging for the production of lentiviruses.

Preparation of lentiviruses containing GFP gene using calcium phosphate sedimentation method

To prepare the lentivirus cells and transfer them to target cells, HBS (2X) and $CaCl_2$ (2.5 M) buffers were used.

Transfection steps are as follows:

1. A total of 1×10^6 HEK-293T cells were placed in a 15 cm^2 plate containing 10% DMEM medium and incubated at 37°C in an incubator containing CO2.
2. After the cell density reached 80%, the medium was replaced with the cell with a serum-free DMEM medium and incubated for 2 hours in an incubator containing CO2 at 37°C.
3. The transfection solution containing three plasmids pTD, psPAX 2, and pMD2.G with concentrations of 22 µg, 15 µg, and 8 µg was mixed with 115 µL of $CaCl_2$ (2.5 M) and distilled water with a volume of 550 µL for 30 minutes in a 15-mL Falcon tube and then was placed in the dark at room temperature.
4. 500 µL of HBS 2X buffer was added to the solution and placed at room temperature for 5 minutes. Then, it was added drop by drop to the plate containing HEK-293T cells.
5. After 24 hours, the medium was replaced with cells with a new 10% serum DMEM.
6. On two occasions, after 48 and 72 hours, the medium was collected and then filtered with a 0.45 µm pore filter and ultrasound centrifugation was performed at 40000 rpm for 1.5 hours.

Confirmation of virus production using ELISA

HIV Diagnostic kit (DIA.PRO, Italy) was used according to the protocol for confirmation of virus production.

Viral concentration using ultracentrifuge

1. The medium was packed on transfected HEK-293T cells containing viruses produced by cells within 48-72 hours after transfection.
2. To isolate HEK-293T cells simultaneously with the removal of the medium, centrifugation was performed at 1200 rpm for 5 minutes.
3. After centrifugation, the medium was collected and then filtered with a 0.45 µm pore filter and ultrasound centrifugation was performed at 40000 rpm for 1.5 hours.
4. After the centrifugation was completed, the culture medium was removed under the hood, and then 1 mL of the fresh medium was added to the viral sediment. The concentrated viruses were then used for transduction of the target cells.

Transduction of AGS cells

1. A total of 5×10^5 AGS cells were added to each well of a 6-well plate and placed overnight in a Co_2 incubator at 37°C.
2. After the cell density reached 80%, 200 µL of the virus produced was added to the AGS and then cultured and incubated at 37°C for 8-12 hours in a CO_2 incubator.
3. After changing the culture medium on AGS cells, 2 mL of 10% serum DMEM was added to each well.
4. For 3 days, the culture medium was replaced with cells and GFP expression was evaluated using a Nikon Multizoom AZ100 multi-purpose microscope.
1. After fluorescence microscopy, the cells were evaluated by flow cytometry to confirm the GFP gene expression in the cells.

Results

Preparation of lentiviruses containing GFP gene

The supernatant of HEK-293T cells was collected 48 and 72 hours after transfection. After initial confirmation of virus production by ELISA, the viruses were condensed with an ultracentrifuge and prepared for transduction. Figure 1 shows the production of the virus within 72 hours of transfection.???

Transduction of AGS cells with viruses containing GFP gene

AGS cells were infected with produced viruses to detect the viral activity and GFP expression was evaluated by fluorescence microscopy and flow cytometry after 72 hours. Figure 2 shows that the pTD-GFP lentivirus has promoted the expression of the GFP gene in AGS cells, indicating the successful performance of the virus in gene transfer. With these two methods, it was found that more than 90% of the AGS cells expressed the GFP gene.

Results obtained from flow cytometry

AGS cells were infected with produced viruses and GFP expression was evaluated by flow cytometry after 72 hours. The pTD-GFP lentiviral vector promoted the expression of the GFP gene in AGS cells, which indicates the successful performance of the virus in gene transfer. More than 90% of the AGS cells expressed the GFP gene. Figure 3 shows the control AGS cells that were not infected with the vector and thus did not express the GFP gene. Figure 4 shows the AGS cells that were infected with the vector and thus expressed the GFP gene.

Discussion

Several different methods have been used to transfer the therapeutic gene. The selection of an appropriate vector for effective expression of the gene in stem cells and its progenitor cells is of great importance (1). Several viral vectors including retroviruses, adenoviruses, herpes viruses, and lentiviruses have been used for this purpose. Studies have shown that the recent vector made up of type 1 human immunodeficiency virus, which is capable of infecting a wide range of dividing and non-dividing cells, is one of the best carriers for gene transfer. This vector can enter the desired gene into the host genome, thereby causing long-term expression of the transgene (2,3).

Gastric cancer is the second most common cancer in the world, which is in fact one of the main causes of cancer deaths. It is estimated that 900 000 new cases are diagnosed each year. Gastric cancer is one of the important factors in mortality worldwide, which is more prevalent in developing countries than in industrial societies (7). In this study, after the construction of the vector lentivirus, which was performed using pTD, psPAX 2, and pMD2.G plasmids, transfection of them in HEK-293T cells was performed. The structure was also confirmed using the ELISA method (standard protocol). The fluorescence microscopy showed that the vector expressed the GFP marker in the AGS cell line. Moreover, based on flow cytometric results, this vector caused the expression of the GFP gene in more than 90% of AGS cells, indicating the acceptable function of the vector in transmitting this gene.

In 2010, Jiang et al showed that transgenes transmitted by lentivirus can be stably expressed in hematopoietic progenitor cells and endothelial progenitor cells derived from embryonic stem cells using lentiviruses in fundamental research, clinical trials of gene therapy, and regenerative

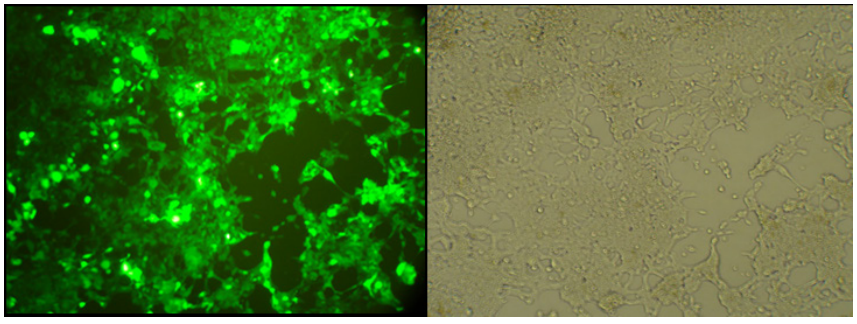

Figure 1. GFP gene expression 72 hours after transfection in HEK-293T cells. Image **a** of HEK-293T cells is observed using fluorescence microscopy. Image **b** of the same cells is observed using a bright field microscope.

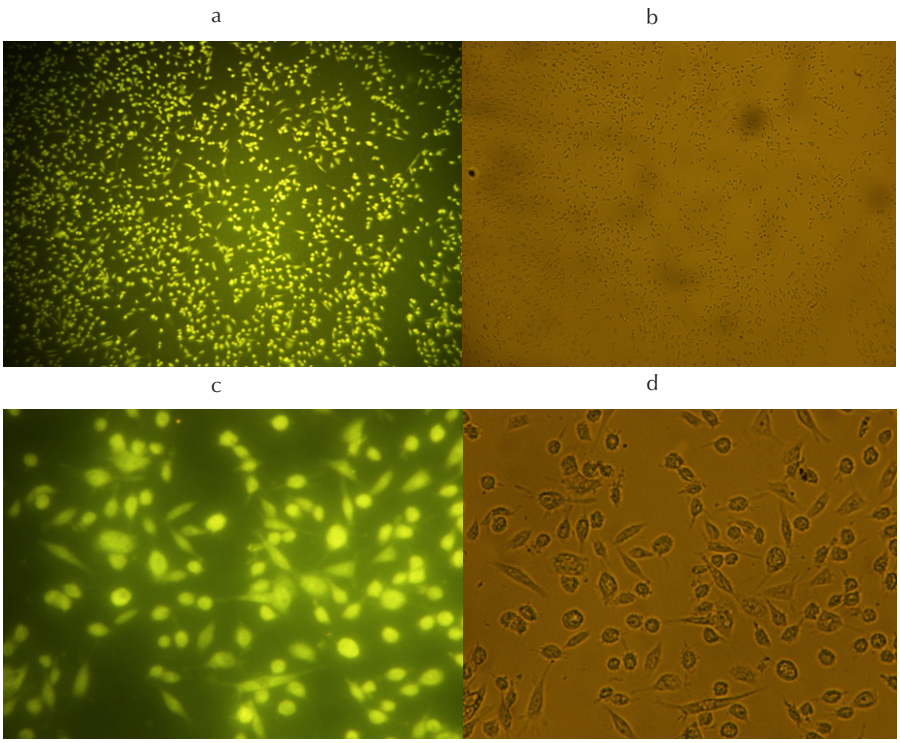

Figure 2. AGS Cells Transduced with pTD-GFP Lentiviral Vector After 72 Hours. Images **a** and **c** of AGS transduced cells are observed using fluorescence microscopy. Images **b** and **d** of the same cells are observed using a bright field microscope.

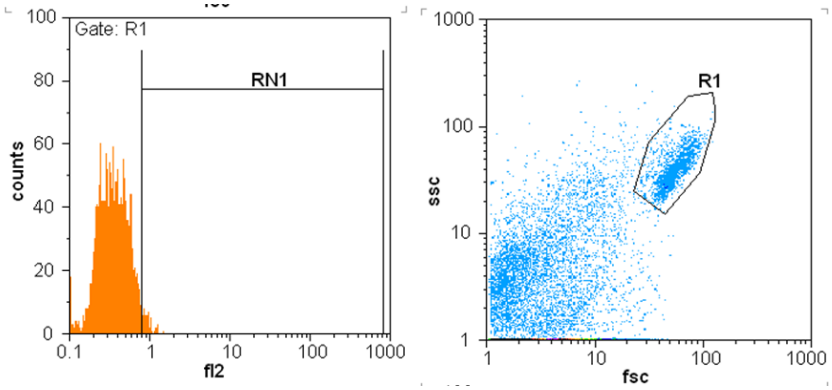

Figure 3. The Results obtained from flow cytometry for the control sample.

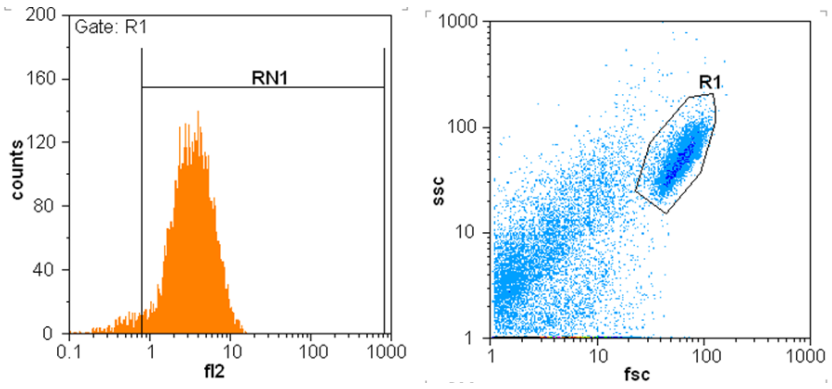

Figure 4. The results obtained from flow cytometry of AGS cells expressing the GFP gene.

technology (4). This is largely in line with the results of this study. Moreover, Zhang et al used lentiviruses in 2010 to kill cancer cells. Expressing high levels of HER-2, prostatic cancer cells, are resistant to monoclonal antibodies. They showed that the lentiviruses with envelope proteins destroyed the resistant prostate cancer cells by binding to the therapeutic antibody (17). In 2011, Balaggan and Ali used lentivirus in ocular diseases and transmitted the gene into retina cells (18).

In 2011, Varma et al used hematopoietic stem cells for gene therapy. In this method, lentivirus is used to transfer the gene to these cells. In a therapeutic genetic study, a lentiviral vector containing this gene was transmitted to cord blood-derived stem cells, where these cell lines were able to express this gene for a long time (19). The results of a study by Allahbakhshian-Farsani et al in 2014 indicated successful production of TetO-FUW-OSKM lentiviral vector. The efficacy of gene-transfer method using lentiviruses and appropriate expression of transcription factors in HDF cells were examined after transduction (20). Nasri et al conducted a study in which they transmitted GFP gene to CHO, Jurkat, HepG2, MCF-7, and MEF cells using lentiviral vector. The gene expression was observed in about 96% of the cells (21). In 2012, Liu et al proved that lentiviral vector is a good tool for transferring SiRNA and silencing genes (22).

In studies that focused on transferring siRNA to gastric (23-27), liver (28), and lung (29) cancer cells, the basis for the transfer of siRNA from the lentivirus was to induce apoptosis. The study we conducted was a preliminary and one in which we were looking at how to measure the transduction and expression of the GFP gene in AGS cells. The results of this study showed high level of transduction, and the expression of the GFP gene in AGS cells was more than 90%. This study would help us design a research project that induces the transfer of siRNA from apoptosis to lentiviruses in AGS cells. However, further studies in this regard are required.

Taking together the above-mentioned studies as well as the current one, it can be concluded that the use of the lentiviral vector for gene transfer to most cell lines is possible, but more studies are needed. This study also helps future studies on the application of lentivirus for shRNA transfer to change the expression of genes involved in the development of gastric cancer.

Conclusion

What is certain today is that cancer is a worldwide disease, and unfortunately most cancers do not have a good prognosis. Such studies have led to an increase in information on ways of transferring the gene to cancer cells. As we can increase the efficiency of the gene transfer by lentiviruses, we could improve the therapies. Based on the results of this study as well as those of the other studies conducted in this field, it can be concluded that the use of lentiviral vector is possible for the transfer of various genes and shRNAs to most cell lines, but, further studies are required.

Conflict of interests

No potential conflicts of interest are disclosed.

Ethical considerations

The ethical protocol of the research was approved by the Ethics Committee of Shahrekord University of Medical Sciences with code: IR.Skums.Rec.1394.34.

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