Iranian Journal of Virology 2020;14(2): 23-28

Original Article

Human Reovirus Serotype 3 Effectively Targets Huh-7 Cells

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Abstract

Background and Aims: Huh-7 is a cell line that was derived from a liver tumor of a Japanese man. Hepatocellular carcinoma (HCC) is considered as a primary liver cancer. Highly resistant tumor to treatment which causes the death of many patients annually. Thus, targeting the cancer cells by using a new method could be effective in therapy of this cancer. Reoviruses are oncolytic viruses that can infect and kill tumor cells, which have an activated Ras signaling pathways, while normal cells are resistant to infection and replication of these viruses. The aim of this study was to evaluate the effect of oncolytic human reovirus on Huh 7 cell line in vitro.

Materials and Methods: Human reovirus serotype 3, Huh-7 cell line, and normal human fibroblasts were used in this study. After virus purification and plaque assay, human reoviruses were inoculated into the Huh-7 cells and human normal fibroblasts as negative control. Virus cytopathic effect, cell viability, and viral RNA replication were assessed at the different time of post-infection.

Results: Virus cytopathic effects and cell lysis were clearly observed and reovirus RNA replication was detected in the Huh-7 cells, whereas normal human fibroblasts were resistant against reovirus infection.

Conclusion: The result of the present study showed that human reoviruses serotype 3 can destroy the Huh-7 cells. Accordingly, the use of human reovirus could be considered as a potential therapy for HCC and liver cancer.

Keywords: hepatocellular carcinoma (HCC); Huh-7; oncolytic virus; reovirus

Introduction

Huh-7 cell line that was introduced by Nakabayshi and Sato at first was taken from a liver tumor of a 57-year-old Japanese male in 1982 (1). The most common primary liver cancer is Hepatocellular carcinoma (HCC) (2). HCC is known as the fourth most important cause of cancer death (3). This cancer is affected by various risk factors such as hepatitis B virus (HBV), hepatitis C virus (HCV), Cirrhosis, alcohol abuse, tobacco smoking, aflatoxin, pesticides, alcoholic liver disease, Nonalcoholic Steato-hepatitis (NASH), obesity, diabetes, fatty liver disease hemochromatosis and alpha1-anti-trypsin deficienc (4). HCC is more common in men tha women. It is the fifth and seventh most commo cancers among men and women, respectively (5).

Due to the differences between the time of formation and acquisition of important risk factors for liver disease and HCC, the incidence of HCC varies among different populations living in the same area (6).

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Owing to late detection of HCC, the death rate of this cancer is the same as its incidence (7). Therefore, due to the limitations of HCC treatment (such as patients aging, low number of the liver donors, metastasis, cancer reversion and tumor progression), it is important to use an effective technique to kill these cancer cells (8, 9).

Reovirus is the acronym of Respiratory Enteric Orphan virus that belongs to the family Reoviridae and the genus Orthoreovirus (10).

These viruses are divided into four serotypes: type 1 Lang (T1L), type 2 Jones (T2J), type 3 Abney (T3A), type 3 Dearing (T3D) and type 4 Ndelle (T4N) (11). The genome of human reovirus consists of 10 segments of double-stranded RNA that classified according to their size in three groups: large (L1, L2, L3), medium (M1, M2, M3), and small (S1, S2, S3, S4) (12).

The overexpression of Ras signaling pathway is seen in more than 30% of cancer cells causing stimulate tumor growth, angiogenesis and metastasis (13). Reovirus serotype 3 (T3D) can infected both normal and cancer cells but in normal cells, because of inactivity of the Ras pathway, the replication of virus is stopped and the cell remain survive. While, reovirus are able to destroy cancer cells with activated Ras pathway and recur cell lysis cycle in adjacent cells (14).

The purpose of this study was to evaluate the effects of oncolytic human reovirus serotype 3 on Huh-7 cells in vitro.

Methods

Cells. L-929, Huh-7 and normal human fibroblast cells were from Pasteur Institute of Iran. These cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin at 37 ºC in a humidified 5% CO2 incubator. The cells were passaged when they reached approximately 80% confluence.

Reovirus propagation. Reovirus serotype 3 strain Dearing (T3D) (provided by Dr. Ataei-Pirkooh) was propagated in L-929 cells. Briefly, the cell culture medium was poured off and monolayered cells were washed two times with phosphate buffered saline (PBS). Then, cells were infected by reovirus and incubated at 37°C for 90 minutes.

After incubation, medium without serum was added and cells were returned to 37°C until observation of cytopathic effects (CPE) in more than 80% of cells.

Afterwards, cell culture flask subjected to three rounds of freeze-thawing and the supernatant were gathered as virus seed for the subsequent steps. All steps of working with reoviruses were done in biosafety level 2 laboratory.

Plaque assay. Following virus purification (15), viral titers were determined by plaque assays technique using L-929 cells. Briefly, L-929 cells were seeded into a 6-well plate. After monolayer cells formation, they were infected by purified reovirus and incubated at 37ºC for 90 minutes. Then overlay medium containing DMEM and 0.3% agarose was added onto each well. The plate was incubated at 37°C for 4 days. At the fourth day, cells were fixed in formalin for 10 minutes and plaques were visualized by using crystal violet staining. Finally, plaques were counted and PFU/mL calculated. Plaque assay was done in duplicate.

Reovirus-RNA replication in Huh-7. To evaluate the replication of reovirus-RNA, Huh-7 and normal human fibroblast cells were harvested at the various time of post infection (0, 2, 4, 6, 24, 48 and 72 hours) and real-time PCR was performed as follow: Reovirus-RNA extraction was carried out by FavorPrep Viral Nucleic Acid Extraction Kit I (Favorgen Biotech, Taiwan) according to the manufac-turer's instruction. Extracted RNAs were preserved at -70°C till subsequent phase.

cDNA synthesis was performed using Revert-Aid First Strand cDNA Synthesis Kit (Thermofisher, USA) according to the manu-facturer's instruction. Synthesized cDNA was stored at -20ºC until real-time PCR step.

Real-time PCR reaction was contained 10 µl of 2x SYBR Green master mix (BioPCR, England), 0.3 µl 10 pM of forward and reverse primers, 2 µl of the template and 7.4 µl of DNase-free water. Each run was concluded negative control and done in duplicate.

Reovirus primer sequences were as 5'-CATATGACTACCACTTTCCCG-3' (forward) and 5'-GCTATGTCATATTTCCATCCG-3' (reverse) (16) and picked up from L1 gene.
Human β-globin gene was used as an internal control (17). The real-time PCR program repeated for 40 cycles (table1). The real-time PCR curves were confirmed by the melting curve analysis.

Viability of Huh-7 cells post-infection by human reovirus. Huh-7 cells viability were estimated by trypan blue staining. Viability level was determined at different time of post-infection by human reovirus (0, 24, 48, and 72 h). As well as, the viability percent of normal human fibroblasts were calculated for comparison.

Table 1. Real-time PCR program for detection of the reovirus RNA.

denaturation	94°C	15 s
annealing	58°C	30 s
extension	72°C	30 s

Results

Plaque assay. Following crystal violet staining, viral plaques appeared as bright and clear areas. The best plaque counting was performed for well which had 10^{-6} dilution of viral stock. There were approximately 25 plaques (Fig. 1) and the amount of reovirus was calculated 5×10^{7} PFU/ml.

CPE of human reovirus. Normal human fibroblast and Huh-7 cells were infected by reovirus at an MOI of 10 PFU/cell. As shown in Fig. 2, no CPE was detected in the normal human fibroblasts, even at 72 hours post-infection. In contrast, Huh-7 cells infected with reovirus clearly exhibited CPE. CPE manifestations were like rounding and clumping, appearance of inclusion bodies, granulation and sloughing.

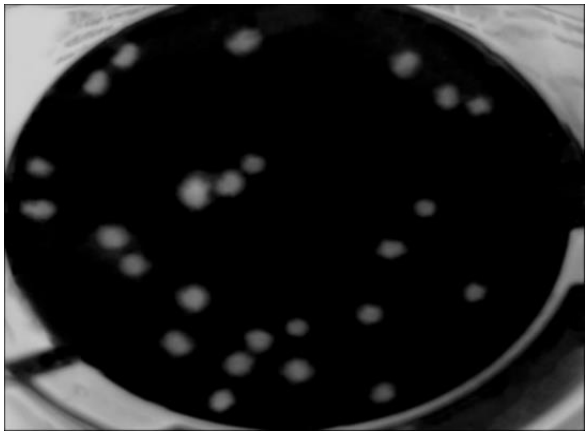

Fig. 1. Results of plaque assay. The plaques disappeared after 4 days. Bright areas are consequences of cell lysis that are not able to absorb color.

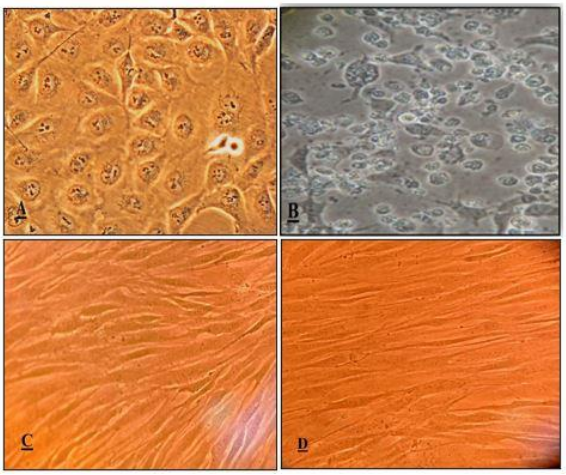

Fig. 2. The effects of reovirus infection (MOI=10) on normal human fibroblast and Huh-7 cells. A: Huh-7 cells before infection by reovirus. B: Huh-7 cells after infection by reovirus at 72 hours. C: Normal human fibroblasts cells before infection by reovirus. D: Normal human fibroblasts cells after infection by reovirus at 72 hours.

Cell viability. After inoculation of reoviruses into the cells, samples were collected at 0, 24, 48 and 72 hours, and cell counting was done. The results (Table 2) showed that viability level in normal human fibroblasts was about 100% at different sampling times, while the viability rate of Huh-7 cells was reduced by increasing the time of post-infection.

Table 2. Viability levels of different time of post-infection.

Sampling times (hour)	**0**	**24**	**48**	**72**
Normal human fibroblasts (viability %)	100	100	96	94
Huh-7 cells (viability %)	100	50	25	10

Real-time PCR. The real-time PCR results showed that there was a direct relation between reovirus RNA level and its duration time of cell infection by reovirus; while this relation was not observed for normal human fibroblast cells (Fig 3). Melting curve analysis was carried out to confirm the assay specificity and two separate peaks were detected (83.3°C for the L1 gene reovirus and 79°C for the beta-globin gene).

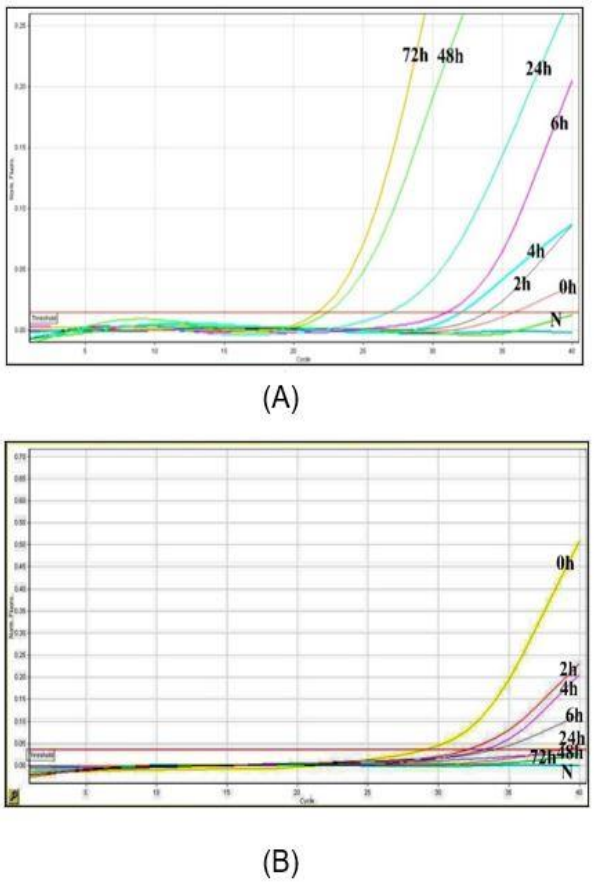

Fig. 3. Replication of the L1 reovirus gene in different samples by real time-PCR. Each curve corresponds to one reaction. (A) Real time-PCR curves of Huh-7 cells samples at the different time of post infection. An increasing trend was clearly seen in the reovirus-RNA levels. (B) No significant difference was seen in the reovirus-RNA quantity for human normal fibroblast samples.

Discussion

Huh-7 cell line was derived from a liver tumor of a Japanese man in 1982 (18). HCC is the most common liver cancer that reports one million deaths in the worldwide each year (19). Furthermore, the survival of HCC patients depends on a variety of factors, including the stage of disease, tumor size, liver function and disease symptoms (20). Thus, a selective targeting system might be beneficial for reducing the HCC mortality rate.

Recently, many studies have reported using reovirus as oncolytic viruses for the treatment of different cancer cells with overexpression of Ras signaling pathways (13, 21-28).

Prior to human clinical trial, oncolytic properties of a virus are usually tested in vitro using cancer cell lines and then in vivo studies will be progressed using human-tumor-grafted mouse (29). For this, we have evaluated the oncolytic potency of reovirus in Huh-7 cells with hyperactivation of Ras signaling pathway as a pre-study for using in HCC oncolytic viral therapy (30).

Conclusion

The present study showed that that Huh- cell viability rate, virus-RNA levels and viral CPE were increased at the different time of post-infection by human reovirus. In contrast, human reovirus had no significant effects on normal human fibroblasts, which used as control.

Generally, based on the growth inhibition and destruction of cancer cells by human reovirus serotype 3, it could be consi-dered as a potent therapy for liver cancer and HCC. To elucidate the reovirus susceptibility of liver tumor cells, it should be examined the capacity of reovirus replication in tumor-derived primary human liver cancer tissue in future.

Acknowledgment

The authors would like to thank Pasteur Institute of Iran and Iran University of Medical Sciences for their support.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

This study was not financially supported by any individual, agency, or institution.

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