# ORIGINAL ARTICLE

# The Effects of Human Reoviruses on Cancer Cells Derived from Hepatocellular Carcinoma Biopsies

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## SUMMARY

*Background:* Hepatocellular carcinoma (HCC) is the most common type of liver cancer around the world. Since this cancer is highly resistant to the existing treatments, we used a novel method, which selectively targets HCC cancer cells to improve the treatment process. As normal cells are resistant to reovirus replication, we used oncolytic reoviruses, which can infect, replicate in, and destroy cancer cells. In this study, the effects of oncolytic human reoviruses on cancer cells, derived from HCC biopsies, were investigated.
*Methods:* First, reoviruses were purified. Then a plaque assay was performed to estimate the number of viruses and determine the multiplicity of infection (MOI). To evaluate the effects of reoviruses on cancer cells derived from HCC biopsies, replication of reovirus RNA, viral protein production, cytopathic effects (CPE), and cancer cell viability were assessed at different intervals post-infection.
*Results:* Replication of reovirus RNA and viral protein production were detected in cancer cells. Also, different levels of viral protein production, CPE, cytotoxicity, and cancer cell viability were observed at different intervals post-infection with human reoviruses. In contrast, normal human fibroblasts, which were used as negative control, remained unchanged.
*Conclusions:* For the first time, the effects of human reoviruses on HCC biopsies were investigated. The results showed that human reoviruses could replicate in and destroy cancer cells derived from HCC biopsies. Overall, human reoviruses can be potentially used for the treatment of HCC.
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## KEY WORDS

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the fourth most fatal cancer around the world due to its poor prognosis and difficult treatment [1]. The average survival rate in patients with HCC is 6 - 20 months [2,3]. This complex type of cancer is affected by various risk factors and cofactors. It is mainly caused by chronic infection with hepatitis B virus

(HBV) and hepatitis C virus (HCV). Cirrhosis, alcohol abuse, tobacco smoking, aflatoxins, pesticides, obesity, diabetes, and genetic risk factors (e.g., hemochromatosis and alpha-1 antitrypsin deficiency) are other risk factors for the development of HCC [4].

HCC is more common among men than women, with a male-to-female ratio of 2:1. The high prevalence of HCC in men may be related to gender differences in exposure to risk factors, such as HBV and HCV infections, alcohol use, and cigarette smoking [5]. Since HCC is diagnosed at the end stage of liver infection in most patients, its mortality rate is approximately the same as its occurrence. Therefore, early detection of this cancer is highly important to improve survival [6]. There are several methods for the treatment of HCC, including liver resection, liver transplantation, chemoembolization, and radiofrequency ablation. Currently, the most effective treatment is tumor liver resection or liver transplantation. The most important obstacles to the successful treatment of HCC include aging, limited number of liver donors, metastasis, cancer reversion, and tumor progression [7]. Therefore, a new therapeutic approach against HCC is required to overcome these problems.

The respiratory enteric orphan virus (reovirus) belongs to the family *Reoviridae* and the genus *Orthoreovirus*. In 1953, this virus was first discovered by Stanley et al. in Australia and then named by Sabin in 1959 [8]. Reoviruses are divided into four serotypes, based on the inhibition of hemagglutination and neutralization: type 1 Lang (T1L), type 2 Jones (T2J), type 3 Abney (T3A), type 3 Dearing (T3D), and type 4 Ndelle (T4N). In humans, these viruses are isolated from the respiratory and gastrointestinal tracts. However, they are not associated with any symptomatic or acute diseases [9,10].

Reovirus serotype 3 (T3D) is a non-manipulated and wild-type virus with the innate ability to destroy cancer cells. Evidence shows that the activity of Ras signaling pathway increases in more than 30% of all human cancers. In cancer cells, the overexpression of Ras proteins is attributed to a direct mutation in the *ras* proto-oncogene or a mutation in the elements upstream or downstream of Ras. These mutations stimulate tumor growth, angiogenesis, and metastasis [11]. Since the Ras signaling pathway is not upregulated, transcription of dsRNA virus stimulates antiviral defense in normal cells. In contrast, cancer cells with an activated Ras signaling pathway are susceptible to reoviruses through PKR phosphorylation and blocking cellular defense against viral infection [12]. Therefore, the present study aimed to evaluate the effects of oncolytic reoviruses on tumor cells, derived from HCC biopsies.

## MATERIALS AND METHODS

### Preparation of HCC-derived cells
In the present study, three liver biopsies of HCC patients were provided by Tehran Medical Imaging Center. Liver biopsies were taken by sonography-guided FNA (fine-needle aspiration) method. Liver tissue specimens were immediately transferred to the transport medium. Transport medium components were DMEM (Dulbecco's Modified Eagle's Medium), 10% FBS (fetal bovine serum) and 2 x Pen/Strep solution. Then, biopsy samples were transferred to the cell culture laboratory with cold chain storage. HCC-derived cells were obtained from liver biopsies. Briefly, liver biopsy tissue was washed using FBS and then minced into 0.5 - 1 $mm^3$ fragments. Tumor fragments were placed in a 25 $cm^2$ cell culture flask, and a thin layer of DMEM supplemented with FBS, glutamine, antibiotics and non-essential amino acids was added on them. Cell culture flask was incubated at 37°C in a humidified 5% $CO_2$ incubator until the occurrence of migration and outgrowth of the tumor cells. In addition, the L-929 cell line was purchased from the National Cell Bank of Iran (code number C161) and normal human fibroblasts were acquired from Dr. Aghasadeghi at the Pasteur Institute of Iran.

### Virus propagation and purification
Reovirus T3D was kindly provided by Dr. Ataei-Pirkooh (Iran University of Medical Sciences). L929 cells were grown under common cell culture conditions and then infected with reovirus T3D at a MOI (multiplicity of infection) of 20 PFUs/cell. After the observation of CPE (cytopathic effect) in more than 80% of cells, cells and supernatant were frozen and thawed three times. The lysate was used as a virus stock for plaque assay and virus purification. Plaque assay and purification of reovirus T3D were done as described previously [13].

### Real-time PCR for Reovirus-RNA
To evaluate the changes and a possible increase in the reovirus-RNA within HCC-derived cells, real-time PCR was performed at a different time of post-infection. Normal human fibroblasts were applied as negative control of reovirus replication. Briefly, HCC-derived cells were plated onto 6-well cell culture plates at $10^5$ cells per well. The next day and after monolayer formation, the cells were infected with reovirus at an MOI of 10 PFUs/cell. Normal human fibroblasts were used as negative control in separate plates and all tests were performed in duplicate. Sampling was done at 0, 6, 12, 24, 48, and 72 hours post-infection with reovirus. Reovirus-RNA was extracted from each sample using Roche High Pure Viral Nucleic Acid kit (Roche, Germany) according to the manufacturer's instruction. Extracted viral RNAs were kept at -70°C until the next step. cDNA synthesis was done using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Real-time PCR for reovirus-RNA was carried out using SYBR Green method and the reaction contained 2 x qPCRBIO SyGreen Mix (PCR Biosystems, UK), 0.5 micromole (µM) of each primer [14], reovirus-RNA,

and DNase free water. Each run included a negative and internal control (beta-globin). All real-time PCR tests were done in duplicate [15]. The real-time PCR program was initiated by a denaturation step at 94°C for 3 minutes. The amplification step consisted of denaturation at 94°C for 15 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds repeated for 40 cycles. Finally, the specificity of the results was evaluated by the melting curve analysis.

**Viability and cytopathic effects**
To assess the susceptibility of HCC-derived cells toward the reovirus, the cells were plated onto six-well dishes at $10^5$ cells/well and then infected with reovirus at an MOI of 0.1 PFUs/cell. Morphological changes and cytopathic effects were evaluated in 3 days of post-infection, and the number of viable cells was counted by trypan blue staining. Normal human fibroblasts were used as negative control in separate wells and all tests were performed in duplicate.

**Immunofluorescence staining**
To evaluate the production and localization of reovirus proteins in the cytoplasmic area of HCC-derived cells, immunofluorescence staining was performed using mouse anti-human reovirus type 3 monoclonal antibody (MyBioSource, USA). Briefly, HCC-derived cells were plated onto six-well dishes at $10^5$ cells/well and infected with reovirus at an MOI of 0.1 PFUs/cell. At 2 - 3 days after infection, the cells were fixed in 4% paraformaldehyde followed by three washes with PBS (phosphate-buffered saline). After permeabilization with 0.1% Triton X-100, unspecific binding of antibodies was blocked with 1% BSA, 22.52 mg/mL glycine in PBST (PBS + 0.1% Tween 20). Then, the cells were incubated in 1:500 diluted mouse anti-human reovirus type 3 antibody in a humidified chamber. Finally, the cells were immunostained with 1:1,000 diluted FITC (fluorescein isothiocyanate) goat anti-mouse IgG (BioLegend, USA) followed by three washes with PBS. Immunofluorescence staining was assessed by the ultraviolet microscope.

## RESULTS

**HCC-derived cells**
Tumor biopsies, used for the HCC-derived cell cultures, were histologically classified as Edmondson-Steiner grade II HCC. The addition of a thin layer of the culture medium to the cell culture flask prevented floating and movement of biopsy fragments and made it easier for adhesion, cancer cell outgrowth, and monolayer formation. After almost two weeks, the tumor cells began to outgrow (Figure 1), and a tumor cell monolayer was formed after almost one month.

**Virus propagation and purification**
Mouse L-929 cells are the best cell line to propagate and purify reovirus T3D at high titers. The reovirus CPE was detected in more than 80% of cells at 48 - 72 hours post-infection. The CPE manifestations included rounding and clumping, emergence of inclusion bodies, granulation, and sloughing. Following the purification process, the gray band of the purified reovirus was aspirated and used to estimate the number of purified reovirus virions in a plaque assay. The number of purified reovirus virions was calculated to be 3 x $10^{13}$ PFUs/mL.

**Real-time PCR assay**
The results of real-time PCR assay showed a direct relationship between the reovirus RNA level and duration of cell infection by the reovirus; however, no relationship was observed in normal human fibroblasts (Figure 2). A melting curve analysis was conducted to confirm the assay specificity, which detected two separate peaks (83.3°C for reovirus *L1* gene and 79°C for beta-globin gene). The human beta-globin gene was used as an internal control to evaluate the accuracy of nucleic acid extraction and determine the absence of PCR inhibitors in the reaction. All samples had a cycle threshold (Ct) of 20 - 25, which confirmed the quality of the extraction process and the absence of PCR inhibitors.

**Viability and cytopathic effects**
Morphological changes and virus-induced CPEs were observed one day post-infection in HCC-derived cells, but not normal human fibroblasts even after seven days. The reovirus CPEs included rounding, clumping, and inclusion bodies. To evaluate cell viability after inoculation of reoviruses, samples were collected at 0, 6, 12, 24, 48, and 72 hours. The number of viable cells was counted by a hemocytometer after trypan blue staining. As shown in Figure 3, the results showed that the viability of HCC-derived cells clearly reduced after 24 hours and dramatically decreased after 72 hours post-infection. In contrast, the viability of normal human fibroblasts was not significantly different at different sampling intervals.

**Immunofluorescence staining**
Immunofluorescence staining, which uses specific anti-human reovirus antibodies, is used to determine the presence of reovirus antigens and proteins within the cytoplasm of infected HCC-derived cells (but not the negative control or normal human fibroblasts) (Figure 4). Confirmation of the presence of reovirus proteins indicated the reovirus replication ability and production of viral proteins in HCC-derived cancer cells.

## DISCUSSION

HCC accounts for approximately 85 - 90% of primary liver cancers. Nearly 550,000 to 600,000 new HCC cases are reported annually around the world [4,16].
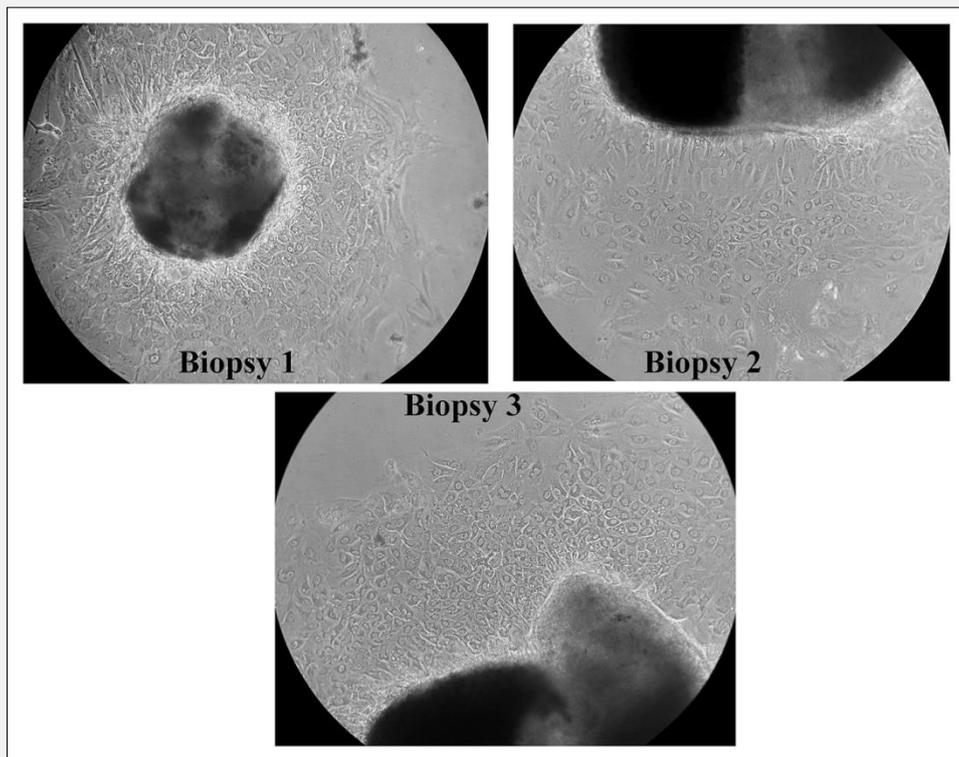
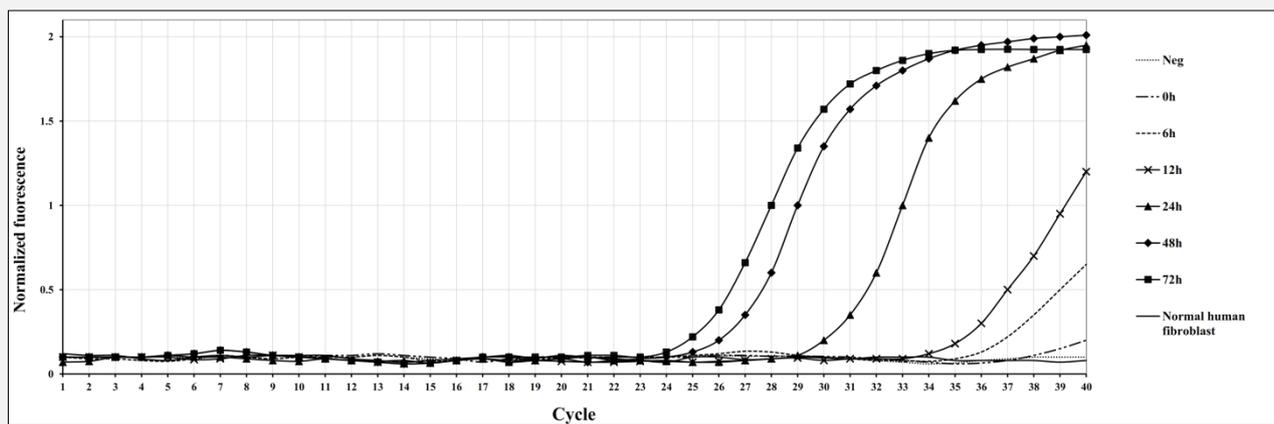**Figure 1. Outgrowth and monolayer formation of cancer cells derived from three HCC biopsies.**



**Figure 2. Amplification plot of reovirus real-time PCR assay for HCC-derived cells of a biopsy.**

**To evaluate the reovirus RNA level, HCC-derived cells were collected at 6, 12, 24, 48, and 72 hours post-infection. The curves indicated the reduction of Ct value over time, showing the increased amount of reovirus RNA and its replication. This mode was not detected in normal human fibroblasts ($p < 0.05$).**
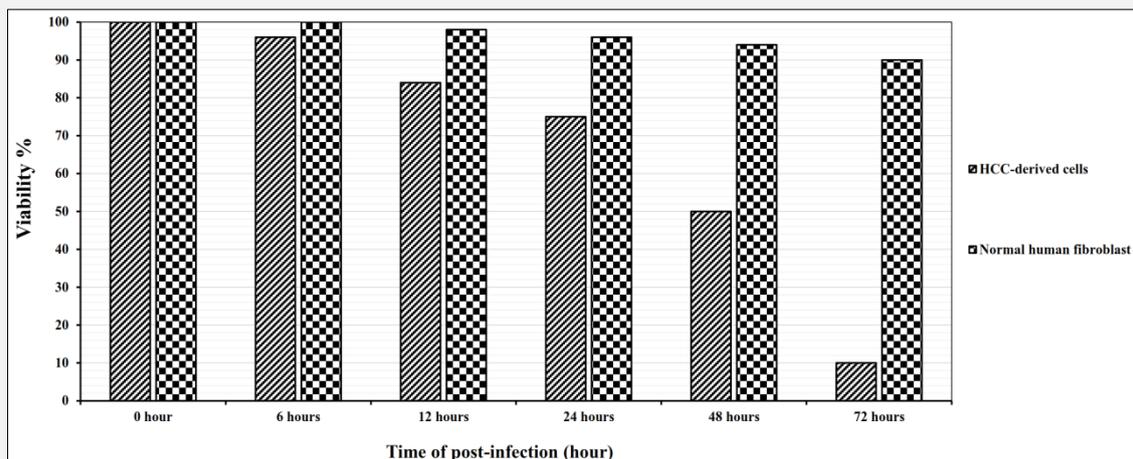
**Figure 3. Viability levels at different intervals post-infection with reoviruses.**

The viability percentage of HCC-derived cells was significantly lower than normal human fibroblasts over time post-infection ($p < 0.05$).



**Figure 4. Immunofluorescence staining of infected cells derived from one HCC biopsy.**

Luminous spots (white arrow) represent the virus production factories within the cytoplasm of infected HCC-derived cells.

Iran is located in the Middle East, where HCC can be detected in patients with intermediate or advanced stages of the disease [17]. Almost five cases of HCC per 100,000 people are diagnosed in Iran [18]. This cancer is often clinically silent until it is well-developed. Due to its poor diagnosis, the survival of patients is short,

causing 1,000,000 deaths annually. Today, the long-term survival of HCC patients can be increased through surgical procedures, and about 20% of patients with early HCC symptoms are candidates for surgery [19,20]. Therefore, use of a novel method of treatment, which can affect cancer cells without affecting normal cells, can be beneficial [21].

Recently, numerous studies have reported the use of reoviruses for destroying different cancer cells. Reoviruses have selective and potential oncolytic properties for a variety of cancer cells, especially those with an active Ras signaling pathway [22]. The results of previous research have shown the increased activity of the Ras signaling pathway in HCC tumor cells. Therefore, this cancer may be treated via reovirus therapy [23]. Numerous experimental studies have also reported the anticancer effects of reoviruses against various human cancers, including colon cancer, ovary cancer, breast cancer, head and neck cancer, pancreatic cancer, malignant gliomas, and medulloblastoma. Moreover, further phase I, II, and III clinical trials are underway [24-36].

In the present study, the effects of reoviruses on HCC-derived cells and normal human fibroblasts were evaluated *in vitro* to determine their potential use for the treatment of HCC. Since fibroblasts are the most common connective tissue cells in various human organs, including the liver, and the number of normal hepatocyte passages in the cell culture is very limited, we used normal human fibroblasts as normal cell controls in the experiments, similar to previous studies [24,29]. HCC cells, derived from three tumor biopsies, were cultured and monolayered. To investigate the replication of reoviruses in HCC cancer cells, replication of reovirus genome and synthesis of viral proteins were studied. We observed prominent morphological changes and viral CPE in HCC-derived cells, infected by reoviruses.

Moreover, our results showed that the viability of HCC-derived cells was significantly lower than normal human fibroblasts. The level of these changes was dependent on the sampling time post-infection. The lowest percentage of viability was observed at 72 hours post-infection in HCC-derived cells. The results of real-time PCR assay showed that the level of reovirus RNA replication increased with time post-infection. Also, reovirus proteins were detected in tumor cells by immunofluorescence staining, which is similar to the results of a study by Wilcox et al. [25]. Also, Samson et al. recently focused on reovirus-induced immunity responses in patients with HCV- or HBV-related HCC. They showed that reoviruses could induce innate immune responses in the absence of cytotoxicity and then suppress HCV or HBV replication, thereby increasing the patient survival [36]. These changes led to the inhibition of growth and destruction of cancer cells. Therefore, reoviruses can be considered as a potential therapy for HCC and liver cancer.

Overall, it is necessary to examine reovirus replication in patients with liver cancer in the future to elucidate the reovirus susceptibility of liver tumor cells. Also, the effects of reoviruses on a large series of clinical samples must be examined in the clinical setting. Our findings suggested the potential clinical use of reoviruses as a combination antitumor therapy against liver cancer.

## CONCLUSION

In the present study, the effects of human reoviruses on cancer cells, derived from HCC biopsies, were investigated. The viral effects were assessed by CPE observation, real-time PCR assay, cell viability assessment, and immunofluorescence staining. The results showed that human reoviruses could replicate in and destroy cancer cells, derived from HCC biopsies. Therefore, we suggest human reoviruses as a potential therapy for HCC, although further research is essential.

**Statement of Ethics:**
This work has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). The ethics committee of the Pasteur Institute of Iran approved this study (registered number 833), and before the sampling began, informed consent was obtained from all patients.

**Declaration of Interest:**
The authors have no conflicts of interest to declare.

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