**Iranian Journal of Virology 2015;9(4): 24-28**
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## Original Article

# Rotavirus NSP4 protein as a viral biotoxin selectively promotes cytotoxicity

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## Abstract

**Background and Aims:** The number of new cancer cases with considerable mortality is increasing worldwide. Since the inability of current therapies in treatment of patients and prevention the progress of tumors with fewer side effects, implementation of new methods is needed. Gene therapy has widespread systemic cytotoxic effects against tumor cells. Rotavirus NSP4 has been shown to elicit extensive cytotoxic activities in transfected or infected cells. In this study, the biological cytotoxic effect of NSP4 rotavirus protein was investigation on TC-1 tumor cell integrity.

**Materials and Methods:** NSP4 gene of rotavirus was cloned into the pCDH plasmid and then TC-1 tumor cells were transfected with plasmids. After reviewing the presence of this protein by SDS-PAGE and confirming NSP4 expression by western blotting using anti-NSP4 antibody, the cytotoxic effect of NSP4 expression in TC-1 tumor cells was measured by the MTT assay.

**Results:** Significant differences were observed in the cell viability between the control groups and the group of cells that received NSP4 gene.

**Conclusion:** Rotavirus NSP4 gene posses specific cell cytotoxicity and is potentially effective in tumor destruction.

**Keywords:** Rotavirus, NSP4, Enterotoxin, Cytotoxicity, Cancer Therapy

## Introduction

Cancer is a major public health problem worldwide. Because of its high morbidity and mortality in the world, new therapeutic strategy are needed to improve cancer treatment (1).

Tumor therapy has been much improved in recent decades but there are some problems that limits the use of these methods such as drug resistance in cancer cells, ability of tumor cells to evade apoptosis, serious side effects of these treatments associated with non -specific cytotoxicity(2). Cancer gene therapy includes a wide range of treatments. The effectiveness of many types of gene therapy in the laboratory and animal models has been demonstrated. Gene therapy has been used to direct cell microenvironment, development of cancer vaccine, the use of viruses for death of cancer cells, transferring genes into cancer cells to kill them or restore normal phenotype (3).

Rotaviruses are nonenveloped viruses that causes acute gastroenteritis in young children (4). The viral genome Contains 11 segments that encodes 6 structural proteins (SPs) and 6 nonstructural proteins (NSPs) (5). NSP4 is a multifunctional protein that is coded by gene segment 10 and consists of 175 amino acids. This protein is identified as a virus enterotoxin and induces an immune response during

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[ DOI: 10.21859/isv.9.4.24 ]

rotavirus infection. NSP4 cytoplasmic domain exhibits important biological functions (6). Laboratory investigation in animal models has shown that NSP4 (114–135) and NSP4 (112-175) induces diarrhea in mice. NSP4 consisting of three hydrophobic regions, an α-helix and a cluster of basic residues which are participate in the formation of viroporin domain (7). Viroporins are small proteins that forms a hydrophilic pore and modify cell's permeability to ions or other small molecules(8). NSP4 activity disrupts calcium homeostasis by viroporin domain (4). Recombinant NSP4 expression has cytotoxic effect in Sf9 cells expressing NSP4 protein caused by increasing concentrations of cytoplasmic Ca2+ (9). Expression of recombinant NSP4 in mammalian cell, decreases membrane stability and leads to cell death (10). Alysine-rich region of NSP4 which is not related to the enterotoxic peptide sequence is responsible for the induction of cytopathic effects (11). In this study, the biological properties of this protein were considered to destroy TC-1 tumor cell. Rotavirus NSP4 gene was cloned into the pCDH plasmid and TC-1 cells were transfected with target plasmids. After reviewing the presence of this protein by SDS-PAGE, NSP4 expression was confirmed by western blotting using anti- NSP4 antibody. The cytotoxic effect of NSP4 in TC-1 tumor cells was measured by MTT assay. Significant differences were observed in the cell viability between the control groups and the cells that received NSP4 gene. We showed that rotavirus NSP4 gene posses specific cell cytotoxicity and potentially effective in tumor studies.

## Methods

**Constructs.** TA vector containing Rotavirus NSP4 (RF strain) gene was preserved in our laboratory. The expression vector pCDH were kindly provided by Dr. E. Arefian from Stem Cell Technology Research center of Iran. All the enzymes obtained from Fermentas (Germany). The presence of the NSP4 gene in the TA vector was confirmed with restriction enzymes. Because the TA cloning site does not coincide with the pCDH vector, at first the NSP4 gene was sub-cloned from TA into the unique *XbaI* and *ApaI* cloning sites of the pcDNA3.1(-) vector. The recombinant plasmid pcDNA3.1 (-)-NSP4 was amplified in E.coli strain DH5α and plasmid preparation was performed using GeneAll Plasmid Miniprep Kit. The presence of the NSP4 gene in the constructed vector was determined using restriction enzyme analysis. pcDNA3.1 (-)-NSP4 was digested with *EcoRI* and *NheI* andNSP4 fragment was purified and ligated into plasmid pCDH. Restriction enzyme analysis was performed to confirm NSP4 ligation into the pCDH.

**Cell line.** TC-1 cell line was obtained from a cell bank (Pasteur Institute of Iran). They were grown in RPMI 1640, supplemented with 10% FCS and was incubated at 37°C in 5% CO2 (12).

**SDS-PAGE and Western blot analysis.** TC-1 cells were cultured in a 6-wells plate and incubated overnight in complete medium without antibiotics. When they reached to 80% confluency, transfection of pCDH-NSP4 construction into the target cells was performed using Lipofectamin2000 (Invitrogen, USA) according to the manufacturer's instruction. Cells were harvested within 24 hours after transfection; the cell lysates were prepared by multiple freeze-thawing cycles and sonication (60Hz, 0.5 amplitude). The cell lysates were loaded on 12% SDS-PAGE gel and followed (by coomassie blue staining. To monitor expression of NSP4, Western blot analysis was performed.

**MTT assay.** Cell viability was measured by the MTT assay using the manufacturer's instructions. Approximately $2\times10^4$ TC-1 cells per well were cultured in 96- well plate and incubated at 37°C. The test was done on three groups, control cells, cells transfected with empty vector and cells transfected with plasmids containing NSP4. Transfection was performed using 2.0 micrograms of plasmids and 5.0 micro liters Lipofectamin according to Invitrogen protocol. Twenty four hours following transfection, 20 ml of 5 mg/ml MTT was added to the wells and incubated at 37°C

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for 3 hours, DMSO was added and the absorbance at 560 nm was measured.

## Results

**Identification of pcDNA3.1 (-)-NSP4 and pCDH-NSP4 by enzymatic digestion.** The accuracy of constructed plasmids was confirmed by restriction enzyme analysis. Competent E.coli strain DH5α was transformed with ligation product of digested NSP4 fragment into linearized pcDNA3.1 (-) during the first cloning and into linearized pCDH in the second cloning. Plasmids extracted using Plasmid Miniprep Kit. Constructed plasmids were digested with responsible enzymes and the results showed that the extracted plasmid contains NSP4 gene.

**Characterization of NSP4 protein by Western blot analysis:** The transfected cell lysates were prepared and total protein bands were separated on 12% SDS-PAGE gel and stained with Coomassie blue. The proteins were transferred to a PVDF membrane and electrophoresis was performed for 1 hour at 90 V. The membrane was incubated with HRP-conjugated mouse polyclonal anti-NSP4 antibody and specific bands were detected

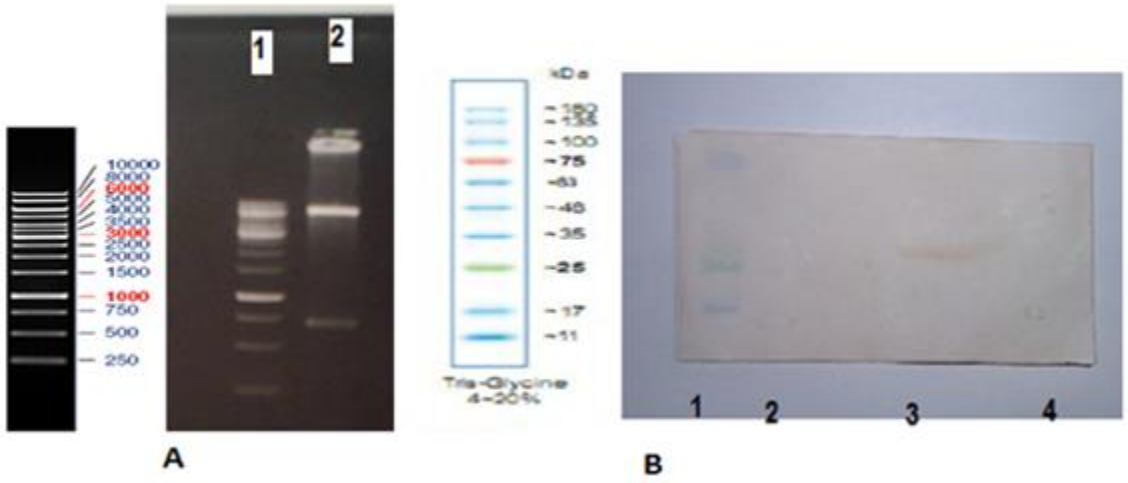



**Fig. 1.** A) Enzymatic digestion of NSP4 fragment and linearized pCDH ligation product. Lane 1: 1kb DNA marker Lane 2: Recombinant pCDH-NSP4 vector. B) western blot analysis of NSP4 using polyclonal antibody. Lane 1: protein size marker, lane 2: control cells transfected with pCDH plasmid, lane 3: the cells transfected with pCDH-NSP4 Construct and lane 4: control TC-1 cells.

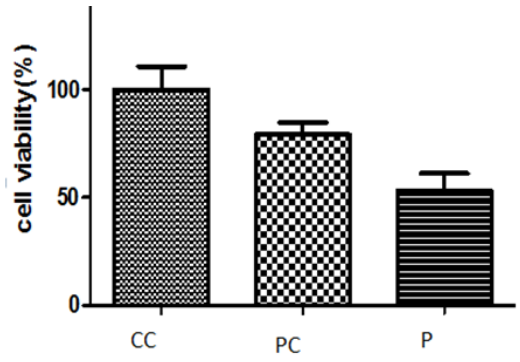



**Fig. 2.** MTT assay result. CC Lane: TC-1 cells, PC lane: cells transfected with pCDH and P lane: cells transfected with pCDH-NSP4 construct.

using Diamino Benzidine substrate (Biogene, Iran). This protein was absent in untransfected cells and cells transfected with pCDH.

**Viability assay.** Viability determined by tetrazolium bromide (MTT) test using 2×104 TC-1 cells per well in 96-well plates.MTT assay was performed 24 hours after transfection cells with empty pCDH and pCDH containing NSP4. The results of the test and control groups were analyzed with Prism software and using one-way ANOVA and TUKEY method. Significant differences in cell viability were observed ($p$ value $< 0.0001$) between both groups of control and the cells transfected with pCDH- NSP4.

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## Discussion

NSP4 is the most important factor in the pathogenesis of rotavirus diarrhea(13). NSP4 has naturally cytolytic activity in infected cells and its cytopathic and enterotoxic activity caused by several mechanisms(11). Peptides derived from the cytoplasmic domain of NSP4 (114-135) residues have enterotoxin activity in vitro and cause the liposome membrane permeability(14); On the other hand, NSP4 proximal domain including basic amino acids is important for its cytotoxicity in MA104 infected cells(10). Tian et al. (15) have confirmed selective membrane destabilizing activity that promoted by purified NSP4 or synthetic peptide corresponding to residues (114 to 135). Residues(54-74) create an amphipatic helix which has cytopathic effect on ER, leads to increasing in intracellular Ca2+ level and in vivo membrane disrupting activity of NSP4(11). The rotavirus NSP4 protein is a biotoxin. Biotoxins produced by living organisms and can kill other organisms or damage them(1). Some biotoxins are present great potential as anti-tumor agents. The medicinal properties of these materials can be used in the production of new compounds that may be useful in cancer therapy in the near future. Due to the biological function of this protein full length NSP4 gene was used to destroy TC-1 cells in vitro. NSP4 gene expression in a eukaryotic tumor cell was studied and confirmed its cytotoxicity effect in tumor cells, by increasing concentrations of cytoplasmic Ca2+(16). The results show that rotavirus NSP4 proteins can be consider as a biotoxin for cancer immunotherapy and have the potential to kill tumor cells as an anti-cancer agent, especially in the case of cancer cells that are resistant to the drug or when other treatment are not effective.

In conclusion, developed qRT-PCR in the current study is a simple, less time-consuming, and highly sensitive and specific. It also requires less manipulation for quantification of Rubella virus RNA. This study confirmed that our designed qRT-PCR experiment is a well appealing technique in the field of research and development (R&D) in biopharmaceutical industries as well as IPQC tests for vaccine manufacturing.

## Conclusion

Rotavirus NSP4 has cytotoxic effectes on TC-1cells and this protein could be considered as an antitumor agent in cancer studies.

## Acknowledgement

This study was supported partly by a grant from the Research Deputy of Tarbiat Modares University and partially was supported by a grant (89002201) which was sponsored by the Iran National Science Foundation (INSF). We gratefully acknowledge their supports.

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