Original Article

**Autophagy knock down: as a booster for the replication of viruses in cell culture**

Mahdieh Talebi¹, Taravat Bamdad¹*, Ehsan Arefian², Habib Ahmadi³, Hesam Karimi¹, Hamzeh Choobin¹

¹Department of Virology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, IR Iran
²Department of Microbiology, College of Science, University of Tehran, Tehran, IR Iran
³Department of Genetics, College of Medical science, University of Shahid Beheshti, Tehran, IR Iran

Received: 12 May 2016, Accepted: 25 September, 2016

**Abstract**

**Background:** Autophagy suppression recently has been known to have a remarkable effect for cellular adjustment and viability in the final stages of cancer. On the other hand, autophagy has the potential effect in preventing many viruses from replication. Beclin1 is the most substantial constituent in autophagy apparatus regulation. This study was intended to investigate the beclin1 siRNA knockdown effect on the extent of activity of the oncolytic vesicular stomatitis virus (VSV) as a model in cell culture. **Materials and Methods:** In the current study, the cancer cell line, HeLa (cervical squamous cancer cell line) was infected by VSV, followed by beclin1 siRNA vector transfection. The potential change in the expressions of gene beclin1 in transfected cells, as well as untransfected ones were examined by real time PCR, and also the titer of viruses was compared in cells with and without transfection. **Results:** The results revealed that the amount of putative gene beclin1 expression in HeLa cells decreased greatly due to siRNA suppressive impact, and also the sensitivity of the cells to VSV oncolytic effect increased upon decrease in beclin1gene expression. **Conclusion:** It seems that autophagy suppression by using siRNA with VSV is a substantial aid for increase in virus titer in cancer cell lines. **Keywords:** Autophagy, Vesicular Stomatitis Virus, Beclin1, Virus therapy

*Corresponding Author: * Taravat Bamdad, Department of Virology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, IR Iran. E-mail: bamdad_t@modares.ac.ir, P. O. Box 14115-331.


**Introduction**

Autophagy machine has been suggested to act as a cell surviving constituent. It functions to maintain cellular homeostasis in conditions such as nutrient deprivation and micro environmental pressures (1, 2). Though, autophagy provides eukaryotic cells the required energy by recycling DNA and RNA material, Protein aggregates, as well as cellular organelles, thus promoting persistence of established malignancies (3, 4).

From the molecular point of view, autophagy is governed by a pannel of genes, particularly ones encoding yeast Atg-related proteins. The most substantial protein coding gene in this category is Beclin-1(mammalian homologue of Atg6). It is a gene encodes for a protein, which has a major role in coordinating autophagy machine operation. Beclin1 interferences with Ambral1, BIF1, UVRAG proteins promote autophagy and with Bcl2, and Rubicon. Also, its interaction with Vps34 (PI3KIII) creates the two-layer membrane of autophagosome (5).

Remarkably, autophagy is active in fighting against viruses. Autophagy activation in virus infected cells leads to form the two layer membrane vesicles, consequently getting rid of virulent invaders. Also, it functions as an innate and adaptive immune response
trigger to fight against viruses. Excluding some viruses like HCV and other flaviviruses that take advantage of autophagy in favor of their replication, reducing the autophagy can help the viruses in infected cells to propagate more efficiently and cause more oncolytic effects in cells. This can be taken as a strategy to increase virus titer in producing cells and also to augment the efficacy of oncolytic viruses in cancer cell therapy (3, 6-8).

Many viruses are striking examples of major public health concerns in modern era. They create health problems for the millions of people throughout the world every year, such as severe infections, neoplasm, etc. (9). Vaccination for such infections is one of the main ways to control the disorders which demand high production of attenuated viruses in vaccine producing companies.

On the other hand, in contrast to the fact that some malignancies like hepato cellular carcinoma, burkit lymphoma and cervical carcinoma induce by viruses; oncolytic viruses could have anti-mitotic qualities and cell destruction activity which are desired in fighting against cancer. Moreover, Medical advances in cancer therapy have developed a set of therapeutic option including chemotherapy, surgery interferences, immunotherapy, radiotherapy and so on. However, challenging complications for sick people facing severe side effects of mentioned therapies has prompted medical scientists to look for more safe potential cure options. Therefore, viruses have gained remarkable attention in treatment of several cancers due to their good spread in malignant cells the low side effects for patients (10, 11).

VSV is one of the oncolytic viruses which have attracted many attentions in the field of cancer virotherapy. It has been shown that in VSV sensitive cancer cells, the expression of Beclin1 has reduced in comparison with VSV resistance tumor cells (12).

In the present study, we aimed on evaluating relation between autophagy knockdown and the rate of VSV replication in a suitable cervical cancer model (HeLa cells).

**Methods**

**Plasmid preparation and siRNA ligation.**
The vector used in this study was a pCDH3.0. Beclin-1 siRNA was designed with sticky ends compatible to BamH1 and EcoR1at 5´ and 3´ends, respectively and synthesized by Tag Copenhagen company (Denmark) in shRNA formulation. That targets from position of 584nt to 608nt of Beclin 1 mRNA.

The siRNA ligation reaction to the pCDH3.0 plasmid vector was accomplished, based on T4 ligase action on BamH1 and EcoR1 at the linearized plasmid ends. Clony PCR was performed by universal primers to amplify the ligated siRNA, after propagating the vector in Escherichia coli DH5α medium culture. Then, PCR product was undergone running on 2% agarose gel including Red green dye, followed by a digital CCD camera picture taken of the gel to documentation. Also, PCR product was subjected to Sanger sequencing in ABI sequencer 3130 equipment, using universal primers.

**Transfection of HeLa cell.** After culturing HeLa cells (cancer cells from cervical tumor), 5% fetal bovine DMEM (Gibco,UK) medium- antibiotic free- was aliquoted to every single well of a 24-well plate. The process followed 24 hrs by adding the contents of two transfection reagent solution, one containing (lipofectamine 2000, invitrogen, pCDH vector and free-serum medium) and the other same but containing mock siRNA were added drop-wise in different concentrations in to the wells. Then, treated medium cultures were incubated for 72 hrs in 37˚C, 10% CO2. Transfected cells were examined by fluorescent invert microscopy, and the presence of GFP gene expression on the penetrated plasmid was identified for cell transfection.

**RNA extraction & cDNA preparation.** HeLa cell cultures in 96-well plate were kept in 5% CO2 incubator in 37˚C for 24 hours till they were subjected to either siRNA transfection or mock vector transfection in separate rows, while leaving one row intact as control. HeLa cells, corresponding to each well, were harvested after 48 hours of incubation in 37˚C by shoveling well bottoms up before micro tube centrifugation. Next, using the high quality RNA extraction kit (Roche, Basel, Switzerland),total RNAs of precipitated cells were extracted. RT- PCR reaction was performed to synthesize beclin-1 complementary DNA for each sample tube using cDNA synthesis kit (Qiagen, Hilden, Germany), which included hexamer primers.
Real-time PCR reaction. The rate of beclin1 expression in siRNA and mock transfected cells were evaluated by ABI realtime instrument using Taq Man assay (Qiagen, Hilden, Germany), based on comparison with GAPDH gene as base level of mRNA. Data analysis was done by REST program with respect to realtime PCR output.

Cell Culture and Virus Titer Determination via TCID50. HeLa cells were used to propagate VSV virus, New Jersy strain. Logarithmic dilutions (10^{-1} to 10^{-12}) of the virus were inoculated on HeLa cells in a 96-well culture plate, with being one row uninoculated as control. After 72 hour the microplates were examined for CPE and the Karber formula was exploited to calculate virus titer.

The same procedure was performed with putative cells, transfected with siRNA 24 hrs before virus inoculation. Both of the experiments were performed in triplicates.

Results

Construction of siRNA-pCDH vector. Beclin1 siRNA fragment with two sticky ends was inserted in to a pCDH plasmid in MCS under the control of a CMV promoter, so this vector can express the gene of interest in mammalian cells. Incorporated SV40 origin can ease replication in human cell lines and an ori, elsewhere, replication in prokaryotes. The designed siRNA included a potential loop motif sequence, intended to function as a site for cleaving function of nuclear enzyme, Dorsha (Rnasen), and sense and anti-sense strands, which are corresponding to coding regions on beclin1 mRNA transcripts.

The results obtained by colony PCR in screening for recombinant plasmids, included siRNA fragment, confirmed the ligation procedure accuracy. Illustration of PCR products run on gel electrophoresis was given (Fig1).

Real-time PCR. Quantitative PCR assay for beclin1 gene expression 72 hrs after siRNA suppression was performed. Melting point curve was verified for PCR accuracy (Fig 2). CT values for real-time PCR reactions were determined regarding to amplification plots. Beclin1 gene expression for siRNA treated cells in comparison to untreated cells showed a great decrease of 1000 time fold (p<0.0001), as evaluated by REST program analyzer.

Virus titration in HeLa cells. To estimate and compare titer of VSV in the presence and absence of Beclin 1expression, 72 hours after virus inoculation VSV titer was calculated. The survey presented 10^{8.66} TCID50/ml for VSV in HeLa cell and 10^{10.59} TCID50/ml in siRNA transfected cells.

Discussion

Cancer treatment has attracted many researches in the new world. Due to its complicated nature, many approaches in experimental studies have been exploited to pickup new path for more efficient cure, including attempting various signaling pathways which may confer new strategies for gene therapies.

Autophagy is a cellular mechanism for maintaining homeostasis. It plays an important role in preventing cellular events due to environmental pressures from progression to dysplastic changes. However, autophagy machine in established malignant cells is out to compensate for nutrients shortage and genome instability to lengthen survival time (13).

Several previous studies showed a positive effect of autophagy knockdown in ameliorating malignancy phase in different types of cancers. Accordingly, results obtained by the current study presented strong effect of beclin1 siRNA in silencing autophagy response (14-16).

On the other hand, autophagy reduction can help many viruses to replicate more efficiently. Data obtained from previous studies showed VSV immediately activates autophagy formation upon its surface glycoprotein attach, called VSVG. The activated autophagy controls VSV replication (6).
Autophagy knock down: as a booster for the replication of viruses in cell culture

Talebi et al.

Vol 2, No 2, Spring 2016

Because of the defect in innate immunity in tumor cells, many transformed cells are sensitive to viral replication. This is the base of using some viruses as oncolytic agents. Since autophagy is a part of innate immune response, depriving from autophagy machine could be a good device to facilitate viral trafficking in to such cancerous cells. This suggests autophagy can benefit new strategies of viral therapy for cancer. A remarkable number of studies have showed autophagy blocking drugs such as 3MA (PI3KIII inhibitor) is effective in sensitizing cancer cells in treatment approaches like hormon, radiotherapy etc. In the context of inhibiting autophagy viruses can evade from being trapped in cellular xenophagy, hence protecting themselves to replicate more efficiently in cancerous cells (3, 6, 13, 17, 18).

The current study was to evaluate autophagy suppression through beclin1 gene knockdown in a scenario where VSV would reproduce more vigorously. As notified, beclin1 gene silencing could have a substantial effect on increasing titer of the virus in HeLa cells. This confers autophagy suppression in combination with VSV can be influential in cervical squamous cell therapy.

Besides, this data from VSV as a model virus can be applied to increase the rate of virus replication and production of virus particles in cell culture.

Finally, gene therapy in combination with virotherapy have shown appropriate results in the cell line level (19, 20). It is suggested that the future studies address the effects of this combinatorial therapy in proper animal models (21). On the other hand, reducing autophagy in cells can be considered as a benefit approach to increase the titer of many viruses when high titer of virus production is desired.

Conflicts of Interest

There is no conflict of interest to the study.

Acknowledgment

We wish to thank Bonyakhteh Institute staff.

Funding/Support: This work was supported by Faculty of Medical Sciences, Tarbiat Modares University.

References