Original Article

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

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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

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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 Ambra1, BIF1, UVRAG proteins promote autophagy and with Bcl2, and Rubicon. Also, its interaction with Vps34 (PI3KIII) creates the two-layer membrane of autophagosom (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 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% CO_2. 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% CO_2 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 beclin 1 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 TCID$_{50}$. 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.
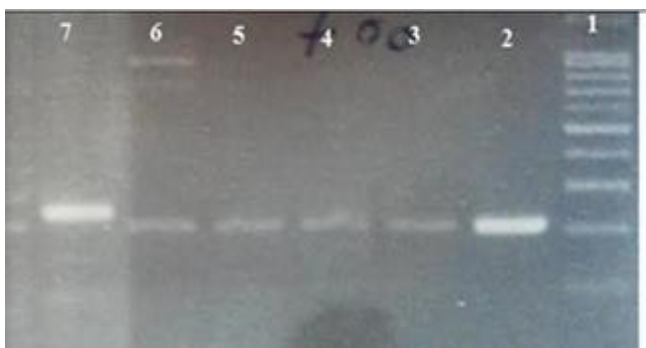

Figure 1. PCR cloning products run on 2% agarose gel electrophoresis to confirm the ligation process, using universal primers. The lane 7 represents a ligated plasmid pCDH, compared with the other lanes showing unligated plasmids.

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).
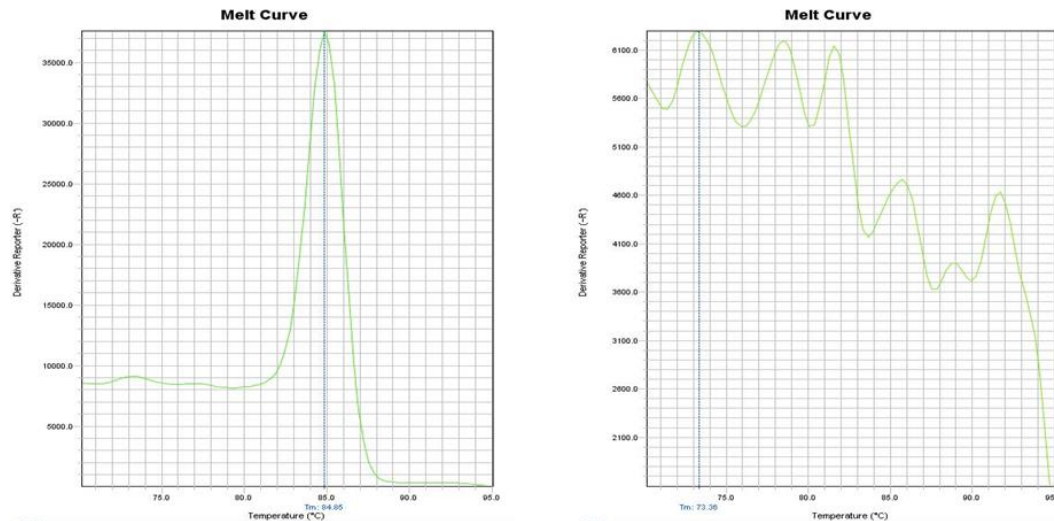
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Figure 2. Diagrams show melting curves for beclin1 gene real-time PCR. The left curve represents untreated cells, compared with the beclin1 siRNA treated cells at the right.

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 beclin1gene 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

References

1. Taneike M, Yamaguchi O, Nakai A, Hikoso S, Takeda T, Mizote I, et al. Inhibition of autophagy in the heart induces age-related cardiomyopathy. Autophagy. 2010;6(5):600-6.

2. White E, Karp C, Strohecker AM, Guo Y, Mathew R. Role of autophagy in suppression of inflammation and cancer. Current opinion in cell biology. 2010;22(2):212-7.

3. Choi KS. Autophagy and cancer. Experimental & molecular medicine. 2012;44(2):109-20.

4. Mah LY, Ryan KM. Autophagy and cancer. Cold Spring Harbor perspectives in biology. 2012;4(1):a008821.

5. Kang R, Zeh H, Lotze M, Tang D. The Beclin 1 network regulates autophagy and apoptosis. Cell Death & Differentiation. 2011;18(4):571-80.

6. Shelly S, Lukinova N, Bambina S, Berman A, Cherry S.

Autophagy is an essential component of Drosophila immunity against vesicular stomatitis virus. Immunity. 2009;30(4):588-98.

7. Wang HY, Yang GF, Huang YH, Huang QW, Gao J, Zhao XD, et al. Reduced expression of autophagy markers correlates with high‑risk human papillomavirus infection in human cervical squamous cell carcinoma. Oncology letters. 2014;8(4):1492-8.

8. Jing K, Lim K. Why is autophagy important in human diseases? Experimental & molecular medicine. 2012;44(2):69-72.

9. Siegel R, DeSantis C, Virgo K, Stein K, Mariotto A, Smith T, et al. Cancer treatment and survivorship statistics, 2012. CA: a cancer journal for clinicians. 2012;62(4):220-41.

10. Wong HH, Lemoine NR, Wang Y. Oncolytic viruses for cancer therapy: overcoming the obstacles. Viruses. 2010;2(1):78-106.

11. Patel MR, Kratzke RA. Oncolytic virus therapy for cancer: the first wave of translational clinical trials. Translational Research. 2013;161(4):355-64.

12. Nabizadeh A, Bamdad T, Arefian E, Nikoo SHR. Autophagy Gene Activity May Act As a Key Factor for Sensitivity of Tumor Cells to Oncolytic Vesicular Stomatitis Virus. Iranian Journal of Cancer Prevention. 2016;9(1).

13. Chen N, Karantza-Wadsworth V. Role and regulation of autophagy in cancer. Biochimica et Biophysica Acta (BBA)- Molecular Cell Research. 2009;1793(9):1516-23.

14. Takahashi Y, Coppola D, Matsushita N, Cualing HD, Sun M, Sato Y, et al. Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. Nature cell biology. 2007;9(10):1142-51.

15. Itakura E, Kishi C, Inoue K, Mizushima N. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. Molecular biology of the cell. 2008;19(12):5360-72.

16. Liang C, Feng P, Ku B, Dotan I, Canaani D, Oh B-H, et al. Autophagic and tumour suppressor activity of a novel Beclin1- binding protein UVRAG. Nature cell biology. 2006;8(7):688-98.

17. Hippert MM, O'Toole PS, Thorburn A. Autophagy in cancer: good, bad, or both? Cancer research. 2006;66(19):9349-51.

18. Meijer AJ, Codogno P. Regulation and role of autophagy in mammalian cells. The international journal of biochemistry & cell biology. 2004;36(12):2445-62.

19. Stanford MM, Barrett JW, Nazarian SH, Werden S, McFadden G. Oncolytic virotherapy synergism with signaling inhibitors: Rapamycin increases myxoma virus tropism for human tumor cells. Journal of virology. 2007;81(3):1251-60.

20. Sung CK, Choi B, Wanna G, Genden EM, Woo SL, Shin EJ. Combined VSV oncolytic virus and chemotherapy for squamous cell carcinoma. The Laryngoscope. 2008;118(2):237-42.

21. Schache P, Gürlevik E, Strüver N, Woller N, Malek N, Zender L, et al. VSV virotherapy improves chemotherapy by triggering apoptosis due to proteasomal degradation of Mcl-1. Gene therapy. 2009;16(7):849-61.