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Original Article

Adjunct Autophagy Suppression May Be a Promising Way to Eliminate the Resistance of Cancer Cells to Treatment

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Abstract

Background and Aim: Autophagy is a highly conserved mechanism in eukaryotic cells which removes the dysfunctional organelles from the cell. Autophagy has immense physiological and pathological roles in cells. There are a variety of reports showing the dual function of autophagy as a tumor suppression and promotion phenomenon. Therefore, targeted therapy approaches like virotherapy can be a promising cancer treatment. Vesicular stomatitis virus (VSV) is a well-known oncolytic virus which mutations in its matrix (M) protein including M51R, make it a better candidate for oncotargeting. Moreover, beclin-1 is one of the key regulators of autophagy and also apoptosis.

Methods: In the present study, the level of autophagy markers such as beclin-1 and LC3 were investigated concerning the apoptosis process induction by VSV M-protein. Two colorectal cancer cell lines HCT116 and SW480 and one normal colon epithelial cell line (FHC) which expressing VSV M51R mutant M-protein were compared regarding autophagy versus apoptosis. All experiments were conducted at least in triplicate.

Results: The results showed that the elevated level of caspase 3 and reduced amount of beclin-1 in transfected SW480 cells may be an acceptable description for apoptosis.

Conclusion: In HCT116 cells domination of autophagy, plays a supportive mechanism for the cells to survive in response to M51R M-protein stress. Suppression of autophagy as an adjunct can be a promising way to eliminate resistance to cancer treatment. We have quantitively evaluated the Beclin-1 and LC3-II as autophagy markers, Future evaluation of these two along with other markers like P62 will reduce the limitations of this study.

Keywords: Apoptosis; Autophagy; Colorectal Cancer; M-protein; Oncolytic Virus; VSV.

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Introduction

Autophagy is a cellular regulatory mechanism that removes unnecessary components of the cell (1). Although autophagy may appear to be a destructive process, it is a natural cell defense mechanism (2). In response to stressful situations such as nutrients shortages, autophagy is responsible to remove unnecessary organelles and reduce cell consumption to use their raw materials to produce new components (3). Recent reports have declared a spectrum of physiological to pathophysiological roles for autophagy. Based on the progression of autophagy, it can lead to cell survival or cell death.

Autophagy is involved in many physiological processes such as cell growth, aging, cell death, stress response, and cancer (4).

Autophagy plays an important role in preventing the onset and early growth of cancer cells. This process has two functions in tumor cells: in the early stages of the tumor, it suppresses cell proliferation, and in advanced stages, it causes the survival of tumor cells (5). An increase in the progression of cancer cells was reported in mice that their process of autophagy was genetically impaired. This phenomenon has two responsibilities, ensures that the manipulated components of the cells are removed before they cause any problems, and ensure that in case of nutrient shortage, any unnecessary components are recycled (6).

Along with autophagy, apoptosis is a cell damage controlling phenomenon in which the unwanted cell's program to be degraded. In the situation of any lethal stress to cells, the cell tries to dissolve the unnecessary organelles as much it can initially. These dissolving intracellular organelles can lead to provide more energy for the cell and so far, the cell survival and pass the stress situation (7). However, the prolonged autophagic process will result in cell death and apoptosis. The balance between autophagy and apoptosis will define the destiny of the cell (8).

Approximately 30 genes regulate the autophagy process, all of which have been discovered in yeast, and 16 of their homologs have been identified in humans (9). Among these genes, the beclin-1 gene, also known as Atg6, is found in eukaryotes. Beclin-1 is strictly preserved (10). It has been reported that very high levels of BECN1 are expressed in malignant colorectal tissue, while is very lower in the normal colorectal epithelium (11).

Autophagy is controlled by class 3 phosphatidyl inositol-3 kinase complex, which is an autophagy-induced complex that regulates phagosome formation. Becklin-1 is one of the key components for phagosome formation, which acts as a base for its assembly (10).

Furthermore, chemotherapeutic agents, as well as viral oncolytic therapeutics, are active harmful stress for a cancer cell. The oncolytic virus approach is a promising treatment that has recently become known as a new method of cancer treatment (12). These viruses preferably infect and multiply in cancer cells without harming healthy cells. Over the decades, several studies have reported the potential for treatment using oncolytic viruses (13). Some viruses, such as the Newcastle disease virus (NDV), myxomavirus, reovirus, and vesicular stomatitis virus (VSV), naturally have oncolytic properties (14). VSV which belongs to the rhabdovirus family has recently been studied as an oncolytic agent (15).

VSV is effective in the treatment of various cancers such as endometrial cancer (16), malignant melanoma (17), pancreatic adenocarcinoma (18), hepatocellular carcinoma (19), prostate cancer (20), and colorectal cancer (21). In the case of VSV, the matrix protein (M) is responsible for inhibiting cell antiviral effects by suppressing the overall expression of the host gene and therefore induction of apoptosis via the mitochondrial pathway. M protein mutation prevents the virus from suppressing antiviral responses in normal tissue but does not harm the virus's ability to multiply in cancer cells that violate their antiviral responses (22).

Autophagy was reported to be activated through the identification of glycoprotein (G protein) by TLR4 directly (23). Moreover, the nuclear factor erythroid 2-related factor 2 is involved in regulating autophagy, which may enhance the enzymatic properties of various VSV genetic variants such as VSVΔ51 M protein (24). However, it is said that autophagy is due to VSV occurs through the interaction of VSV-G protein with TLR4, but the role of M VSV protein is not yet known (25,26). The current study is designed to evaluate the relationship of VSV mutant M-protein which is approved as a potential threat for cancer cells with autophagy machinery.

Methods

Cell lines preparation: Two human colorectal cancer cell lines (HCT116 and SW480) and a non-cancer, Fetal human colon (FHC) cell line (CRL-1831) was selected.

HCT116 and SW480 cell lines were cultured in their recommended media RPMI 1640, enriched with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin. FHC Cells were cultured in Dulbecco modified Eagle medium (DMEM, Gibco, Invitrogen) supplemented with 25 mmol/L HEPES, 10 ng/mL cholera toxin, 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 100 ng/mL hydrocortisone (Sigma-Aldrich), and 10% FBS. Cells were grown at humidified 37°C and 5% CO2 atmosphere. Cells were passaged and sub-cultured to 90% confluence twice a week. The normal colon cell line FHC was passaged once per week.

Plasmid preparation and transfection: Site-directed mutagenesis was performed to generate the pCDNA3.1 (Invitrogen, San Diego, CA, USA) containing M51R mutant M-protein. During this mutagenesis, the methionine was changed at position 51 to arginine. The primers were designed using the NEB web server as:

Forward: "GTTGACGAGAaGGACACCTATG"

Reverse: "TCCAAAATAGGATTTGTCAATTG"

Bacterial strain Escherichia coli DH5α (Pasteur Institute) was used for the propagation and preparation of constructed plasmids. Transfection experiments were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were transfected with different amounts of plasmids and Lipofectamine to determine the appropriate concentration of constructs and Lipofectamine. To determine the appropriate dose of plasmid and lipofectamine we tried different doses to perform transfection efficiently. In the experiment, the amount of lipofectamine was 1 µL and DNA was 200 ng in 96 plates. In 6 well plates, the amounts of DNA and lipofectamine used per well were 6 µL and 5000 ng respectively. Forty-eight hours post-transfection, the number of live SW480 cells reduced to 60% while yet approximately 80% of HCT116 cells were alive. Upon monitoring SW480 and HCT116 cells in the duration of 72h post-transfection, the SW480 live-cell decreases to 30% while 80% of HCT116 and FHC cells were alive. Having said that since 24 h after seeding, cells reach 80-90% confluency, the medium was contained 2% FBS in the time course of the experiment to control the proliferation.

Western blot analysis: The expression of VSV M51R in the transfected cells was investigated by the western blot method. Cells with a density of 10000 were seeded onto 6-well plates. Once reaching 80% confluency after 24 hrs, they were transfected with M51R containing plasmids. After 72 hrs, cell lysate was prepared by lysis buffer for SDS-PAGE and Western blotting. Following SDS-PAGE, proteins were transferred from the gel to nitrocellulose membrane (Roche, Germany), blocking was performed by dry milk (3%) in a solution of TBS and Tween-20% at pH 7.6 for 30 min. Specific mouse monoclonal antibody 23H12 was used against the VSV M51R protein and detected by ECL reagents kit according to the manufacturer's instructions. MCF7 cells transfected with plasmid pCDNA3.1 (empty plasmid) considered as negative control while as a positive control following the standard protocol, we infected MCF7 cells with VSV wild type at an MOI of 10PFU per cell. After 24h, the cells were harvested and lysed. Supernatant that contains M-protein was used for western blotting assay as a positive standard. Proper reference was included (56).

Cell viability assay: The survival of transfected cells was evaluated by MTT colorimetric assay. Approximately 5000 cells were seeded in a 96-well microplate and transfection was operated with 200 ng/µL and 1 µL Lipofectamine-2000 each well. The MTT assays were performed three times, at 24, 48, and 72 hrs post-transfection. To do this, the media was aspirated, then 20 µL of MTT reagent (5 mg/mL) was added to each well. The plates were incubated for a standard time of 4 hours at 37°C. Subsequently, the supernatant was removed from the wells and 100 µL of DMSO was added to each well. The OD of the solution was measured at 570 nm using an ELISA microplate reader. MTT assays were conducted at least in triplicates.

Evaluation of caspase 3: Evaluation of human caspase 3 activity was performed to investigate the apoptosis phenomenon using Caspase-3 colorimetric kit (Abcam, UK), regarding the manufacturer's instructions as following: Transfected cells (106 /ml), as well as untransfected

cells (as control), were harvested and lysed with chilled lysis buffer, followed by incubation on ice for 10 minutes. The lysates were centrifuged at 10000 g for 1 minute. The supernatant (cytosolic extract) was transferred to a fresh clean tube on an ice container for immediate assay. Total protein concentration was assessed by the Bradford test and adjusted to 50–200 µg protein concentration per well. Reaction Buffer in an amount of 50 µL of 2x (containing 10mM DTT) was supplemented to samples, 5 µL of the 4 mM DEVD-p-NA substrate was added to each well. Following incubation at 37°C for 60 -120 minutes, the results were read at 405 nm using an ELISA microplate reader. These experiments have also been repeated independently three times.

Morphologic evaluation of apoptosis by TUNEL and DAPI staining: Due to investigate the effect of M51R containing plasmid in HCT16, SW480, and FHC cells 72 hrs post-transfection double staining of cells by DAPI and TUNEL assay were performed.

The "terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling" (TUNEL) assay is a common method used to detect the DNA fragments that resulted in cell death since apoptotic cells are characterized by DNA fragmentation. The method is based on the fluorochrome labeling of free 3-OH DNA ends (57).

The TUNEL assay was performed by the TUNEL assay kit (Roche, Switzerland). Briefly, 72 hours after transfection of HCT116, SW480, and FHC cells with a plasmid containing M51R or with empty plasmid considered as a negative control, the cells were fixed with 4% paraformaldehyde and made permeabilize with 0.5% Triton X100. Then the cells were stained with a special kit for In Situ Cell Death Detection (Roche, Switzerland). Fragmented DNA in dead cells was labeled according to the manufacturer's instructions and observed by fluorescence under a fluorescence microscope.

DAPI (4′,6-Diamidino-2-Phenylindole) is a fluorescent stain that is extensively used as a nuclear marker such as DNA fragmentation or condensed nuclei which are common phenomena in apoptotic cell death (58). After fixing by 4% paraformaldehyde, the cells were stained with DAPI dye (100 ng/mL) (Sigma-Aldrich, Germany) and observed by a fluorescent microscope.

Measurement of beclin-1: Beclin-1 is a major regulator among numerous proteins involved in the autophagy process. Beclin-1 is the main regulator of autophagy which promote the formation of autophagosome in the autophagy process. Meanwhile, it plays an important role in cancer cell death and other biological phenomena (59). Thus the level of Beclin-1 was measured to investigate the process of autophagy (Human BECN1 / Beclin-1 ELISA Kit Catalog No. LS-F7208). Briefly, 100µl of diluted lysate in the microplate was incubated for 1 hour at 37°C. Then, 100µl of Detection Reagent-A were added to each well. Following incubation for 1 hour at 37°C and washing with wash buffer, 100µl detection reagent was added to each sample and were incubated for 30 minutes at 37°C. After adding TMB and stop solution according to the protocol, the OD values of each sample were read using a microplate reader at 450 nm. These measurements were conducted in triplicates.

Measurement of LC3II: Among various proteins that directly or indirectly regulate autophagy, LC3 is an important autophagosomal marker that has been widely used to elucidate the mechanism of autophagy. (60) In this study, the quantification of LC3-II was determined by Autophagy ELISA Kit (Cell Biolabs). Briefly, cell lysate samples were transferred to the anti-LC3 antibodies Coated Plate. After an incubation period of at least 2 hrs at 37°C, the micro-wells were washed three times with 250 µL 1X Wash Buffer. Then 100 µL of the diluted anti-LC3 antibody was added to each well and the microplate was shacked for 2 hrs at room temperature. After one time washing, each well was covered with100 µL of the diluted HRP conjugated secondary antibody. Subsequently, the microplate was incubated at room temperature for 1 hour. In the next step, after washing each well, 100 µL of Substrate Solution was added to each well and was incubated for 30 minutes. The amount of 100µL of Stop Solution was added to each well and absorbance was detected on a spectrophotometer at

450 nm. LC3II measurements were conducted in triplicates, independently.

Results

Western blot: Western blotting was performed to confirm the expression of VSV-M51R M-protein in transfected cells. As shown in fig.1, the VSV-M51R M-protein was detected in transfected SW480, HCT116, and FHC cells.

MTT assay: MTT assay reconfirmed the cytotoxicity effect of M51R on two human colorectal cancer SW480, but not on HCT116 and normal colon cells FHC. The MTT assay was performed three times at 24, 48, and 72 hrs post-transfection with M51R M-protein plasmids. The results indicated that the cell viability of transfected SW480 cells reduced after 48 and 72 hrs and approximately 80 % of cells were killed 72 hrs post-transfection. While HCT116 as well as FHC cells expressing M51R protein did not show any decrease in cell viability and were alive.

Measurement of Caspase 3: Caspase 3 is one of the executioner caspases which following either intrinsic or extrinsic apoptosis pathways, can trigger apoptosis once activated (27). Caspase 3 is the most important caspase responsible for apoptotic morphological changes such as DNA fragmentation.

In the current study, the level of caspase 3 was analyzed 48 and 72 hrs post-transfection. SW480 cells showed a remarkable increase in caspase 3 level compared to the cells transfected with empty plasmids as control (fig.4). Meanwhile, HCT116 and FHC cells expressing M51R protein, showed no detectable changes in the amount of caspase 3 comparing to untreated or transfected cells with empty plasmids.

Apoptosis detection through TUNEL/DAPI staining: TUNEL/DAPI staining was performed to investigate the apoptosis 72 hours after M51R plasmid transfection in HCT116, SW480, and FHC cells. Untransfected cells were regarded as a negative control. As shown in fig. 3, the TUNEL assay indicated the DNA breaks in SW480 transfected cells as a strong green fluorescence which implied cells undergone apoptosis while these spots were not observed in control cells, transfected HCT116, and FHC cells. These data were further confirmed with DAPI staining exhibited condensed chromatin in transfected SW480 cells which revealed the occurrence of apoptosis in cells. These findings are corroborant with our previous findings that the level of caspase 3 in transfected SW480 cells was increased after 72 hrs compared to Transfected HCT116 and FHC cells.

Induction of autophagy by beclin-1 and LC3II: The levels of beclin-1 and LC3 were measured to investigate the activation of autophagy in FHC, SW480, and HCT116 cells expressing M51R as well as control cells. As showed in fig.4 both colorectal cancer cell lines SW480 and HCT116 showed a high level of beclin-1 and LC3II rather than normal cells. The amount of beclin-1 and LC3II were almost at the same levels in transfected and untreated normal FHC cells. The level of both beclin-1 and LC3-II considerably decreased in SW480 cells transfected with M51R after 72 hrs. Meanwhile, HCT116 cells expressing M51R exhibited barely changed in the amount of beclin-1 and LC3-II concentrations.

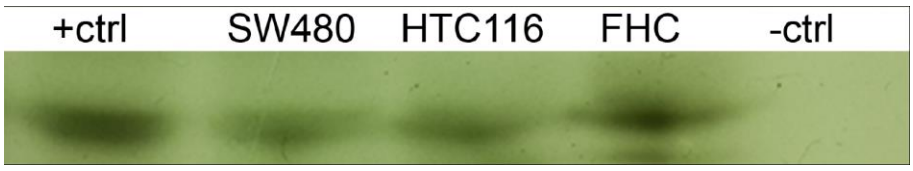

Figure 1. Western blot analysis. Expression of M51R protein in transfected SW480, HCT116, FHC, compared to MCF7 cells transfected withwild-type VSV and empty plasmid as a positive and negative control respectively.

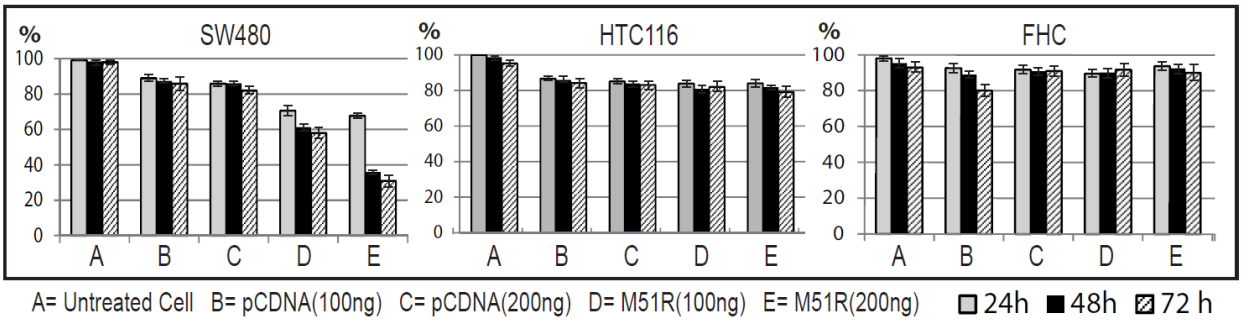

Figure 2. MTT assay. Effect of M51R protein on cell viability of colorectal cancer cell HCT116, SW480, and normal colon cell FHC. MTT assay was performed with two 100 and 200 ng/µl concentrations for 24, 48, and 72 hours.

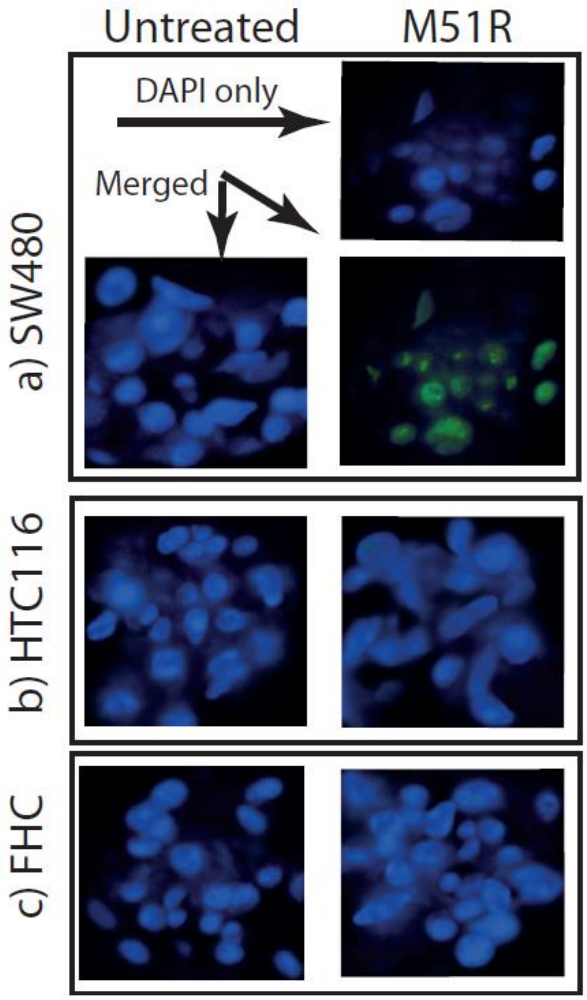

Figure 3. Townie DAPI-TUNEL staining of untreated versus M51R M-protein expressing cells. Only treated SW480 cells show apoptosis where the nuclei stained green with TUNEL (a, right).

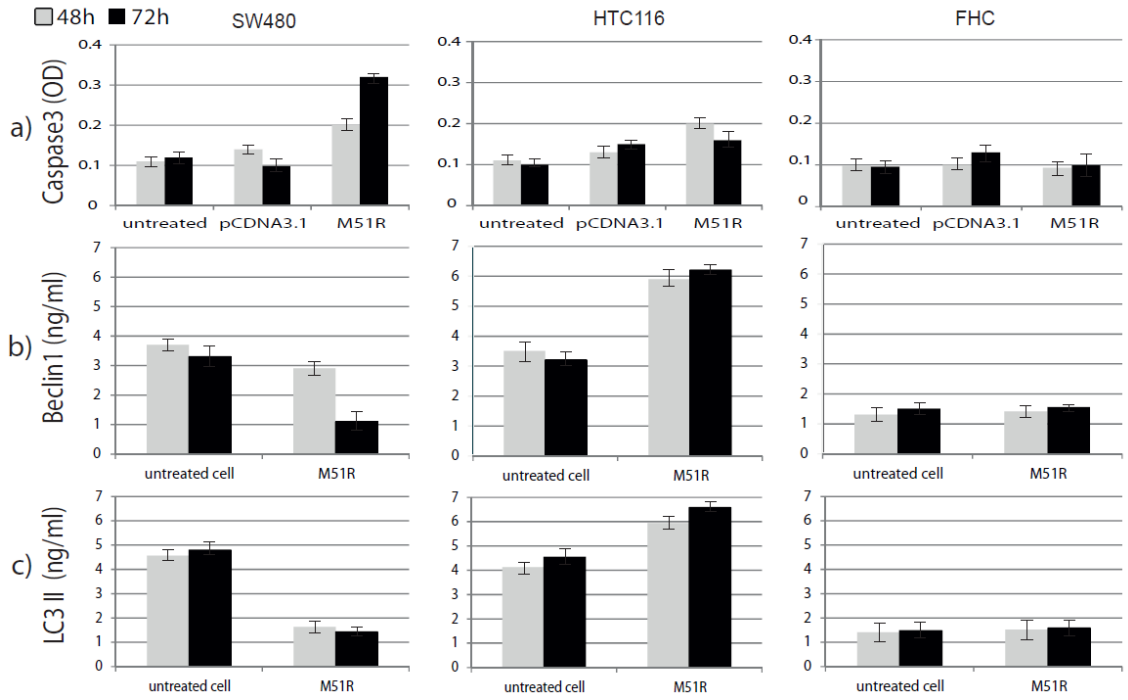

Figure 4. Caspase 3/Beclin-1/LC3 II measurements in Transfected HCT116, SW480, and FHC cells.
a. Caspase 3 assay in three case study cell lines represented an increased level of caspase 3 in SW480 cells compared to transfected HCT116 and FHC cells.
b. Beclin-1 measurement in three case study cell lines. Enhanced levels of beclin-1 were observed in treated HCT116 cells. Untreated cells were considered as control.
c. LC3 II levels in three case study cell lines. The elevated amount of LC3II was shown in Transfected HCT116 cells rather than two cell lines.

Discussion

Evidence has been linked autophagy with a variety of diseases including tumors such as lung cancer, breast cancer, pancreatic cancer, and GI cancers (esophageal carcinoma, and colorectal cancer (CRC) (28). Autophagy is a regulated physiological process in cells that eliminate damaged organelles, none/low functional long-lived proteins, and macromolecules (29). Autophagy starts with the formation of autophagosomes (the vacuoles with double-layer membrane) which capture the components to break down and deliver to lysosomes to be recycled (30). Beclin-1 and LC3 are the most widely investigated proteins involved in autophagy pathways (31,32). During the initiation of the autophagy pathway, a cytosolic form of LC3 (LC3-I) binds to phosphatidylethanolamine phospholipid molecules (POPE) and forms the LC3II known as a marker for autophagosomes (33). In addition to LC3II, Beclin-1 is the main constituent of the class III phosphatidylinositol 3-kinase (PI3K-III) complex which plays an important role to govern autophagy pathways (34,35). The autophagy process is a biological double-edged sword were based on the conditions may establish an adaptation response leading to cell survival, versus a type of programmed cell death known as a type II or autophagic cell death. However, the conditions and factors which direct autophagy to play a protective or lethal role remain obscure (36). Likewise, in the context of cancer, several reports describe a progressive effect for autophagy through an unknown mechanism (37).

Furthermore, VSV with an M51R mutated M-protein is well known as an oncolytic virus through inducing apoptosis in cancer cells but not in normal ones. Shelly et al reported that VSV can also provoke autophagy in cultured cells, likewise, Schache et al who validated that VSV can prompt autophagy in a variety of malignant cells (13,25,38,39). In this study, we have analyzed the autophagy process by investigating the level of

beclin-1 and LC3 in two Colorectal cancer cell lines (SW480 and HCT116) and one normal colon cell line (FHC), transfected with M51R VSV M-protein delivering plasmids. Our results indicated that the levels of beclin-1 and LC3II in colorectal cancer cell lines were remarkably high comparing to FHC normal colon cells. Correspondingly, previous experiments reported that beclin-1 and LC3 are overexpressed in several cancers (40). Fuji et al detected a high level of LC3 protein in pancreatic cancer patients (41). Schmitz et al revealed both beclin-1 and LC3 are overexpressed in CRC (42). Our observations are also in line with Shen and Sato's study that reported an increased LC3II and beclin-1 levels in CRC compared to normal cells in the surrounding tissue (43,44). There has been controversy about the role of beclin-1 in CRC. There are studies suggesting carcinogenesis promoting the effect for expression of beclin-1, while others imply CRC tumor development suppression (45,46).

Interestingly, we observed a difference between the expression of beclin-1 and LC3 in the two cases study CRC cell lines transfected with M51R delivering plasmids. In SW480 cells the concentration of Beclin-1 and LC3 was decreased after 48 and 72 hrs while transfected HCT116 cells showed a considerable increase of beclin-1 and LC3 expression in the same period. Meanwhile, the MTT test revealed that SW480 cell survival was reduced within 72 hours after transfection, nevertheless HCT116 cells normally survived after 48 and 72 hours. Moreover, increased level of caspase 3 in SW480 cells but not in HCT116 and FHC normal cells can be correlated to the cell death observed in MTT assay, since caspase 3 is apoptosis common key executor (47). Then we validated the apoptosis phenomenon observation by DAPI and TUNEL dual staining that further supported the enhanced level of caspase 3 accounts for apoptosis induction in the transfected SW480 cells. The activation of caspase 3 can lead to the DNA fragmentation known as a common hallmark of apoptosis, and TUNEL can visualize it (48). Researchers suggested that autophagy induced by beclin-1 is crucial for the survival and maintenance of the cancer cells, somehow knocking down the beclin-1 makes cells susceptible to apoptosis (49). In case of exposure of the cells to apoptotic stimuli, beclin-1 breaks down to beclin-1-N and beclin-1-C fragments. This beclin-1 breaking mediates by caspase 3 or 7 that makes beclin-1 unable to induce autophagy cascade. Beclin-1-C can be transferred to mitochondria and make cells more sensitive to apoptosis (50). Therefore, it is likely beclin-1 is a key switching mediator between autophagy and apoptosis (49). However, apoptosis or autophagy can be triggered depending on the cellular condition, in response to stress. It is likely that the cells' toleration to the new stress condition, differs in either autophagy or apoptosis threshold (51).

Moreover, it is well established that autophagy can exhibit paradoxical roles as supportive or promoting tumor cells in CRC depending on genetics and the stages of the cancer cells (52). Colorectal cancer cell lines HCT116 and SW480 belong to stages IV and II respectively. As CRC cells are very heterogeneous and show numerous gene mutations, each cell line may differ in its autophagic response to stress. The response of HCT116 cells to the M-protein expression is assumed like a survival autophagy mechanism and may be explained by the fact that these cells are hyper-mutated belong to the higher CRC stage (31,51,53,54). Previous studies have reported the expression of beclin-1 in CRC cases especially in higher-stage tumors (55).

Conclusion

In conclusion, autophagy is a complex cascade with several proteins involving in each step of the pathway. The explicit and effective mechanisms that influence the suppression or enhancement of tumor survival are controversial and have not yet been elucidated. Researchers found that the level of beclin-1 can play an important role in several tumors and mediates crosstalk between autophagy and apoptosis in higher stages of cancer. Our results also support this hypothesis in colorectal cancer as a survival mechanism in higher stages. This can make the beclin-1 a potential efficient therapeutic target for colorectal cancer. We suggest further research on the response of the cancer cells to autophagy and apoptosis cross-talk in different cancer stages regarding new therapeutic approaches. Although in

the present study, we have quantitively evaluated the Beclin-1 and LC3-II as autophagy markers, Future evaluation of these two along with other markers like P62 will reduce the limitations of this study.

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Conflict of Interest

The authors declared that they have no conflict of interest associated with this study.

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Ethics

This study was approved by the Research Ethics Committee of the Golestan University of Medical Sciences (35678).

Individual consent agreements were obtained from 120 CRC patients for using a piece of the resected tumor mass in this project.

Consent for publication: No person's data was included in this project.

Availability of data and materials: All data generated or analyzed during this study are included in this published article and any more questions can be forwarded to MRK.

Author's contribution

Experiments, analysis, manuscript preparation (ZM and MRK), intellectual, planning the project, supervision, manuscript editing (AM and MRK), scientific consultant (AM, MRK, MMSD, and MP).

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