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The Relationship between Autophagy Process and Expression of MicroRNA-146a-5p in MKN-45 and MCF-7 Cell Lines

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ABSTRACT

After chemotherapy or radiation therapy, autophagy activity increases in tumor cells for the adaptation of the tumor cells to stress. Thus, disturbance in autophagy can enhance the effectiveness of anticancer drugs. On the other hand, recent findings highlight the importance of microRNAs (miRs) in autophagy, including miR-146a-5p. In gastric and breast cancer miR-146a-5p is frequently reduced, and more precise identification of its function in these cancers is needed. The aim of this study was to evaluate the relationship between miR-146a-5p and autophagy in MKN-45 (human stomach cancer cell line) and MCF-7(breast cancer cell line).

The expression of miR-146a-5p in MKN-45 and MCF-7 cell lines was measured before and after induction of autophagy using real-time polymerase chain reaction (PCR). A flow cytometry assay was used for the apoptosis assay, and autophagy induction was approved. Also, the formation of autophagic vacuoles was ensured in cells by western blotting and fluorescence microscopy.

Real-time PCR showed that miR-146a-5p level in starvation groups, during autophagy, was significantly lower than in control groups, and also tumor necrosis factor receptor (TNFR)-associated factor 6 *(TRAF6)* level, a key target of miR-146a-5p, in starvation groups, during autophagy, was more than control groups but it was significant only in the MCF-7 group.

According to previous studies and the results of the present study, miR-146a-5p may be considered a negative regulator of autophagy. However, to confirm this, further studies are needed on different cancer cell lines.

Keywords: Autophagy; Autophagy-related Genes; Breast cancer; Gastric cancer; MicroRNA; miR-146a-5p; MKN-45 cell line; MCF-7 cell line

INTRODUCTION

Autophagy is a conserved intracellular pathway maintaining cell homeostasis by recycling damaged intracellular organelles and aggregated proteins. It can act as a tumor suppressor by protecting cells against oxidative stress, genomic mutations, starvation, and the accumulation of abnormal proteins[1] or lead to tumor cell survival, drug resistance, and ultimately, tumor progression after facing chemotherapy, radiotherapy, metabolic stress, hypoxia, and even organelle damage.[2] Therefore, autophagy can be considered a therapeutic target in cancer treatment strategies.[3,4]

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Control and regulation of autophagy are mediated by autophagy-related genes (ATGs).[5,6] Recent studies illustrated that microRNAs (miRs) are involved in regulating autophagy by targeting genes or affecting the autophagy signaling pathway.[7-10] Studies have shown that impaired expression of microRNAs is correlated with pathological processes, such as cancer.[11-13] In contrast, another group of microRNAs was downregulated during malignancy progression. These microRNAs, known as tumor suppressor microRNAs, prevent cancer development under normal circumstances by inhibiting the expression of protooncogenes.[14] Literature has shown that many autophagy-regulating microRNAs are involved in different stages of cancer progression[10] and are even effective in response to treatment.[8]

In this regard, altered expression of miR-146a-5p is a crucial event in the pathogenesis of human illnesses, including autoimmune diseases and cancers.[15] According to recent studies, various levels of expression of miR-146a-5p are reported in different tumors,[16] and it is thought to serve as a tumor suppressor in a variety of cancers via inhibition of interleukin 1 receptor-associated kinase 1 (*IRAK1*)and tumor necrosis factor receptor (TNFR)-associated factor 6 *(TRAF6*) expression.[17] Studies showed that miR-146a-5p is downregulated in most cancers, such as breast and gastric cancer, which is significantly reduced in tumor tissues compared to nontumor tissues.[16,17]

There are a few contradictory studies that believe miR-146a-5p acts as an oncomiR in gastric cancer,[18,19] but most of the evidence supports that miR-146a-5p can be used as a targeted therapy in some cancers to make tumor cells sensitive to medications, such as chemotherapy or radiotherapy.[20] Regarding conducted studies about the effect of miR-146a-5p on increasing the sensitivity of cancer cells to treatment,[21-23] it is assumed that this increased response to treatment is due to the inhibition of the autophagy process in cancer cells. Few studies to date have been done about the effect of miR-146a-5p on the autophagy process, suggesting that miR-146a-5p acts as a negative autophagy regulator in Dengue virus-infected cells and inhibits autophagy in these cells by targeting TRAF6.[24] Due to the few studies that claimed the inverse relationship between autophagy and miR-146a-5p, we decided to investigate the relationship between miR-146a-5p and the autophagy pathway in MCF-7 (breast cancer cell lines) and MKN-45 (human stomach cancer cell line).

MATERIALS AND METHODS

Cell Culture

Human breast and gastric cancer cell lines, including MCF-7 and MKN-45, were prepared from the Iranian Biological Resource Center (Tehran, Iran). MCF-7 and MKN-45 cell lines were cultured in DMEM/F12 (Bio-idea, Iran) and RPMI-1640 (Biosera, France), respectively, which were supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and incubated in a Humidified incubator at 37°C with 5% CO_2 (BINDER, Germany).

Autophagy Induction

Different cell lines induce autophagy after being exposed to starvation, and the time of autophagy induction varies among different cells.[25] To trigger autophagy, we cultured MCF-7 and MKN-45 cells at 1×10^6 cells/mL density in a 6-well plate. After 24 hours of incubation in a humidified incubator with 5% CO_2 at 37°C, the serum-starved medium was added for 8[25] and 6 hours, respectively, to induce autophagy. Then, we used 2 methods to test autophagy in the cell lines as discussed below.

Apoptosis Assay

An apoptosis assay was performed to measure the amount of cell death and confirm the occurrence of autophagy through flow cytometry, as starvation can induce apoptosis as well.[26] An Annexin V-propidium iodide (PI) staining kit (Mab Tag, Germany) was used to assess induced apoptosis in cancer cells. First, cells were harvested and incubated with 5µL of annexin V for 10 minutes at room temperature. Then, 3µL of PI was added and left in the dark. Finally, control and starvation groups were evaluated by flow cytometry (BD FACS, USA). The results were analyzed using Flowing software version 2.4.1 (Turku, Finland).

Fluorescence Microscopy Assay

To detect autophagic vacuoles using a florescence microscope, cells were washed with PBS twice and then stained with 1 µg/mL of acridine orange solution (Merck, Germany). After 15 minutes of incubation at 37°C, cells were washed twice with PBS and suspended with fresh medium. Then, the cells of each well were evaluated by a fluorescence microscope (Ti2-E, China) and analyzed with ImageJ 1.43u software (NIH, USA).

Real-time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (PCR) analysis was accomplished to evaluate the expression of ATG7, ATG12, TRAF6, and miR-146a-5p before and after induction of autophagy. For this purpose, total RNA was extracted using an RNA extraction kit according to the manufacturer's protocol (Qiagen, Japan).

After total RNA extraction, cDNA was synthesized with Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Then, by using particular primers, as shown in Supplementary Table 1, real-time PCR was performed in an Applied Biosystems Real-Time PCR system using SYBR-Green Master Mix (Ampliqon, Denmark) with the following parameters:

For ATG7, ATG12, and TRAF6: predenaturation step at 95°C for 15 minutes, 35 cycles of denaturation at 95°C for 20 seconds, annealing and extension at 60°C for 1 minute. For miR: predenaturation step at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 10 seconds, annealing and extension at 60°C for 1 minute. U6 and HGPRT were used as internal controls for data normalization, and each run was performed in duplicate.

Western Blot for Detection of LC3

Western blotting technique was used to confirm the expression of LC3 protein as an important marker of autophagosomes in the autophagy pathway. To obtain the total protein, cells were washed with PBS twice and lysed by lysis buffer (Enzo Life Science, USA) with 1 mM of protease inhibitor, phenylmethylsulfonyl fluoride (Sigma-Aldrich, USA). The protein concentration was measured using the Bradford assay. Then, equal amounts of protein (15 µg) were run on a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Afterward, the nitrocellulose membrane was treated with a blocking buffer with 4% BSA (Sigma-Aldrich, USA) for 2 hours at room temperature to confirm specific binding sites. After that, the membrane was first washed with TBST (Tris-buffered saline plus 0.1% Tween 20) and incubated with primary antibody, goat anti-human microtubule-associated proteins 1A/1B light chain 3B (LC3) IgG (Dr Ali Mostafaie from Kermanshah University of Medical Sciences kindly provided it as a gift) with dilution 1:100 for 12 hours at 4°C. Following that, the membrane was rewashed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody, rabbit anti-goat, IgG (Abcam, UK) with dilution 1:5000 for 2 hours at room temperature. Finally, the membrane was rinsed as previously described, and protein bands were visualized with an enhanced chemiluminescence (ECL) kit (Bio-Rad, USA).

Statistical Analysis

Date analyses and intergroup comparisons were performed using SPSS version 24.0 (IBM, USA). The *t* test was used for a 2-group comparison analysis. The flow cytometry analyses were accomplished with Flowing software version 1.5.2 (Turku, Finland). The data were presented as the mean ± standard deviation (SD), and statistical significance was considered for *p* values less than 0.05. All experiments were conducted in duplicate.

RESULTS

Flow Cytometry Assay

As shown in Figures 1A and 1B, the apoptosis rate of both control and starvation in MCF-7 and MKN-46 cell line groups revealed no statistical difference.

Fluorescence Microscopy

Figures 2A, 2B, and 2C display that starvation increased the number of autophagic vacuoles in the MCF-7 (p=0.001) and MKN-45 cell lines (p=0.006).

Real-time PCR

miR-146a-5p expression level in starvation groups was significantly lower than in control groups in both cell lines (Figure 3). Furthermore, the expression levels of TRAF-6, ATG-7, and ATG-12 were higher in starvation groups compared with control groups of both cell lines. From a statistical point of view, the results of the increased expression level of *TRAF6* in MKN-45 were not significant (p=0.4), while in MCF-7 was significant (p=0.002). The increased expression level of ATG-12 in MKN-45 was insignificant (p=0.1), and in MCF-7 was significant (p=0.007). The results of the increased expression level of ATG-7 in MKN-45 were significant (p=0.02), but in MCF-7 was not significant (p=0.26).

Western Blot

Western blot analysis confirmed LC3Π expression in 2 cell lines after starvation in both MCF-7 and MKN-45 cell line groups (Figure 4). LC3-II was observed prominently in the starvation groups. The LC3-II/LC3-I ratio in the starvation groups was 2.7 in MCF-7 and 2.5 in MKN-45.

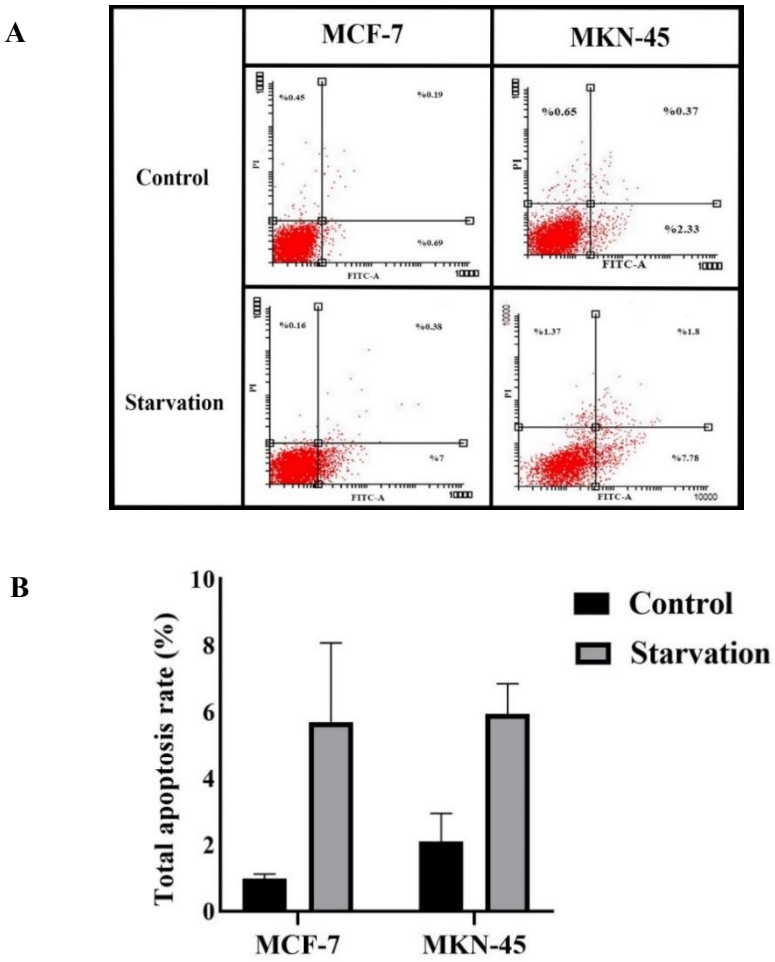
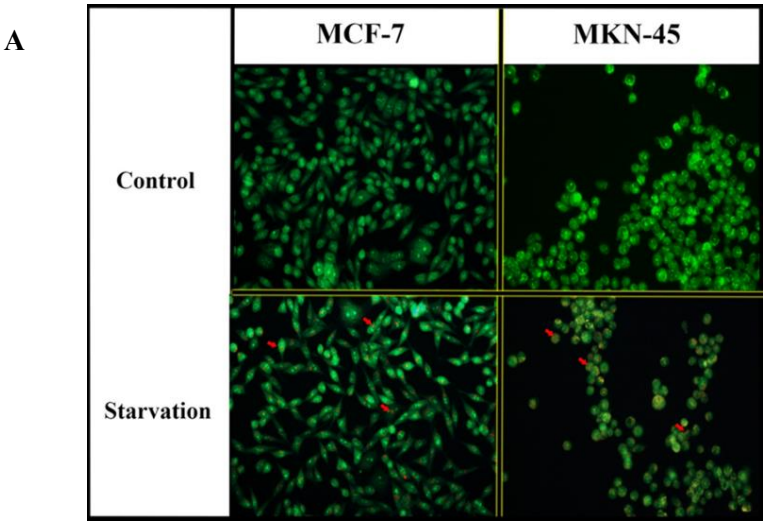


Figure 1. A. Apoptosis was detected by Annexin V-PI staining (flow cytometry) in control and starvation groups in MCF-7 and MKN-45 cell lines. B. Total apoptosis rate ± standard deviation of control and starvation groups in MCF-7 (5.695±2.383) and MKN-45 (5.940±0905) cell lines.



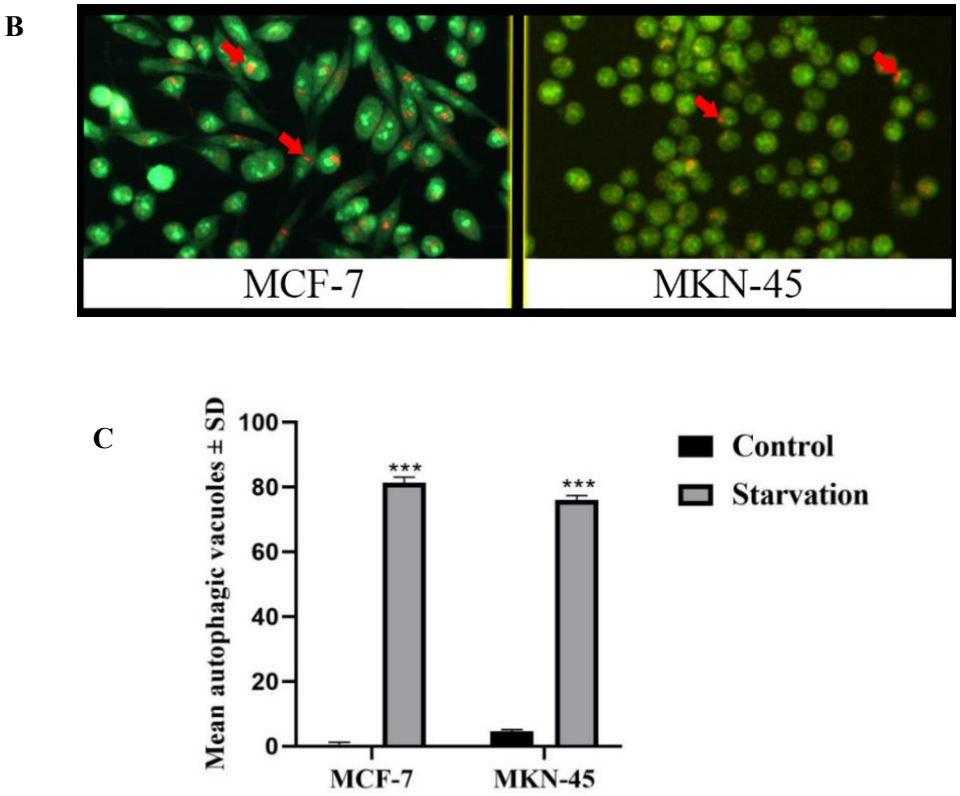
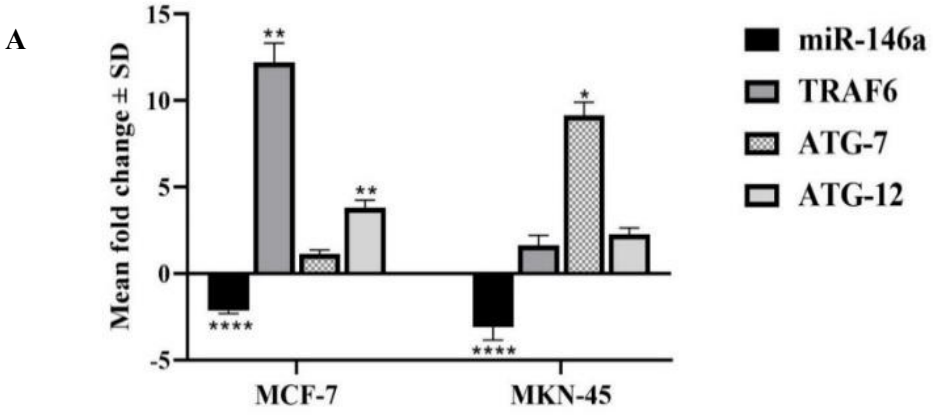


Figure 2. A. Detection of Autophagic vacuole (AVO) in control and starvation groups with acridine orange staining (10×magnification). B. detection of AVO in starvation groups (20× magnification). C. Autophagic vacuoles±SD in control and starvation groups. MCF-7 (%82.5±3.53), MKN-45 (%77±2.82). amount of autophagic vacuoles increased in starvation groups (*p* value<0.05).



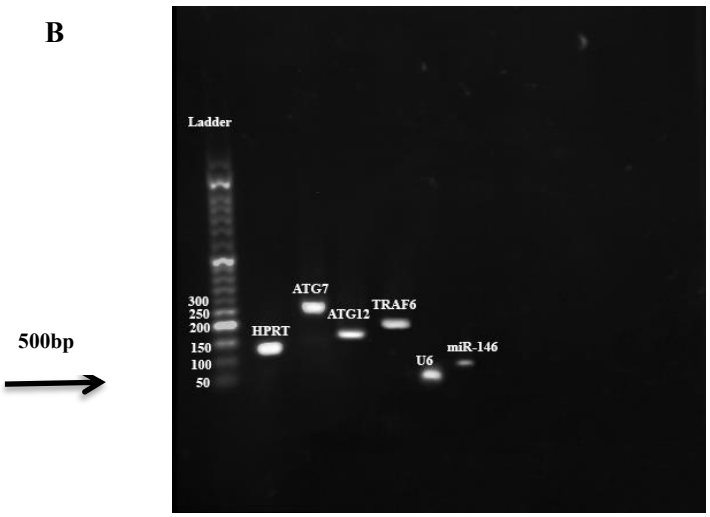


Figure 3. A. Expression changes in miR-146a-5, tumor necrosis factor receptor-associated factor 6 (*TRAF6)*, and autophagy-related genes (*ATG7* and *ATG12*) after starvation. B. Agarose gel electrophoresis image is related to hypoxanthine-guanine phosphoribosyltransferase *(HGPRT), ATG7, ATG12, TRAF6, U6*, and miR-146a-5p, respectively. A DNA ladder (50–800 bp) has run in the first lane of the gel, on the left side.

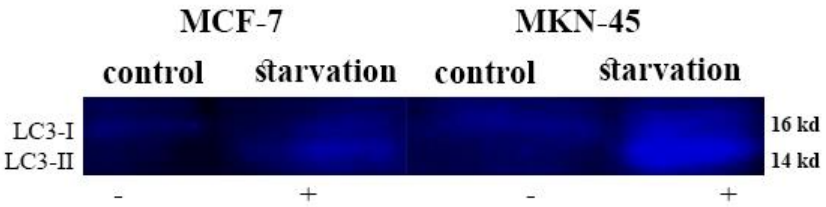


Figure 4. Conversion of microtubule-associated proteins 1A/1B light chain 3B (LC3)-I to LC3-II was examined by Western blot in MCF-7 and MKN-45 cell lines before and after subjecting them to starvation.

DISCUSSION

The study investigates the relationship between miR-146a-5p and autophagy in gastric (MKN-45) and breast (MCF-7) cancer cell lines. Results show that miR-146a-5p expression significantly decreases during autophagy induction in both cell lines. Additionally, the study indicates increased levels of autophagy-related proteins (TRAF6, ATG7, ATG12) in starvation conditions. These findings suggest that miR-146a-5p may act as a negative regulator of autophagy, supporting the potential of targeting miR-146a-5p in cancer therapy.

According to the latest data from 81 clinical trials on autophagy as a potential objective in the treatment of diseases, 53 of them are focused on cancer. As a result, around 70% of clinical research focuses on the role of autophagy in cancer, and autophagy regulation's potential for cancer treatment is promising.[27]

Under tumor-stressing conditions,[29] like anticancer therapies, such as chemotherapy and radiotherapy[30] autophagy activity augments to promote drug resistance in cancer cells.[31]

Although this mechanism appears to be related to cell survival and death,[28,29] its relevance to cell death is still debatable.[30]

Autophagy inhibitors may be able to decrease cancer mortality by preventing metastasis. Therefore, targeting autophagy for cancer treatment can be a promising therapeutic strategy.[31,32]

Studies show that the change in miR-146a-5p expression is a key event in the pathogenesis of human diseases such as cancer, which is thought to act as a tumor suppressor in a variety of cancers.[33] including hepatocellular carcinoma, esophageal squamous cell carcinoma, non-small cell lung cancer, and prostate cancer.[20]

It has been reported that miR-146a-5p expression decreases in breast[34] and gastric cancer,[35] as a result, it can be considered a tumor suppressor in these cancers.[20,35]

Few contradictory studies concluded that miR-146a-5p acts as an oncomiR in gastric cancer,[18] but most evidence suggests that miR-146a-5p can function as an anticancer treatment in some tumors to make cells sensitive to medications.[20]

The goal of the present research was to investigate the relationship between miR-146a-5p and autophagy in MCF-7 and MKN-45 cancer cells. In this study, starvation was used to induce autophagy,[36] Due to the fact that cells react differently to the start of the autophagy process, each cell group was affected by starvation for an unequal period.[25] Additionally, flow cytometry was used to examine apoptosis, as autophagy can lead to apoptosis too,[37] under certain conditions, prolonged or excessive autophagy can lead to cellular stress and trigger apoptosis. This crosstalk occurs through various molecular pathways, including the regulation of Bcl-2 family proteins and the activation of caspases. Autophagy can lead to the degradation of key apoptotic inhibitors, thereby promoting the activation of apoptotic pathways. This dual role of autophagy highlights its complex involvement in cell survival and death, making it a significant area of study for potential therapeutic interventions in diseases such as cancer.[38]

Some studies have demonstrated that miR-146a-5p may inhibit the autophagy process[39,40] which indicates miR-146a-5p as tumor suppressor miR[41] Clinical studies show decreased expression of miR-146a-5p in gastric and breast cancers,[17] which makes this microRNA a therapeutic option in cancer treatment.[42,43]

An investigation about the relevance of miR-146a-5p and the autophagy process suggested that miR-146a-5p acts as a negative autophagy regulator in Dengue virus-infected cells and inhibits autophagy in these cells by targeting *TRAF6*.[24]

Another study discussed that the expression of miR-146a-5p is decreased in some cancers such as lung cancer. It has also been argued that increased expression of the miR-146a-5p in this cancer increases the sensitivity of cells to chemotherapy and inhibits autophagy by targeting ATG12, as well.[44]

Furthermore, finding in a study about the effect of miR-146a-5p expression on the sensitivity of hepatocellular carcinoma cells to radiotherapy, revealed that miR-146a-5p overexpression can raise sensitivity to radiotherapy in hepatocellular carcinoma cells.[43]

A different investigation explored the role of miR-146a-5p in cancer, focusing on its expression in cancer cells and its involvement in autophagy regulation. It highlights that miR-146a-5p can modulate autophagy pathways, which play a crucial role in cancer cell survival and proliferation. By regulating key autophagy-related genes, miR-146a-5p influences the degradation of damaged cellular components, impacting cancer progression and treatment responses.[45]

Based on multiple studies, combination therapies such as the use of autophagy inhibitors including miR-146a-5p along with radiotherapy or other anticancer treatments are almost more effective than single therapies such as chemotherapy or radiotherapy alone, which lead to cancer drug resistance by autophagy induction.[46]

Despite extensive research, there is still no satisfactory approach to modulating autophagy that does not disrupt other cellular functions. Furthermore, it is crucial to regulate autophagy in tumor cells without simultaneously disrupting autophagy in healthy cells.[27]

One of the strengths of this study is the use of well-established techniques such as flow cytometry, western blotting, and real-time PCR, for evaluating autophagy. However, it is recommended to employ techniques such as transfection to directly investigate the role of miR-146a-5p in cancer cells, thereby gaining a clearer understanding of its function.

Based on this study and previous studies, miR-146a-5p may potentially be considered a negative regulator of autophagy, and overexpression of this microRNA may harm cancer cells by reducing autophagy. However, further investigations are required in various cancer cell types to confirm this proposition and explore therapeutic interventions that target miR-146a-5p.

Autophagy is considered a crucial process in cells, and its activity undergoes changes during cancer development and treatment. Therefore, it is necessary to enhance knowledge in this field and take a step towards targeted therapies by identifying the specific microRNAs, compounds, and networks involved in this pathway. Conventional cancer treatments such as chemotherapy and radiation therapy often come with severe side effects and sometimes lead to tumor recurrence and patient loss.

STATEMENT OF ETHICS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Data Availability

Not applicable.

AI Assistance Disclosure

Not applicable.

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