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Original Research Article

MicroRNA-744 induces apoptosis and autophagy in human gastric cancer by targeting heparanase-1

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Abstract

Purpose: *To study the possible anti-cancer effects of miR-744 on human gastric cancer cells, and the underlying mechanism.*
Methods: *The mRNA expression of miR-744 was determined using qRT-PCR, while CCK-8, Annexin V/PI and acridine orange assays were used to determine cell viability, apoptosis and autophagy, respectively. The expressions of target proteins were determined using qRT-PCR and Immunoblotting.*
Results: *Significant suppressions of miR-744 were observed in gastric cancer tissues and cell lines. Overexpression of miR-744 inhibited the proliferation while silencing of miR-744 promoted the proliferation of gastric cancer cells. Furthermore, the tumor-suppressive effect of miR-744 in gastric cancer was due to induction of apoptosis and autophagy. Heparanase-1 (HPSE-1) was identified as the target of miR-744. Silencing of HSPE-1 significantly inhibited cell proliferation, whereas its overexpression significantly restored the growth-inhibitory effects of miR-744 overexpression on gastric cancer ($p < 0.05$).*
Conclusion: *These results indicate that miR-744 exerts a tumor-suppressive effect on the growth and proliferation of gastric cancer cells in vitro by targeting HPSE-1. There is need to further determine the anti-cancer effect of miR-744 expression in animal models.*

Keywords: *Gastric cancer, Micro-RNA, miR-744, Proliferation, Apoptosis, Autophagy, Heparanase-1*

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INTRODUCTION

Gastric cancer, a frequently occurring and the most aggressive cancer worldwide, is the 5th most common neoplasm and the 4th deadliest disease globally [1]. In 2018 alone, there were over one million newly-diagnosed cases of gastric cancer, resulting in more than 700,000 deaths worldwide [2]. Despite advances in gastric cancer diagnosis and treatment options, the overall five-year survival of gastric cancer patients still remains very low (10 % for those in advanced stages of the disease) [3]. Hence, there is a pressing need to identify more effective treatment strategies for the management of gastric cancer.

Micro-RNAs (miRs) are small non-coding RNAs ranging in length from 22 to 25 nucleotides, and

they are transcribed by RNA polymerase II [4]. The miRs usually function in post-transcriptional regulation/repression of protein-coding genes by binding to specific sites within the 3'-UTRs [5]. Approximately 30 % of protein-coding genes in humans are regulated by miRs at the post-transcriptional level [6]. The MiRs play crucial regulatory roles in key biological and physiological processes in man. Thus, dysregulation of their expressions has been associated with different types of pathologies [7]. Indeed, there is growing evidence that aberrant expressions of miRs are responsible for a variety of human cancers [8]. Recent studies have suggested that miRs regulate the important hallmarks of malignancy [9]. Some miRs such as miR-15, miR-16, miR-21 and miR-200 are regulatory in nature, and they control the growth and proliferation of gastric cancer [10].

In particular, MiR-744 is a well-recognized suppressor of human gastric cancer [11]. The present study was aimed at investigating the anti-cancer effect of miR-744 on human gastric cancer cells.

METHODS

Human tissues

Gastric cancer tissues and matching normal tissues (n = 30) were obtained from gastric cancer patients. Written consents were received from all the patients. The recruitment of the specimens was made before commencement of treatment. Histological confirmation of the tissue specimens was made by hospital pathologists. The study received approval from the ethical authority of the Chongqing University Three Gorges Hospital, Chongqing (approval no. 5332/WSH/21). The experiments were performed according to the guidelines in the Helsinki Declaration [12].

Cell lines

The cell lines used (AGC, BGC-823, MKN-45, SGC-7901 and GES-1) were obtained from American Type Culture Collection (ATCC). The cells were grown in a high-glucose DMEM containing 10 % FBS and penicillin/streptomycin, in an incubator.

Transfection

The miR-744 mimic, miR-744 inhibitor, miR-NC, si-HPSE-1 and si-HPSE-1 were obtained from GenePharma Co. Ltd. First, HPSE-1 was amplified with PCR. Then, it was cloned in pcDNA3.1 (pcDNA-HPSE-1). Transfection was performed with Lipofectamine 2000 reagent (Invitrogen).

qRT-PCR

Total RNA was extracted from each cell line with TRIzol reagent. Then, the total RNA was reverse-transcribed into cDNA. Thereafter, RT-qPCR was carried out to determine the mRNA expression levels of miR-44, with human GADPH gene as endogenous control. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

CCK-8 proliferation assay

The transfected cell lines were grown in 96-well plates, each at a density of 2.5×10^4 cells/well, and cultured for different time durations. Then, 10 µL of CCK-8 solution was added to each well, followed by incubation at 37 °C for 2 h. Thereafter, the cells were harvested and resuspended in 100 µL of PBS buffer. Finally, the absorbance of each well was read at 570 nm in a micro-plate spectrophotometer.

DAPI and Annexin V-FITC/PI staining assays

For DAPI staining, each transfected cell line was initially plated in a 24-well plate at a density of 10^5 cells/well. After 24 h incubation at 37 ^oC, the cells were harvested, washed with PBS, and fixed in 4 % paraformaldehyde, followed by the addition of Triton X-100 (0.1 %). Then, the cells were washed and incubated with DAPI (0.5 µg/mL) for 5 - 7 min. Lastly, the cells were photographed under a fluorescent microscope. After 24 h, the transfected cells were processed for staining using an Annexin V-FITC-PI staining kit (Solarbio, Beijing, China). A FACS flow cytometer (Jiyuan, Guangzhou, China) was employed for the analysis of apoptosis of the stained cells.

Western blotting

Total protein was extracted from the transfected cells by lysing with a protein extraction kit. The cell lysates were centrifuged, and the protein contents of the lysate were determined using the BCA method. Then, equal amounts of proteins were resolved using SDS-PAGE, followed by transfer to PVDF membrane (Millipore). Following incubation with specific primary and secondary antibodies, the protein bands were subjected to enhanced chemiluminescence (ECL; GE Healthcare) and their relative expression levels were calculated using QuantityOne software (Bio-Rad).

Acridine orange (AO) autophagy assay

The fluorescent dye acridine orange (AO) was employed for studying cell autophagy. The cells were stained using an AO staining kit (BestBio, Inc., Shanghai, China). Then, the stained cells were examined and photographed under a fluorescent microscope (Olympus).

Online bioinformatics and dual luciferase assay

The target gene of MiR-744 was predicted with TargetScan 7.2 online software. To validate the prediction, luciferase reporter assay was carried out. In this process, BGC-823 cells were co-transfected with luciferase vectors of HPSE-1 3'-UTR with wild-type (wt) or mutant (mut) miR-744 binding sites, and miR-744 mimic or miR-NC. After 24 h of co-transfection, the host cells were trypsinized and subsequently subjected to luciferase assay using Dual-Luciferase Reporter Assay System.

Statistical analysis

Values are presented as mean ± standard deviation (SD) of three biological replicates from at least three experimental replicas. Student's *t*-test was used to estimate the significance of statistical difference. Values of $p < 0.05$ were taken as indicative of statistically significant differences.

RESULTS

miR-744 inhibited gastric cancer cell proliferation

The results showed gastric cancer tissues exhibiting significant down-regulation of miR-744, when compared to normal tissues ($p < 0.05$; Figure 1 A). Moreover, it was found that gastric cancer cell lines expressed significantly lower miR-744 transcript levels than GES-1 cell line ($p < 0.05$; Figure 1 B). The cells were transfected with miR-744 mimics or miR-744 inhibitor to induce over-expression or down-regulation of miR-744, with miR-NC oligos serving as negative control (Figure 1 C). Results from CCK-8 proliferation assays showed that MiR-744 over-expression significantly inhibited proliferation of the two host cell lines, while its down-regulation significantly enhanced the host cell proliferation *in vitro* ($p < 0.05$; Figure 1 C). These results indicate that reversal of miR-744 repression inhibited cellular growth.

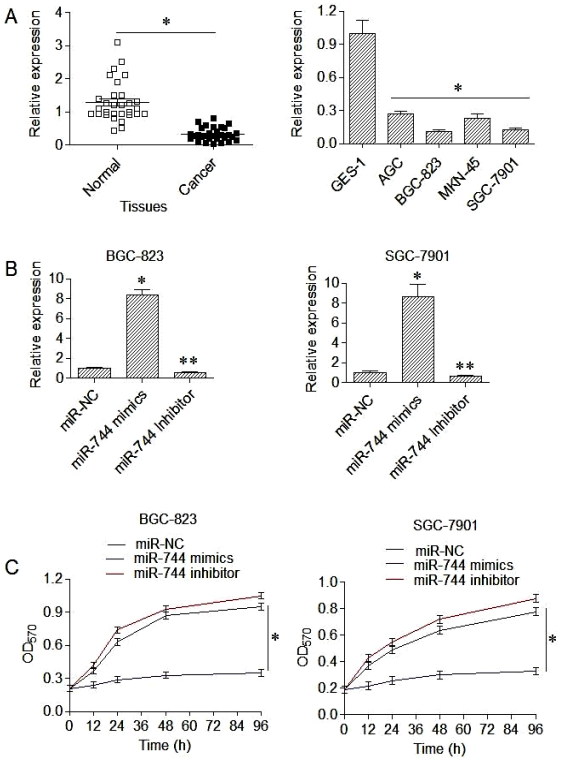

Figure 1: MiR-744 was down-regulated in gastric cancer cells, and its over-expression inhibited gastric cancer cell growth. (A) MiR-744 was repressed in gastric cancer tissues. (B) Gastric cancer cell lines (AGC, BGC-823, MKN-45, and SGC-7901) exhibited significant down-regulation of miR-744, relative to GES-1 and gastric epithelial cells. (C) miR-744 over-expression or down-regulation inhibited or enhanced the proliferation of host BGC-823 and SGC-7901 cells, respectively. *$P < 0.05$; **$p < 0.01$

MiR-744 over-expression enhanced apoptosis

Cells with overexpressed miR-744 showed loss of nuclear integrity which suggested induction of apoptotic cell death (Figure 2 A). To confirm this, Annexin V-FITC/PI apoptosis assay was performed. Results from flow cytometry showed that miR-744 over-expression significantly increased the number of early and late apoptotic cells ($p < 0.05$; Figure 2 B). On the other hand, miR-744 down-regulation significantly reduced the percent of apoptosis of host cells ($p < 0.05$). Moreover, results from western blotting showed that Bax protein expression was significantly decreased by miR-744 up-regulation, while its expression was significantly repressed by inhibiting miR-744 expression in BGC-823 cells ($p < 0.05$; Figure 2 C). The Bcl-2 expression was modulated in a fashion opposite to that of Bax. Therefore, miR-744 knockdown induced apoptosis in gastric cancer cells.

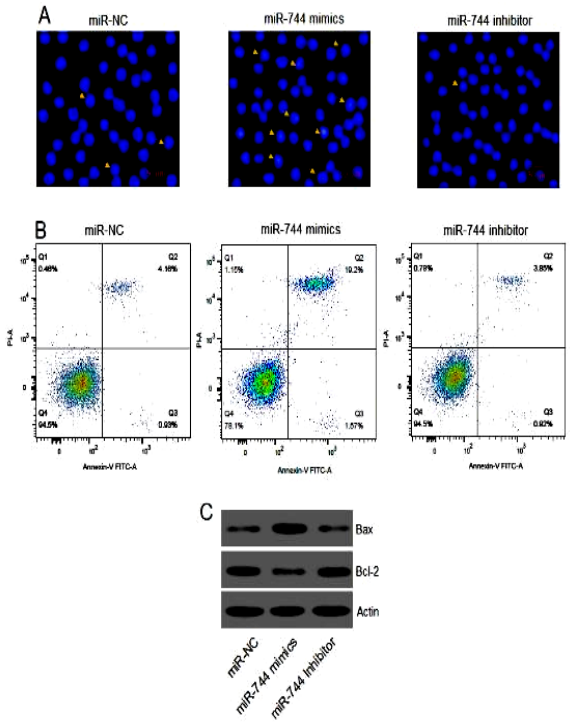

Figure 2: MiR-744 over-expression induced apoptosis. (A) BGC-823 cells showed loss of nuclear integrity after miR-744 over-expression. (B) flow cytometric analysis in miR744 mimic and inhibitor group. (C) Expression levels of Bax and Bcl-2 in miR-744 mimic and inhibitor groups

MiR-744 over-expression-induced autophagy

To study the effect of miR-744 on autophagy in BGC-823 gastric cancer cells, AO staining was used. Red fluorescence was observed in cells that over-expressed miR744, which is suggestive of the presence of acidic autophagosomes and induction of host cell autophagy (Figure 3 A). In contrast, the relative percentage of red-fluorescing cells was significantly lower in the negative control and cancer cells with down-regulated miR-744 ($p < 0.05$). Furthermore, the silencing of miR-744 significantly increased the protein expressions of Beclin 1 and LC3BII, while it decreased the protein expression of LC3BI ($p < 0.05$; Figure 3 B). In contrast, down-regulation of miR-744 produced the opposite effects on the expression levels of these proteins. These results indicate that miR-744 over-expression enhanced the levels of autophagy in gastric cancer cells.

Heparanase-1 was functional target of miR-744 in gastric cancer

The TargetScan analysis predicted that heparinase-1 (HPSE-1) was the specific target of miR744, and identified the miR-744 binding site in the 3'-UTR of HPSE-1 mRNA (Figure 4 A). Dual luciferase assay showed significantly decreased luciferase activity in cells transfected with miR-744 mimic and 3'-UTR of HPSE-1 ($p < 0.05$; Figure 4 B). Moreover, the gastric cancer tissues and cell lines expressed significantly higher levels of HPSE-1 transcript, when compared to the normal adjacent tissues and GES1 cells ($p < 0.05$; Figure 4 C and D). Furthermore, the over-expression of miR-744 resulted in significant repression of HPSE-1 in BGC-823 cancer cells ($p < 0.05$; Figure 4 E). Silencing of HPSE-1 in BGC-823 cells by transfecting the latter with si-HPSE-1 resulted in inhibition of proliferation of the host cells in a fashion similar to that of miR-744 up-regulation (Figure 4 F). Besides, the over-expression of HPSE-1 in BGC823 gastric cancer cells with down-regulated miR-744 attenuated the anti-proliferative effects of miR-744 overexpression in host cells (Figure 4 G). These data confirm that miR-744 exerted tumor-suppressive effect in gastric cancer cells by targeting and reducing the expression level of HPSE-1.

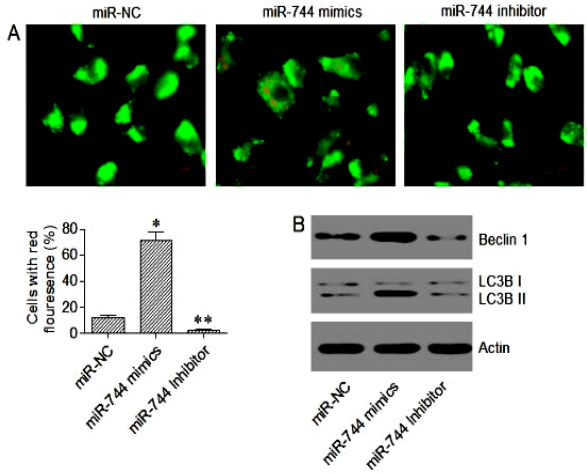

Figure 3: MiR-744 up-regulation enhanced autophagy. (A) Acidic autophagosomes (red dots) seen through AO fluorescent microscopy in BGC-823 cancer cells. (B) miR-744 over-expression increased protein expression levels of Beclin 1 and LC3B II, while it down-regulated LC3B I protein. These effects were reversed by miR-744 knockdown, with respect to protein expressions of Beclin 1, LC3B II, and LC3B I. *$P < 0.05$; **$p < 0.01$)

DISCUSSION

Gastric cancer is one of the devastating health disorders responsible for a significant proportion of cancer-related mortality at the global level. There has been a significant decrease in the overall incidence and annual deaths resulting from gastric cancer in recent years [13].

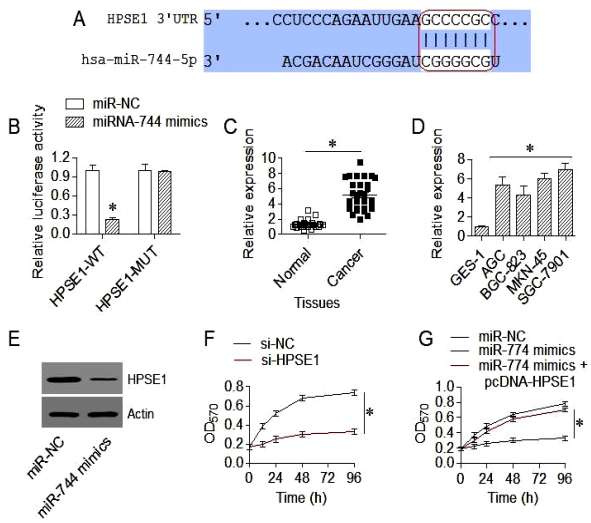

Figure 4: MiR-744 functionally targeted heparinase-1 in gastric cancer. (A) TargetScan predicted the binding site of miR-744 in 3'-UTR of HPSE-1. (B) Results of dual luciferase assay confirmed the sequence-specific binding of miR-744 to 3'-UTR of HPSE-1, as indicated by significant decline in luciferase activity of BGC-823 cancer cells transfected with miR744 mimic and 3'-UTR of HPSE-1 plasmid. (C) Over-expressed HPSE-1 in gastric cancer tissues. (B) Gastric cancer cell lines exhibited significant up-regulations of HPSE-1, relative to GES-1 and gastric epithelial cells. (C) Over-expression of miR-744 enhanced HPSE-1 expression. (D) HPSE-1 silencing inhibited cellular proliferation. (E) Co-transfection of BGC-823 cells over-expressing miR-774 with pcDNA-HPSE-1 attenuated the antiproliferative effects of miR-744 up-regulation. *$P < 0.05$

However, the prognosis of this disease is still very poor, and patients are often diagnosed at advanced stages when distant metastasis has already occurred [14]. At this stage, the traditional therapeutic applications yield less desirable clinical outcomes, thereby necessitating the use of more efficient prognostic and treatment modalities.

Micro-RNAs (miRs) play key roles in cellular processes such as cell division, differentiation, apoptosis, and migration, and hence, in the progression of several types of human diseases, including cancer [15]. Moreover, recent studies have produced evidence for their potential applicability in cancer prognosis [16]. In view of this, the prognostic and therapeutic potential of miR-744 in gastric cancer were studied *in vitro* in cell lines, in the present research. The results showed that malignant gastric tissues and cell lines expressed significantly lower transcript levels of miR744 than normal tissues. Thus, qRT-PCR expression analysis method might possibly be helpful in determination of the prognosis of gastric cancer. Over-expression of miR-744 suppressed the growth of gastric cancer cells by inducing apoptosis and autophagy. The proapoptotic function of miR-744 has also been previously reported. Cancer cells show extended survival because of impaired pathways of apoptosis and autophagy [17]. Thus, restoration of the apoptotic and autophagy signals in cancer cells has been suggested as an efficient therapeutic strategy for human carcinogenesis [18]. However, to realize this, the potential molecular targets need to be identified. The results of the current study showed that miR-744 is the potential therapeutic target for enhancing apoptosis and autophagy in gastric cancer cells. Interestingly, miR-744 was shown to exert an anticancer effect *via* post-transcriptional silencing of heparinase-1 (HPSE-1) which was expectedly up-regulated in gastric cancer tissues and cell lines owing to miR-744 downregulation. Previous studies associated HPSE-1 over-expression with the malignant behaviour of human cancers and gastric cancer cells. Therefore, the results of the present study are in line with previous findings. These results confirm the regulatory involvement of miR-744/HPSE-1 molecular axis in gastric tumorigenesis.

CONCLUSION

The study has shown that miR-744 is down-regulated in gastric cancer tissues and cell lines. Over-expression of MiR-744 inhibits the proliferation of gastric cancer cells by inducing apoptosis and autophagy. Moreover, miR-744 exerts its tumor-suppressive regulatory function through miR-744/HPSE-1 axis.

DECLARATIONS

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None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Dan Zhao and Xiaohong Xie contributed to this work equally. Dan Zhao performed most experiments and Xiaohong Xie drafted the manuscript. Qin Tan collected materials and did part of the experiment. Xin Zhao did the statistical analysis and revised this manuscript. The whole study was designed by Dingpei Xing.

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REFERENCES

1. *Arnold M, Abnet CC, Neale RE, Vignat J, Giovannucci EL, McGlynn KA, Bray F. Global burden of 5 major types of gastrointestinal cancer. Gastroenterol 2020; 159(1): 335-349.*
2. *Jin P, Ji X, Tian Y. Surgical management of oligometastatic disease in gastric cancer. Clin Res Hepatol Gastroenterol 2020; 44(5): 638-645.*
3. *Yan K, Tian J, Shi W, Xia H, Zhu Y. LncRNA SNHG6 is associated with poor prognosis of gastric cancer and promotes cell proliferation and EMT through epigenetically silencing p27 and sponging miR-101-3p. Cell Physiol Biochem 2017; 42(3): 999-1012.*
4. *Weidle UH, Birzele F, Nopora A. microRNAs Promoting Growth of Gastric Cancer Xenografts and Correlation to Clinical Prognosis. Cancer Genomics Proteomics 2021;18(1): 1-5.*
5. *Pu M, Chen J, Tao Z, Miao L, Qi X, Wang Y, Ren J. Regulatory network of miRNA on its target: coordination between transcriptional and post-transcriptional regulation of gene expression. Cell Mol Life Sci 2019; 76(3): 441-51.*
6. *Abdalla F, Singh B, Bhat HK. MicroRNAs and gene regulation in breast cancer. J Biochem Molecular Toxicol 2020; 34(11): e22567.*
7. *Prinz C, Weber D. MicroRNA (miR) dysregulation during Helicobacter pylori-induced gastric inflammation and cancer development: critical importance of miR-155. Oncotarget 2020; 11(10): 894.*
8. *Khafaei M, Rezaie E, Mohammadi A, Shahnazi GP, Ghavidel S, Kadkhoda S, Zorrieh ZA, Forouzanfar N, Arabameri H, Tavallaie M. miR-9: From function to therapeutic potential in cancer. J Cell Physiol 2019; 234(9): 14651-14665.*
9. *Tobias GC, Gomes JL, Soci UP, Fernandes T, de Oliveira EM. A landscape of epigenetic regulation by MicroRNAs to the hallmarks of cancer and cachexia: implications of physical activity to tumor regression. Epigenetics 2019; IntechOpen.*
10. *Xia L, Zhang D, Du R, Pan Y, Zhao L, Sun S, Hong L, Liu J, Fan D. miR-15b and miR-16 modulate multidrug resistance by targeting BCL2 in human gastric cancer cells. Int J Cancer 2008; 123(2): 372-9.*
11. *Xu AJ, Fu LN, Wu HX, Yao XL, Meng R. MicroRNA 744 inhibits tumor cell proliferation and invasion of gastric cancer via targeting brain derived neurotrophic factor. Mol Med Rep 2017; 16(4): 5055-61.*
12. *Crawley FP. The limits of the declaration of Helsinki. Address to scientific session. Helsinki: World Medical Association General Assembly; 2012.*
13. *Kanda M, Shimizu D, Fujii T, Sueoka S, Tanaka Y, Ezaka K, Takami H, Tanaka H, Hashimoto R, Iwata N, et al. Function and diagnostic value of Anosmin-1 in gastric cancer progression. Int J Cancer 2016; 138(3): 721-730.*
14. *Balakrishnan M, George R, Sharma A, Graham DY. Changing trends in stomach cancer throughout the world. Curr Gastroenterol Rep 2017; 19(8): 1-10.*
15. *Xu D, Shao W, Jiang Y, Wang X, Liu Y, Liu X. FTO expression is associated with the occurrence of gastric cancer and prognosis. Oncol Rep 2017; 38(4): 2285-2292.*
16. *Petrek H, Yu AM. MicroRNAs in non-small cell lung cancer: Gene regulation, impact on cancer cellular processes, and therapeutic potential. Pharmacol Res Perspect 2019; 7(6): e00528.*
17. *Zhao J, Li D, Fang L. MiR-128-3p suppresses breast cancer cell progression via targeting LIMK1. Biomed Pharmacother 2019; 115: 108947.*
18. *Adams JM, Cory S. Bcl-2-regulated apoptosis: mechanism and therapeutic potential. Curr Opin Immunol 2007; 19(5): 488-496.*