

Biological functions of miRNA-203b-3p/ZNF324 in laryngeal carcinoma

Liping Wang^{1,2,3}, Xianyang Luo^{1,2,3*}¹ Department of Otolaryngology-Head and Neck Surgery, The First Affiliated Hospital, Medical College, Xiamen University, Xiamen 361003, China² Teaching Hospital of Fujian Medical University, Xiamen 361003, China³ Xiamen Key Laboratory of Otolaryngology Head and Neck Surgery, Xiamen 361003, China

ARTICLE INFO

Original paper

Article history:

Received: June 17, 2023

Accepted: November 10, 2023

Published: December 20, 2023

Keywords:

Laryngeal carcinoma; MiRNA-203b-3p; ZNF324

ABSTRACT

The present study aimed to elucidate the role of MicroRNA-203b-3p (miRNA-203b-3p) in protecting the deterioration of laryngeal carcinoma through targeting ZNF324. Relative levels of miRNA-203b-3p and ZNF324 in laryngeal carcinoma tissues with different tumor node metastasis (TNM) staging and pathological grades were detected. Regulatory effects of miRNA-203b-3p on clonality, viability and 5-Ethynyl-2'-deoxyuridine (EdU)-positive ratio in M2E and TU212 cells were assessed. The binding relationship between miRNA-203b-3p and ZNF324 was evaluated by dual-luciferase reporter gene assay. The involvement of ZNF324 in cell phenotype changes of laryngeal carcinoma regulated by miRNA-203b-3p was explored by rescue experiments. MiRNA-203b-3p was downregulated in laryngeal carcinoma, especially in those with advanced TNM staging or pathological grade. Overexpression of miRNA-203b-3p reduced clonality, viability and EdU-positive ratio in M2E and TU212 cells. In addition, ZNF324 was upregulated in laryngeal carcinoma, which was negatively regulated by miRNA-203b-3p. ZNF324 was verified to be the target binding miRNA-203b-3p. Notably, overexpression of ZNF324 could partially reverse the inhibitory effects of miRNA-203b-3p on laryngeal carcinoma proliferation. MiRNA-203b-3p is downregulated in laryngeal carcinoma, which blocks laryngeal carcinoma cells to proliferate through targeting ZNF324 and thus alleviates cancer progression.

Doi: <http://dx.doi.org/10.14715/cmb/2023.69.14.32>Copyright: © 2023 by the C.M.B. Association. All rights reserved. 

Introduction

Laryngeal carcinoma is the second leading carcinoma in head and neck neoplasm, and its incidence ranks 11th in malignancies globally (1). In China, laryngeal carcinoma is particularly popular in Northeastern regions (2). Although great strides have been made on chemotherapy, radiotherapy and surgery, the therapeutic efficacy of laryngeal carcinoma is unsatisfactory (3). It is urgent to uncover novel strategies and targets for improving the therapeutic outcomes of laryngeal carcinoma.

MicroRNAs (miRNAs) are highly conserved non-coding RNAs. They are able to inhibit mRNA degradation or their translation through complementary base pairing, thereafter downregulating expressions of downstream mRNAs (4). With the advances in sequencing technologies, a great number of miRNAs have been identified to be related to laryngeal carcinoma, which are promising diagnostic or therapeutic biomarkers (5). Through analyzing miRNA profiling in laryngeal carcinoma, Liu et al. (6) screened out 13 differentially expressed miRNAs in laryngeal carcinoma tissues.

The zinc finger structure is composed of multiple cysteines and/or histidine, which in turn binds Zn²⁺ and a stable tetrahedral structure is formed through self-folding. Abundant proteins containing zinc finger structures are found in animals, plants and microorganisms, which are known as zinc finger proteins (7-10). Relative levels of

zinc finger proteins could predict the phenotype state of proliferating or metastatic cancer cells (11). It is reported that zinc finger proteins regulated by miRNAs exert a certain impact on tumor occurrence and progression (12,13). In this paper, we aim to clarify the role of miRNA-203b-3p in influencing the development of laryngeal carcinoma through targeting ZNF324.

Materials and Methods

Sample collection

A total of 15 laryngeal carcinoma tissues and matched adjacent normal ones were surgically resected from 15 laryngeal carcinoma patients. The tumor staging and pathological grade of each patient were recorded. Patients and their families in this study have been fully informed. This study was approved by the Ethics Committee of The First Affiliated Hospital, Medical College, Xiamen University. Signed written informed consent were obtained from all participants before the study.

Quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol (Invitrogen, Carlsbad, CA, USA) was applied for isolating cellular RNA, which was quantified by Agilent BioAnalyzer 2100 (Agilent, Palo Alto, CA, USA). Complementary deoxyribose nucleic acids (cDNAs) were obtained using the miScript II RT and its mRNA level

* Corresponding author. Email: LuoxianyangENT@163.com

was determined using the miScript SYBR Green PCR kit (Qiagen, Hilden, German). U6 was used as the internal reference. The mRNA levels were calculated by $2^{-\Delta\Delta CT}$. Primer sequences were as follows: MiRNA-203b-3p, stem-loop RT: 5'-CTCACAGTACGTTGGTATCCTTGTGATGTTTCGATGCCATATTGACTGTGAGCGAGTGG-3'; Forward: 5'-ACACTCCAGCTGGGTGAAATGTTTAGGACCACT-3'; U6, Forward: 5'-CTCGCTTCGGCAGCA-CA-3'; Reverse: 5'-AACGCTTCACGAATTTGCGT-3'.

Cell culture

Cells were provided by Cell Bank (Shanghai, China). They were cultured in dulbecco's modified eagle medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin in a 5% CO₂ incubator at 37°C. Medium was replaced every three days.

Cell transfection

Cells were inoculated in 6-well plates with 4×10^5 cells/well. Until 70% confluence, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was diluted in serum-free medium with 3 μ L/L and incubated at 37°C for 20 min. Transfection plasmids were dissolved in a serum-free medium for 5 min. Subsequently, cells were cultured with the above mixture for 48 h and the complete medium was replaced at 12 h.

Colony formation assay

Cells were inoculated in 6-well plates with 800 cells per well and cultured for 10 days. Afterward, colonies were fixed in methanol for 20 min and dyed in 1% crystal violet for 30 min. Visible colonies were captured.

Cell counting kit-8 (CCK-8)

Cells were inoculated in a 96-well plate with 8×10^3 cells/well. At the appointed time points, the absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

5-Ethynyl-2'- deoxyuridine (EdU) assay

Cells were inoculated in a 24-well plate with 2×10^4 cells per well. They were incubated in 4% methanol for 30 min, followed by 10-min permeabilization in 0.5% TritonX-100, and a 30-min reaction in 400 μ L of 1 \times ApollorR. Afterward, cells were dyed in 4',6-diamidino-2-phenylindole (DAPI) for another 30 min. EdU-positive cells and DAPI-labeled nuclei were captured.

Western blot

Cells were lysed for isolating cellular protein and electrophoresed. Protein samples were loaded on polyvinylidene fluoride (PVDF) membranes (Roche, Basel, Switzerland). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours. Membranes were reacted with primary and secondary antibodies for the indicated time. Band exposure and analyses were finally conducted.

Dual-luciferase reporter gene assay

Binding sequences in the promoter regions between miRNA-203b-3p and ZNF324 were predicted by bioinformatics. The predicted sequences were amplified and cloned into pGL3-basic vectors for constructing wild-type

and mutant-type luciferase vectors. Cells were inoculated in a 24-well plate with 2×10^4 cells/well. They were co-transfected with 0.5 μ g luciferase plasmid and 20 μ mol/L miRNA-203b-3p mimics/negative control for 48 h. Relative luciferase activity was finally measured.

Statistical analysis

Statistical Product and Service Solutions (SPSS) 20.0 statistical software (IBM, Armonk, NY, USA) was used for data analysis. All data were expressed as mean \pm SD (standard deviation). The paired two-tailed *t*-test was used to compare the differences between the two groups. *P*<0.05 was considered to be statistically significant.

Results

MiRNA-203b-3p was lowly expressed in laryngeal carcinoma

Compared with adjacent normal tissues, miRNA-203b-3p was upregulated in 15 laryngeal carcinoma tissues (Figure 1A). In particular, the miRNA-203b-3p level was lower in laryngeal carcinoma patients with advanced TNM staging (Figure 1B) and pathological grade (Figure 1C). Consistently, miRNA-203b-3p was downregulated in laryngeal carcinoma cell lines (Figure 1D). Among the four tested cell lines, M2E and TU212 cells expressed the lowest abundance of miRNA-203b-3p, and they were selected for the following experiments.

MiRNA-203b-3p suppressed the proliferative ability in laryngeal carcinoma

Transfection of miRNA-203b-3p mimics effectively upregulated miRNA-203b-3p in M2E and TU212 cells, verifying their great transfection efficacy (Figure 2A). Overexpression of miRNA-203b-3p reduced clonality (Figure 2B), viability (Figure 2C) and EdU-positive ratio (Figure 2D) in M2E and TU212 cells. To sum up, miRNA-203b-3p was able to inhibit the proliferative ability in

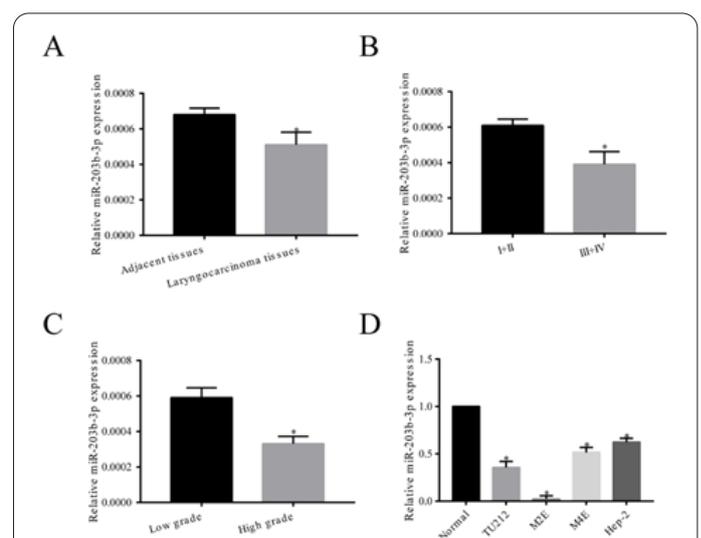


Figure 1. MiRNA-203b-3p was lowly expressed in laryngeal carcinoma. (A) MiRNA-203b-3p levels in 15 matched laryngeal carcinoma tissues and adjacent normal tissues. (B) MiRNA-203b-3p level in laryngeal carcinoma patients in stage I+II or stage III+IV. (C) MiRNA-203b-3p level in laryngeal carcinoma patients in high or low grade. (D) MiRNA-203b-3p levels in normal cell lines and laryngeal carcinoma cell lines.

laryngeal carcinoma.

MiRNA-203b-3p directly bound ZNF324

Compared with adjacent normal tissues, ZNF324 was upregulated in laryngeal carcinoma (Figure 3A). Transfection of miRNA-203b-3p mimics markedly downregulated both mRNA and protein levels of ZNF324 (Figure 3B). Subsequently, we constructed ZNF324-WT and ZNF324-MUT luciferase vectors (Figure 3C). Decreased luciferase activity after co-transfection of ZNF324-WT and miRNA-203b-3p mimics proved the binding between miRNA-203b-3p and ZNF324 (Figure 3D).

MiRNA-203b-3p alleviated the deterioration of laryngeal carcinoma through targeting ZNF324

To further clarify the involvement of ZNF324 in the progression of laryngeal carcinoma, the transfection effi-

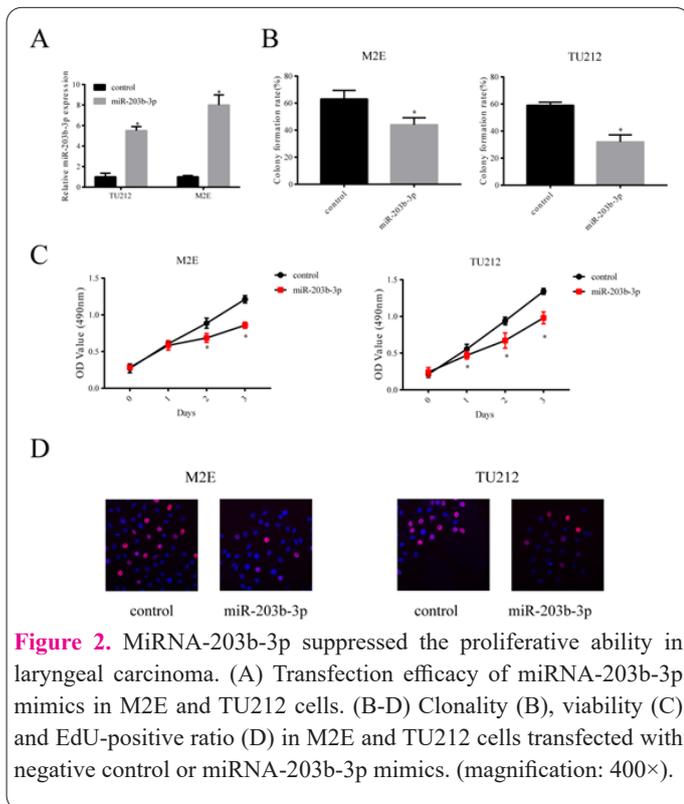


Figure 2. MiRNA-203b-3p suppressed the proliferative ability in laryngeal carcinoma. (A) Transfection efficacy of miRNA-203b-3p mimics in M2E and TU212 cells. (B-D) Clonality (B), viability (C) and EdU-positive ratio (D) in M2E and TU212 cells transfected with negative control or miRNA-203b-3p mimics. (magnification: 400×).

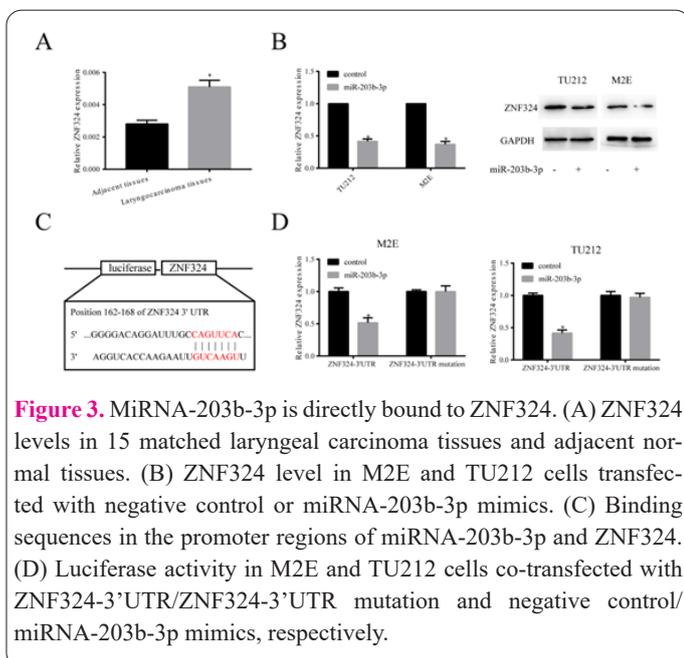


Figure 3. MiRNA-203b-3p is directly bound to ZNF324. (A) ZNF324 levels in 15 matched laryngeal carcinoma tissues and adjacent normal tissues. (B) ZNF324 level in M2E and TU212 cells transfected with negative control or miRNA-203b-3p mimics. (C) Binding sequences in the promoter regions of miRNA-203b-3p and ZNF324. (D) Luciferase activity in M2E and TU212 cells co-transfected with ZNF324-3'UTR/ZNF324-3'UTR mutation and negative control/miRNA-203b-3p mimics, respectively.

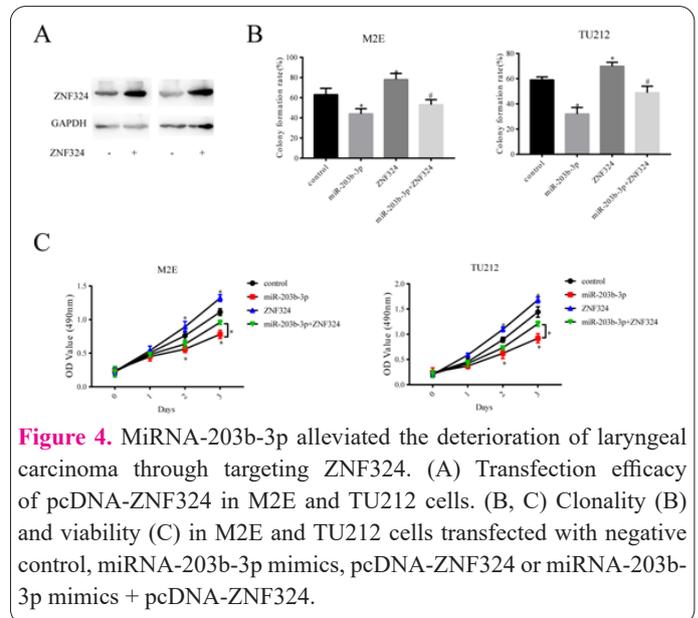


Figure 4. MiRNA-203b-3p alleviated the deterioration of laryngeal carcinoma through targeting ZNF324. (A) Transfection efficacy of pcDNA-ZNF324 in M2E and TU212 cells. (B, C) Clonality (B) and viability (C) in M2E and TU212 cells transfected with negative control, miRNA-203b-3p mimics, pcDNA-ZNF324 or miRNA-203b-3p mimics + pcDNA-ZNF324.

ciency of pcDNA-ZNF324 was tested (Figure 4A). Overexpression of ZNF324 markedly enhanced clonality and viability in laryngeal carcinoma cells. However, the enhanced trends were partially reversed by co-overexpression of miRNA-203b-3p (Figure 4B, 4C). Therefore, it is believed that miRNA-203b-3p alleviated the deterioration of laryngeal carcinoma through negatively regulating ZNF324.

Discussion

Laryngeal carcinoma mainly affects males. Nowadays, its incidence and mortality present an increased trend (14). The high mortality of laryngeal carcinoma is closely linked to strong invasiveness and metastasis. Multiple oncogenes and tumor-suppressor genes are involved in the occurrence of laryngeal carcinoma (15).

MiRNAs are vital regulators at the post-transcription level (16). Current studies have shown that many miRNAs are abnormally expressed in laryngeal carcinoma tissues (17). For instance, miR-519b-3p is lowly expressed in laryngeal carcinoma tissues as a tumor-suppressor gene, which arrests the cell cycle in the G₂/M phase by downregulating HuR and COX-2 (18). Dixon et al. (18) illustrated that miR-519a could bind HuR in the promoter region. HuR, a popular RNA-binding protein, is found to be upregulated in laryngeal carcinoma (19). In Hep-2 cells, miR-34a and miR-34c suppress the development of laryngeal carcinoma through downregulating GALNT7 (20).

In our paper, miRNA-203b-3p was markedly downregulated and it was able to inhibit proliferative ability in laryngeal carcinoma. A relevant study suggested that miRNA-203b-3p could downregulate the protein level of Bcl-xL through binding its 3'UTR, and its level is negatively correlated to the mRNA level of BCL2L1 in breast cancer (21-24). Here, we have verified the binding between miRNA-203b-3p and ZNF324. Interestingly, overexpression of ZNF324 could partially abolish the inhibitory effect of miRNA-203b-3p on proliferative ability in laryngeal carcinoma. Collectively, we have demonstrated that miRNA-203b-3p was a tumor-suppressor gene in laryngeal carcinoma, which alleviated its deterioration through targeting ZNF324.

Conclusions

MiRNA-203b-3p is downregulated in laryngeal carcinoma, which alleviates cancer progression through blocking laryngeal carcinoma cells from proliferating by targeting ZNF324.

Conflict of Interest

The authors declared no conflict of interest.

References

- Purkayastha M, McMahon AD, Gibson J, Conway DI. Trends of oral cavity, oropharyngeal and laryngeal cancer incidence in Scotland (1975-2012) - A socioeconomic perspective. *Oral Oncol* 2016; 61: 70-75.
- Liu Y, Zhao Q, Ding G, Zhu Y, Li W, Chen W. Incidence and mortality of laryngeal cancer in China, 2008-2012. *Chinese J Cancer Res* 2018; 30(3): 299-306.
- Tan J, Jing YY, Han L, et al. MicroRNA-203 inhibits invasion and induces apoptosis of laryngeal cancer cells via targeting LASP1. *Eur Rev Med Pharmacol* 2018; 22(19): 6350-6357.
- Xuan L, Qu L, Zhou H, et al. Circular RNA: a novel biomarker for progressive laryngeal cancer. *Am J Transl Res* 2016; 8(2): 932-939.
- Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat Rev Drug Discov* 2017; 16(3): 203-222.
- Liu M, Wu H, Liu T, et al. Regulation of the cell cycle gene, BTG2, by miR-21 in human laryngeal carcinoma. *Cell Res* 2009; 19(7): 828-837.
- Lyu T, Cao J. Cys(2)/His(2) Zinc-Finger Proteins in Transcriptional Regulation of Flower Development. *Int J Mol Sci* 2018; 19(9): 1867(15-28).
- Dogan B, Najafabadi HS. Computational Methods for Analysis of the DNA-Binding Preferences of Cys2His2 Zinc-Finger Proteins. *Methods Mol Biol* 2018; 1867(15-28).
- Zhao L, Chen S, Jia L, Shu S, Zhu P, Liu Y. Selectivity of arsenite interaction with zinc finger proteins. *Metallomics* 2012; 4(9): 988-994.
- Koczulla AR, Jonigk D, Wolf T, et al. Kruppel-like zinc finger proteins in end-stage COPD lungs with and without severe alpha1-antitrypsin deficiency. *Orphanet J Rare Dis* 2012; 7(29).
- Nikpour P, Emadi-Baygi M, Mohammad-Hashem F, Maracy MR, Haghjooy-Javanmard S. Differential expression of ZFX gene in gastric cancer. *J Biosciences* 2012; 37(1): 85-90.
- Malpeli G, Barbi S, Zupo S, et al. Identification of microRNAs implicated in the late differentiation stages of normal B cells suggests a central role for miRNA targets ZEB1 and TP53. *Oncotarget* 2017; 8(7): 11809-11826.
- Li G, Luo W, Abdalla BA, et al. miRNA-223 upregulated by MYOD inhibits myoblast proliferation by repressing IGF2 and facilitates myoblast differentiation by inhibiting ZEB1. *Cell Death Dis* 2017; 8(10): e3094.
- Cheng ZX, Song YX, Wang ZY, Wang Y, Dong Y. miR-144-3p serves as a tumor suppressor by targeting FZD7 and predicts the prognosis of human glioblastoma. *Eur Rev Med Pharmacol* 2017; 21(18): 4079-4086.
- Luo H, Jiang Y, Ma S, et al. EZH2 promotes invasion and metastasis of laryngeal squamous cells carcinoma via epithelial-mesenchymal transition through H3K27me3. *Biochem Biophys Res Commun* 2016; 479(2): 253-259.
- Tricoli JV, Jacobson JW. MicroRNA: Potential for Cancer Detection, Diagnosis, and Prognosis. *Cancer Res* 2007; 67(10): 4553-4555.
- Guo Y, An R, Zhao R, Sun Y, Liu M, Tian L. miR-375 exhibits a more effective tumor-suppressor function in laryngeal squamous carcinoma cells by regulating KLF4 expression compared with simple co-transfection of miR-375 and miR-206. *Oncol Rep* 2016; 36(2): 952-960.
- Dixon DA, Tolley ND, King PH, et al. Altered expression of the mRNA stability factor HuR promotes cyclooxygenase-2 expression in colon cancer cells. *J Clin Invest* 2001; 108(11): 1657-1665.
- Lopez DSI, Fan J, Yang X, et al. Role of the RNA-binding protein HuR in colon carcinogenesis. *Oncogene* 2003; 22(46): 7146-7154.
- Li W, Ma H, Sun J. MicroRNA-34a/c function as tumor suppressors in Hep-2 laryngeal carcinoma cells and may reduce GALNT7 expression. *Mol Med Rep* 2014; 9(4): 1293-1298.
- Azizi Dargahlou, S., Iriti, M., Pouresmaeil, M., Goh, L. P. W. MicroRNAs; their therapeutic and biomarker properties. *Cell Mol Biomed Rep* 2023; 3(2): 73-88. doi: 10.55705/cnbr.2022.365396.1085
- Kanwal, N., Al Samarrai, O., Al-Zaidi, H. M. H., Mirzaei, A., Heidari, M. Comprehensive analysis of microRNA (miRNA) in cancer cells. *Cell Mol Biomed Rep* 2023; 3(2): 89-97. doi: 10.55705/cnbr.2022.364591.1070.
- Sasani S, Rashidi Monfared S, Mirzaei AR. Identification of some *Echinophora platyloba* miRNAs using computational methods and the effect of these miRNAs in the expression of TLN2 and ZNF521 genes in different human body organs. *Cell Mol Biomed Rep* 2024; 4(1): 43-53. doi: 10.55705/cnbr.2023.386145.1100.
- Aakko S, Straume AH, Birkeland EE, et al. MYC-Induced miR-203b-3p and miR-203a-3p Control Bcl-xL Expression and Paclitaxel Sensitivity in Tumor Cells. *Transl Oncol* 2019; 12(1): 170-179.