Reports of Biochemistry & Molecular Biology
Vol.10, No.4, Jan 2022

Original article

www.RBMB.net

Evaluation of Radiotherapy on *miR-374* Gene Expression in Colorectal Cancer Patient Blood Samples

Azam Ahmadi[1], Mohammad Reza Bayatiani[2], Fatemeh Seif*[2], Jamshid Ansari[2], Parisa Rashidi[2], Mona Moghadasi[3], Mobarakeh Etemadi[3]

Abstract

Background: Current cancer treatments include surgery, radiotherapy, chemotherapy, and immunotherapy. Despite these treatments, a main issue in cancer treatment is early detection. microRNAs (miRNAs) can be used as markers to diagnose and treat cancers. This study investigated the effect of radiotherapy on *miR-374* expression, and *APC* and *GSK-3β*, two of its target genes, in the WNT pathway, in peripheral blood samples from radiotherapy-treated colorectal cancer (CRC) patients.

Methods: Peripheral blood was collected from 25 patients before and after radiotherapy. RNA was extracted from the blood and cDNA synthesized. *miR-374, APC,* and *GSK-3β* expression was determined by real-time polymerase chain reaction (RT-PCR) and the amplicons were sequenced. Finally, the data were statistically evaluated.

Results: Quantitative RT-PCR revealed significant down-regulation of *miR-374* (0.63-fold) and up-regulation of *APC* (1.12-fold) and *GSK-3β* (1.22-fold) in CRC patients after five weeks of radiotherapy. Sequencing of PCR-produced amplicons confirmed the conservation of mature and precursor sequences encoding *miR-374*. *miR-374* expression changed with time after radiotherapy treatment and related tumor grading. Increased age and tumor grade positively correlated with decreased miR-374 expression.

Conclusions: *miR-374* expression, and that of its two target genes, *APC* and *GSK-3β*, changed after radiotherapy. These genes can likely be used as diagnostic radiotherapy markers in CRC.

Keywords: Biomarker, Colorectal cancer, *Mir-374*, Radiotherapy.

Introduction

Colorectal cancer (CRC) is one of the most common high-mortality-rate cancers. Types of known CRCs include adenocarcinoma, which originates from mucus-producing cells, carcinoid tumors, which derive from specific hormone-producing cells, gastrointestinal stromal tumors (GISTs), lymphoma, which originates from immune cells in colorectal tissue, and sarcoma, which originates from blood vessel cells (1, 2). These various CRCs result from changes in several genes. Generally, genes involved in CRC are classified into several groups, including oncogenes and proto-oncogenes, such as transcription factors, growth factors and their receptors, tumor suppressor genes such as *Rb*, and *APC*, and those involved in metastasis, repair, and apoptosis. The products of these genes are present in various signaling pathways, including the p53, TGFB, JAK/STAT, PI3K, and WNT pathways (3-8). Several key gene products are present in each pathway. One component in the WNT pathway is beta-catenin, which is normally surrounded and degraded by scaffolds from *APC*, *GSK3B*

1: Infectious Diseases Research Center (IDRC), Arak University of Medical Sciences, Arak, Iran.
2: Department of Medical Physics and Radiotherapy, Arak University of Medical Sciences and Khansari Hospital, Arak, Iran.
3: Students Research Committee, Arak University of Medical Sciences, Arak, Iran.
***Corresponding author: Fatemeh Seif; Tel: +98 86 34173506; E-mail: sahar_s59@yahoo.com.**
Received: 19 Jan, 2021; Accepted: 24 Oct, 2021

[DOI: 10.52547/rbmb.10.4.614]

(glycogen synthase kinase-3 beta*)*, and *Axin2* gene products. After degradation, beta-catenin enters the nucleus and activates proliferative genes including *C-myc* and *cyclin D*. Gene deregulation caused by mutations in the cancer process lead to the survival of beta-catenin and expression of proliferative genes (9-12). One group of regulatory RNA molecules involved in recent oncology topics is miRNAs. Currently, the role of miRNAs as diagnostic biomarkers is being considered by researchers (13). Peter Jo et al. in 2017 showed that miRNAs in the blood plasma of CRC patients can be used as biomarkers for detection of response rate to treatment (12). Currently, biomarkers for CRC tumors used in chemotherapy include *KRAS*, *BRAF*, *MSI,* and *SMAD4*. Common treatments for CRC include surgery, radiation, chemotherapy, and immunotherapy. Each of these methods has side effects. Despite these treatment options, a major challenge in cancer treatment is early defect diagnosis. This study investigated the effect of radiotherapy on expression of *miR-374*, and *APC*, and *GSK-3β,* two of its target genes, in the WNT pathway in peripheral blood samples from radiotherapy-treated CRC patients.

Materials and Methods

Collection of blood samples

Two ml of peripheral blood were collected from 25 CRC patients referred to Khansari Hospital, Arak, Iran (2018-2020) before, and in the first, third, and fifth weeks after starting radiation therapy. All patients treated with radiotherapy were treated using an Elekta Precise linear accelerator with a daily dose of 1.8 Gy (dose per fraction: 1.8 Gy). The radiation treatment dose in treatment phases I and II were 45 Gy with 5.4 Gy boosts to a total of 50.4 Gy for all patients (Table 1). Radiotherapy was performed as a 3D-conformal protocol with 18 MV photon beams five days per week.

Table 1. Details of radiotherapy doses in this study

Phases of treatment	number of sessions	dose per fraction (Gy)	dose in per phase (Gy)
Phase I	25	1.8	25*1.8= 45
Phase II (Boost)	3	1.8	3* 1.8= 5.4
Total	28	1.8	28 * 1.8= 50.4

In addition to radiation therapy, 15 (60%) of the patients in the study also received FOLFOX, an anti-cancer drug containing folinic acid, fluorouracil, and oxaliplatin. The study was approved by the Ethics Committee of Arak University of Medical Sciences with a Code of Ethics (IR.ARAKMU.REC.1397.52).

Quantitative RT-PCR

The expression of *miR-374*, *APC,* and *GSK-3ß* was evaluated by quantitative RT-PCR. RNA was extracted from the blood samples using an RNX kit (Sinaclone, Iran), and cDNA was synthesized using M-MuLV enzyme (YTA, Iran), random hexamers, and stem-loop primers. cDNAs were used as templates in RT-PCRs using master mix, SYBR green (YTA, Iran), and 10 pmol of forward and reverse primers with an annealing temperature of 54 °C in a thermocycler (Roche). *GAPDH* and *SNORD47* (*U47*) were used as internal controls.

Evaluation of miR-374 sequences

miR-374 amplicons were produced by real-time PCR using two series primers (14, and Fig. 1) PCR Products were sequenced on an ABI-Biosystem xl100 (Macrogen, South Korea). Data were analyzed with BioEdit and Chromas software.

Measurement of the number of eosinophils, neutrophils, lymphocytes

Three observers calculated eosinophil,

[DOI: 10.52547/rbmb.10.4.614]

neutrophil, and lymphocyte on BAL fluid with a blind test using a light microscope with a magnification of 400x, then averaged the results.

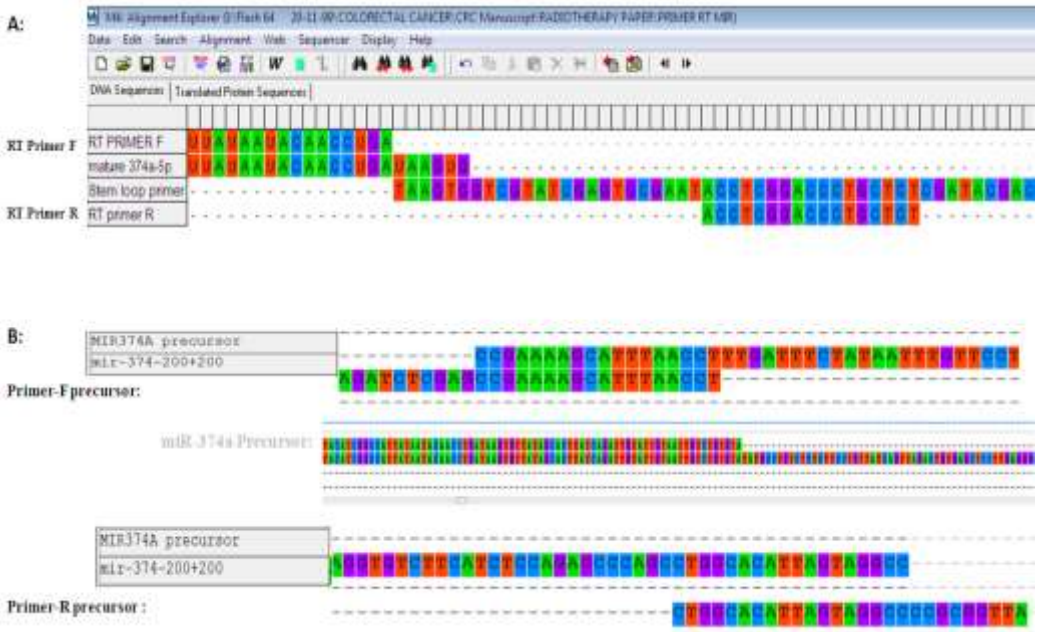

Fig. 1. Schematic overview of primers used to amplify the *miR-374* mature and precursor sequences. RT primers F and R (A) and precursor primers F and R (B).

Statistical analysis

Expression data was analyzed using Excel 2007 and two-way ANOVA in GraphPad Prism 7.0 software. Differences between the groups were considered statistically significant at P less than 0.05. Sequence alignments were performed using MEGA4 and BioEdit alignment tools. Sequencing results were analyzed with Blast, BioEdit, and Chromas Lite version 2.01.

Results

miR-374, APC, and GSK-3ß expression in samples from radiotherapy-treated CRC patients

Peripheral blood was collected from each of the 25 radiotherapy-treated CRC patients before, and one, three, and five weeks after the start of therapy. Patient demographic data are shown in Table 2.

Table 2. Patient demographics and tumor sample characteristics.

Characteristic	Detail	Patients (%)
Gender	Male	64
	Female	36
Median age range	≤ 50	28
	> 50	72
BMI	< 27	56
	> 27	44
History of cancer	No	85
	Yes	15
Tumor grade	T2 & T3	68
	T1 & unknown	32
Differentiation	poorly	33
	moderately	40
	well	27
Anatomical distribution	colon	54
	rectum	54
	sigmoid	27
Taking drugs	FOLFOX	60
	other	40

[DOI: 10.52547/rbmb.10.4.614]

miR-374 expression was significantly down-regulated, while *GSK- 3β* and *APC* expression were significantly up-regulated, one, three, and five weeks after the start of radiotherapy (Fig. 2 and Table 3).

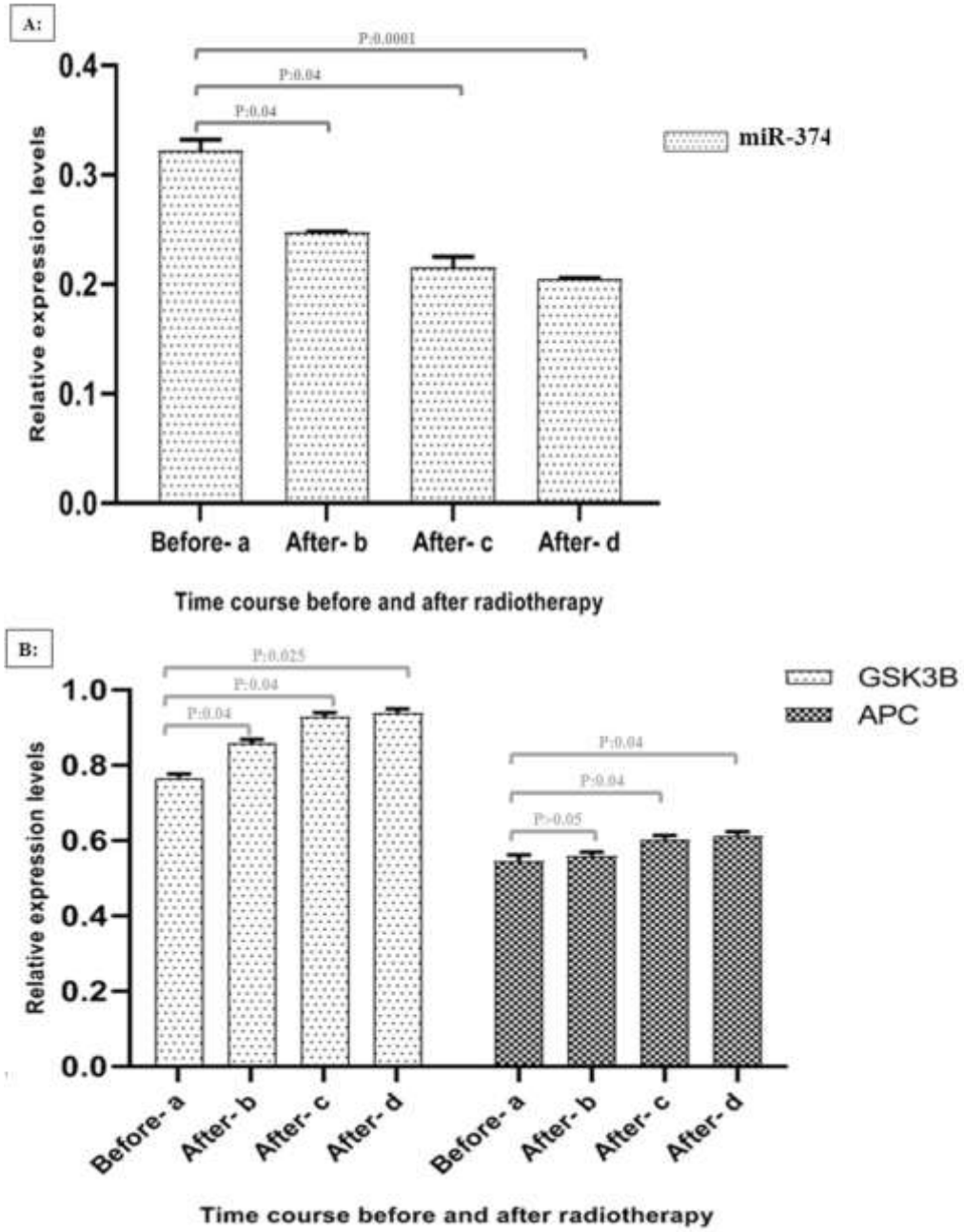

Fig. 2. Expression of *miR-374*, GSK-3β, and APC before, and 1, 3, and 5 weeks after radiotherapy. GraphPad prism, Two-way ANOVA method.

Table 3. Fold-change of *APC*, *GSK-3β*, and *miR-374* expression one, three, and five weeks after radiation therapy.

Gene Expression Changes	Fold-change of *APC* expression	Fold-change of *GSK-3β* expression	Fold-change of *miR-374* expression
First week after starting radiation therapy	1.02↑	1.11↑	0.76↓
Third week after starting radiation therapy	1.10↑	1.21↑	0.66↓
Fifth week after starting radiation therapy	1.12↑	1.22↑	0.63↓

[DOI: 10.52547/rbmb.10.4.614]

Conservation of the miR-374 precursor coding sequence

RT-PCR with mature and precursor *miR-374* primers resulted in 66- and 291-bp amplicons (Figs. 3A and 3B, respectively). Comparisons of the PCR products and Nucleotide Blasts of the amplicon sequences confirmed their identities (Fig. 3C).

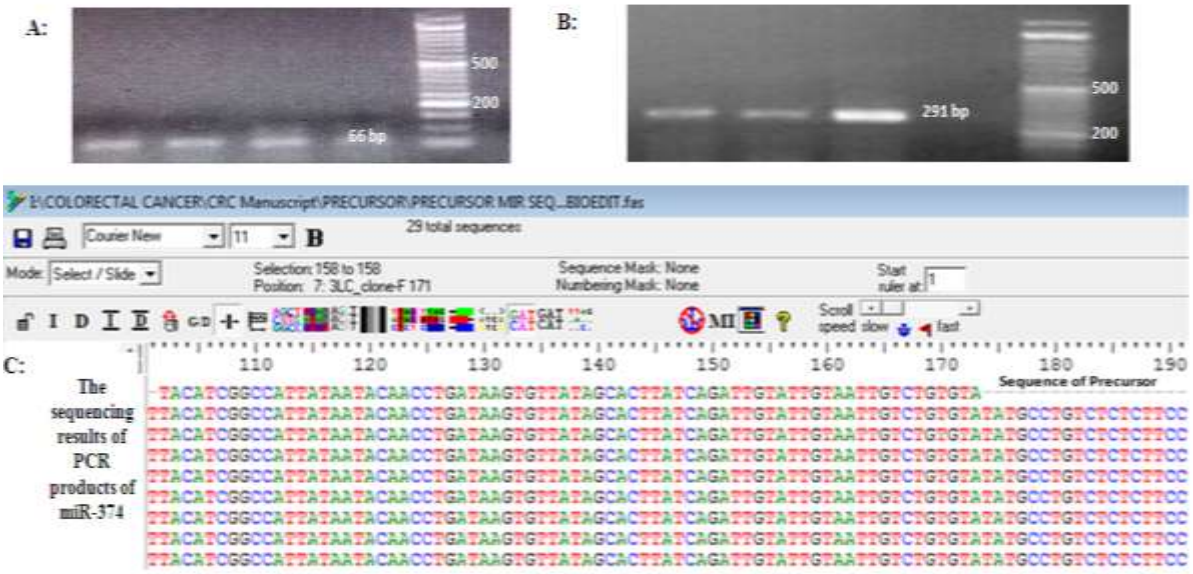

Fig. 3. A and B: Agarose gels of PCR products of 66-bp amplicon of *miR-374* mature sequences and 291bp amplicon of *miR-374* precursor sequences from CRC patient peripheral blood samples. C: Amplicon sequences from real-time PCR and conservation of precursor sequence encoding *miR-374* from CRC patient samples (BioEdit).

Relationship between demographic characteristics and gene expression changes

Increased BMI and age were associated with increased tumor grade and decreased differentiation. In addition, with increased age in cases where the tumor grade increased and differentiation decreased, the expression of *GSK3B* and *APC* genes is not significant as the number of radiotherapy sessions increased. Although with increased age and grade and decreased differentiation, *miR-374* expression was significantly down-regulated. The correlation showed the *miR-374* expression decrease over treatment time positively correlated with increased patient age, tumor grade, and BMI (Pearson r, p= 0.0005, r= 0.08).

Discussion

Colorectal cancer is the second-leading cause of death by cancer after only lung cancer (1, 2, 15). Colorectal cancer stages include stage A, in which the cancer is confined to the mucous membrane, stage B, in which the cancer spreads to the intestinal wall but the lymph nodes are not involved, stage C, in which the lymph nodes also become cancerous, and stage D, the metastatic form. Existing screening methods cannot detect CRC in the early stages; however, early detection and diagnosis is necessary for effective treatment, and the need for new biomarkers for early detection is urgent. The molecular studies of signaling pathways involved in CRC have revealed that multiple pathways, including the WNT pathway, are altered (16). Components of the signaling pathways in humans can be controlled by miRNAs. Two decades after miRNAs were first identified much remains unknown regarding their roles in cancer (17-20). The nucleotide sequences of the genes encoding miRNAs may be conserved. In our study, conservation of the *miR-374* mature and precursor coding sequences in blood samples from CRC patients was demonstrated (Fig. 3). We also investigated *miR-374, APC,* and *GSK-3β* expression in blood samples from radiotherapy-treated CRC patients.

[DOI: 10.52547/rbmb.10.4.614]

Numerous studies have reported altered expression of various miRNAs in CRC. *miR-144* was shown to reduce migration and proliferation of colorectal tumor cells by reducing *ROCK1* levels (21). In another study *miR-30b* was shown to suppress colorectal tumors via targeting the oncogenes *KRAS*, *PIK3CD*, and *BCL2* (22). In addition to being diagnostic markers, miRNAs can also be used as markers for CRC treatment and its radiation resistance. A study in 2017 showed that miRNAs can be used as biomarkers to predict treatment responses. Based on the results of that study, *miR-125b-1*, *miR-1183*, *miR-130a*, and *miR-375* had different expression levels before and after treatment, and these miRNAs target key genes including *ATM* and *CHEK1*, which are involved in chemo-radiation of rectal cancer (23). Peter Jo et al. showed in 2017 that miRNAs in the blood plasma of patients with CRC can be used as biomarkers of treatment response. Their study indicated that *miR-30c* and *miR-31* may be differentiated between cancerous and non-cancerous individuals, and could be used as CRC biomarkers (12). De-regulation of *miR-374* in lung, pancreatic, and gastric cancers, melanoma, and atherosclerosis have been demonstrated in other studies (24-26). In gastric cancer, up-regulation of this molecule was responsible for the proliferative phenotype in a mouse model of gastritis transformation to gastric cancer (27). In 2019, Li et al. showed that *miR-374* was down-regulated in pancreatic cells. They reported this transcript by targeting JAM-2, which leads to growth suppression in pancreatic tumors (28)**.** Baek SJ et al. (2016) using the microarray technique, evaluated 1265 microRNAs and found that four molecules, including *miR-374*, were down-regulated in carbon beam-resistant cells, and this molecule is considered as a possible biomarker for optimal cancer treatment (29). Our results, in which *miR-374* expression was reduced in CRC patients after radiotherapy, were consistent with his study.

In previous work from our laboratory, bioinformatics, and in vitro studies of *miR-374*, *APC*, and *GSK-3β* expression changes were identified in formalin-fixed paraffin-embedded (FFPE) CRC specimens when compared to non-tumor specimens. Increased *miR-374* reduced expression of its target genes in cancer cells, leading to activation of proliferative genes (14).

Our present study found decreased *miR-374* expression and increased expression of its two target genes following radiotherapy, contrary to what occurs in tumor cells in CRC. Our results also showed increased expression of the target genes with increasing radiotherapy over time. It should also be noted that 60% of our patients also received FOLFOX, a chemotherapy drug containing folinic acid, fluorouracil, and oxaliplatin, often used to treat CRC. Therefore, these genes' altered expression might be used as a marker to predict the effects of various therapeutic approaches.

CRC risk factors included aging, diet, obesity, smoking, and alcohol. Our data agreed with other studies that cite age and obesity as CRC risk factors. In our samples, increased BMI and age were associated with increased tumor grade and decreased differentiation. Also, with increased age, in cases where the tumor grade increased and the degree of differentiation decreased, *miR-374* expression decreased significantly, while the *GSK-۳β* and *APC* expression might indicate a need for more chemotherapy sessions. It is possible that expression of these genes might be a marker to follow to determine the effectiveness of chemotherapy. We believe more studies with larger sample sizes are needed to verify this hypothesis.

Given the side effects of CRC treatments, predicting their effects is valuable. Considering their effects on expression of *miR-374* and its target genes, *APC* and *GSK-3β*, two major components of the WNT pathway in CRC, these genes may be useful as predictive markers of patient outcomes.

Acknowledgements

This work was supported by the Research Council of Arak University of Medical Sciences.

[DOI: 10.52547/rbmb.10.4.614]

References

1. Peedikayil MC, Nair P, Seena SM, Radhakrishnan L, Sadasivan S, Naryanan VA, et al. Colorectal cancer distribution in 220 Indian patients undergoing colonoscopy. Indian J Gastroenterol. 2009;28(6):212-5.
2. Mirkarimi HS. Nutritional characteristics in patients with colorectal cancer in Golestan Province of Iran, A case-control study. J Gorgan Univ Med Sci. 2009;10;11(2):38-44.
3. Pourhoseingholi M A, Fazeli Z, Fazeli-Bavandpour F S, Abadi A. Study of mortality trends of colorectal cancer in Iran between 1995 and 2004. Medical Sciences. 2014; 23 (4 and 1) :16-20
4. Rahimi Pordanjani S, Baeradeh N, Lotfi MH, Pourmohammadi B. Epidemiology of colorectal cancer: incidence, mortality, survival rates and risk factors. Razi Journal of Medical Sciences. 2016;10;23(144):41-50.
5. Safaei A, Moghimi DB, Fatemi SR, Ghiasi S, Zali MR. Epidemiology of colorectal Cancer: Study the recorded cases in 1379-86. 2007;9(3):209-216.
6. Fakheri H, Janbabai GH, Bari Z, Eshqi F. The epidemiologic and clinical-pathologic characteristics of colorectal cancers from 1999 to 2007 in Sari, Iran. Journal of Mazandaran University of Medical Sciences. 2008;10;18(67):58-66.
7. Mirmiran P, Azizi F, Janghorbani M, Hatami H. Epidemiology and Control of Common Disorders in Iran. Tehran. Khosravi Publishing; 2009.
8. Center MM, Jemal A, Smith RA, Ward E. Worldwide variations in colorectal cancer. CA Cancer J Clin. 2009;59(6):366-78.
9. Asghari-Jafarabadi M, Hajizadeh E, Kazemnejad A, Fatemi SR. Recognition of the factors affecting survival in colon and rectal cancer patients referred to RCGLD center of Shahid Beheshti University of Medical Sciences: accelerated failure time parametric survival analysis with frailty. Journal of Shahrekord University of Medical Sciences. 2010;12(2):51-64.
10. AR SN, Sh G, Mirshafiei A, Alimohamadian MH. Assessment of TPS tumor marker with ELISA for early detection and monitoring of gastrointestinal cancers. Tehran University Medical Journal. 2006;10;64(11):25-31.
11. Saebnia N, Sadeghizadeh M. The main factors involved in the recurrence of colorectal cancer and therapeutic methods against them. Police Medicine. 2016;10;5(2):87-95.
12. Jo P, Azizian A, Salendo J, Kramer F, Bernhardt M, Wolff HA, et al. Changes of microrna levels in plasma of patients with rectal cancer during chemoradiotherapy. International journal of molecular sciences. 2017;18(6).
13. Massard C, Deutsch E, Soria J. Tumour stem cell-targeted treatment: elimination or differentiation. Ann Oncol. 2006;17(11):1620-4.
14. Bayatiani MR, Ahmadi A, Aghabozorgi R, Seif F. Concomitant Up-Regulation of Hsa-*Mir-374* and Down-Regulation of Its Targets, GSK-3β and APC, in Tissue Samples of Colorectal Cancer. Reports of Biochemistry & Molecular Biology. 2021;9(4):408-416.
15. Rothschild SI. microRNA therapies in cancer. Molecular and cellular therapies. 2014;2(1):1-8.
16. Ishiguro H, Kimura M, Takeyama H. Role of microRNAs in gastric cancer. World J Gastroenterol. 2014;20(19):5694-5699.
17. López JA, Alvarez-Salas LM. Differential effects of miR-34c-3p and miR-34c-5p on SiHa cells proliferation apoptosis, migration and invasion. Biochem Biophys Res Commun. 2011;10;409(3):513-9.
18. Zhou R, Gong AY, Chen D, Miller RE, Eischeid AN, Chen XM. Histone deacetylases and NF-kB signaling coordinate expression of CX3CL1 in epithelial cells in response to microbial challenge by suppressing miR-424 and miR-503. PLoS One. 2013;28;8(5):e65153.
19. Zhao Y, Liu H, Li Y, Wu J, Greenlee AR, Yang C, et al. The role of miR-506 in transformed 16HBE cells induced by anti-benzo [a] pyrene-trans-7, 8-dihydrodiol-9, 10-epoxide. Toxicol Lett. 2011;10;205(3):320-6.

[DOI: 10.52547/rbmb.10.4.614]

20. Incoronato M, Urso L, Portela A, Laukkanen MO, Soini Y, Quintavalle C, et al. Epigenetic regulation of miR-212 expression in lung cancer. PloS one. 2011; 6(11):e27722.
21. Cai SD, Chen JS, Xi ZW, Zhang LJ, Niu ML, Gao ZY. MicroRNA 144 inhibits migration and proliferation in rectal cancer by downregulating ROCK 1. Mol Med Rep. 2015; 12(5):7396-7402.
22. Liao WT, Ye YP, Zhang NJ, Li TT, Wang SY, Cui YM, et al. MicroRNA-30b functions as a tumour suppressor in human colorectal cancer by targeting KRAS, PIK3CD and BCL2. J Pathol. 2014;232(4):415-27.
23. Lim SH, Ip E, Chua W, Ng W, Henderson C, Shin JS, et al. Serum microRNA expression during neoadjuvant chemoradiation for rectal cancer. Journal of clinical Oncology. 2017;35(15).
24. Miko E, Czimmerer Z, Csánky E, Boros G, Buslig J, Dezső B, et al. Differentially expressed microRNAs in small cell lung cancer. Exp Lung Res. 2009;35(8):646-64.
25. Wang W, Ma F, Zhang H. MicroRNA-374 is a potential diagnostic biomarker for atherosclerosis and regulates the proliferation and migration of vascular smooth muscle cells. Cardiovasc Diagn Ther. 2020;10(4):687-694.
26. Li XJ, Li ZF, Xu YY, Han Z, Liu ZJ. microRNA-374 inhibits proliferation and promotes apoptosis of mouse melanoma cells by inactivating the Wnt signalling pathway through its effect on tyrosinase. J Cell Mol Med. 2019;23(8):4991-5005.
27. Ji R, Zhang X, Qian H, Gu H, Sun Z, Mao F, et al. *miR-374* mediates the malignant transformation of gastric cancer-associated mesenchymal stem cells in an experimental rat model. Oncology reports. 2017;38(3):1473-1481.
28. Li W, Tang N, Tao J, Zhu Z, Liu L, Fang Q, et al. MicroRNA-374 targets JAM-2 regulates the growth and metastasis of human pancreatic cancer cells. Am J Transl Res. 2019;11(10):6454-6461.
29. Baek SJ, Sato K, Nishida N, Koseki J, Azuma R, Kawamoto K, et al. MicroRNA *miR-374*, a potential radiosensitizer for carbon ion beam radiotherapy. Oncol Rep. 2016; 36(5):2946-2950.

[DOI: 10.52547/rbmb.10.4.614]