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Concomitant Up-Regulation of *Hsa- Mir-374* and Down-Regulation of Its Targets, *GSK-3β* and *APC*, in Tissue Samples of Colorectal Cancer

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Abstract

Background: The *WNT*-pathway is involved in several cancers, including colorectal cancer (CRC). Many cell signaling components and pathways are controlled by microRNAs. The main purpose of the present study was to investigate the expression of *hsa-miR-374*, and its two target genes of the *Wnt*-pathway in CRC clinical samples.

Methods: In this study, we predicted the miRNAs targeting key genes of *WNT*-pathway using bioinformatics algorithms. The expression levels of *hsa-miR-374*, *APC* and *GSK-3β* on 48 pairs of Formalin-Fixed Paraffin-Embedded (FFPE) CRC tumors and marginal-tumors were evaluated using real time-PCR. Additionally, the *hsa-miR-374a-5p* precursor sequence was amplified by whole-blood DNA as a template. This amplicon was cloned into pEGFP-c1 expression vector and transfected into SW742 cells. Aside from this, MTT assay was performed to evaluate the effect of *miR-374* on cell viability.

Results: The bioinformatics analysis indicated that *hsa-miR-374* binds to the regulatory region the key components of *WNT*-pathway, including *APC* and *GSK-3β* considering the recognition elements and mirSVR scores. Our results revealed significant down-regulation of *GSK-3β* (0.94 times, p= 0.0098) and *APC* (0.96 times, p= 0.03) and up-regulation of *miR-374* (1.22 times, p= 0.0071) on tumor samples compared with their normal pairs. Meanwhile, the results of the over-expression of *miR-374* showed down-regulation of *APC* and *GSK-3β*. MTT-assay also indicated that the *miR-374* increased cell survival.

Conclusions: The results of our study indicated a concomitant change in the expression of *miR-374* and its two related target genes, in clinical samples of CRC. *Hsa-miR-374* might be as a helpful biomarker or therapeutic target in CRC.

Keywords: Colorectal cancer, *GSK-3β*, *miR-374*, *WNT*.

Introduction

The genetic causes of colorectal cancer (CRC) are the occurrence of changes in several components in different signal transduction pathways, including *P53*, *TGFB*, *JAK*/*STAT*, *PI3K*/*Akt*, and *Wnt* pathways. These pathways are linked together in many cases. One of the important pathways associated with CRC cancer is the *Wnt*/*β-catenin* (1–6). *Wnt* ligands can trigger two pathways, cononical *Wnt*-pathway and non-cononical *Wnt*-pathway which are also referred to as *β-catenin* independent pathways (7). In cononical *Wnt*-pathway, following binding *Wnt* signal to the receptor, *β-catenin* (gene ID: 1499) is released, entered the nucleus and activated transcription factors (TFs) such as *C-myc* (gene ID: 4609) and *cyclin D* (gene ID: 595) etc. In the absence of the *Wnt*, *β-catenin* normally is destroyed by scaffolds of *APC* (gene ID: 324), *GSK-3β* (gene ID: 2932) and *Axin2* (gene ID: 8313). The collapse of the genetic regulations

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caused by mutations in the cancer process leads to the survival of *β-catenin* and the expression of the proliferative genes (8), (9). Previous studies have investigated the deregulations of some components of the *Wnt*-signaling pathway in colon cancer samples (10–12). Recently, scientists have proposed the usage of molecular methods such as microarrays for analysis and identification of cancer molecular biomarkers. All components of signal transduction pathways are controlled by molecules called microRNAs (miRNAs) (13). The molecular network of carcinogenesis was determined by a systematic analysis between mRNAs and the expression of miRNAs. In some studies, based on their expression changes, classified tumors. miRNAs often interacted with their binding site in the 3'un-translated region (UTR) of target genes and affected their expression (14, 15). The purposeful study on miRNAs in cancer requires a candidate gene. The aim of our study was to determine the expression change in miRNAs that are selected by bioinformatics studies and its association with two components of the *Wnt* pathway in CRC samples.

Materials and Methods

Bioinformatics analysis

Bioinformatics analysis using different software including microRNA.org, miRwalk, DIANA-microT, Targetscan and NCBI, were employed to predict miRNAs that target 3'-UTR region of 7 key component of *WNT*-signaling pathway including *APC*, *GSK-3β*, *β-catenin*, *Axin1*, *Hnf4a*, *TCF*, *CyclinD* and *C-myc*. We selected miRNAs that simultaneously target several upstream and downstream compounds of *APC* and *GSK-3β* in the *WNT* pathway. Furthermore, the main components of other KEGG signaling pathways including *MAPK*, *PI3K/AKT* and *ErbB* pathways, which are involved in CRC, were determined using miRwalk software.

Ethics statement

The clinical sample collection procedure was approved by the ethics committee of Arak University of medical sciences (Ethics committee ID: *IR.ARAKMU.REC.1397.51*).

Preparation of FFPE tissue samples

In the present research, 48 pairs of formalin-fixed embedded (FFPE) tissue samples with ICD-O code 18 and 19 collected during the last 5 years, used from the archive of pathology departments of Khansarinejad hospital (Arak, Iran). Tumor and non-tumor samples obtained from the same CRC patients were confirmed using hematoxylin and eosin (H-E) staining.

RNA extraction, cDNA synthesis

In our study, 5-10 pieces cut from FFPE samples were trimmed and deparaffinized with xylol (Merck). In the following, digestion with proteinase-K (Cinnagen, Iran), extraction of RNA using a kit (RNX-Plus, Cinnagen, Iran) were performed as explained before (16). In the next step, extracted RNA (50 ng-1 µg) was used as a template for cDNA synthesis. The cDNA synthesis step was performed with 1 µl reverse transcriptase M-MuLV 100 Unit enzyme (Cinnagen, Iran), 3 µl RT Buffer 10 X, 2 µl random hexamer RH 10 µM, 0.75 µl stem loop (ST) primers 10 µM (Copenhagen, Denmark) and 3 µl dNTP 10 mM (Yekta Tajhiz Azma, Iran).

The qPCR reaction

The synthesized cDNAs were used as a template for quantitative-PCR on a light Cycler 96 real-time PCR machine (Roche, Germany) with SYBR green master-mix kit (Yekta Tajhiz Azma, Iran). In this reaction addition to *GSK-3β*, *APC*, and *miR-374* was evaluated the expressions of *GAPDH* and *SNORD47* (*U47*) RNAs for normalization. The sequences of primers showed in Table 1.

Plasmid construction for functional analysis of miR-374

The genomic DNA was extracted from human blood cells using PZP DNA extraction kit (Iran). In order to cloning *pri-miR-374* in pEGFP-c1 vector, primers were designed using specific software (Table 2). The amplification reaction was carried out using these primers and PCR master-mix (Amplicon, Denmark) in a thermocycler (Eppendorf, Germany). The

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fragments were cloned in the vector using *XhoI* and *SacII* restriction enzymes (Fermentas, USA) and T4-ligase (Cinagene, Iran). Recombinant vectors were extracted by a plasmid extraction kit (Takara, Japan). In addition, a scramble fragment used as control. Verification of positive construct containing miR-374 precursor was performed by the sequencing method with an ABI Apply Biosystem 3730xl (Macrogen, South Korea).

Table 1. The primer sequences that used in present study.

Primer ID	Sequence (5'-3')
ST-374 New	GTCGTATCGAGAGCAGGGTCCGAGGTATTCGCACTCGATACGACCACTTA
F-374	TTATAATACAACCTGA
ST-U47	GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACAACCTCA
F-U47	ATCACTGTAAAACCGTTCCA
F-GAPDH	GAAGGTGAAGGTCGGAGTC
R-GAPDH	GAAGATGGTGATGGGATTTC
R-universal	AGAGCAGGGTCCGAGGT
New F- *APC*	TTTGTCTTGGCGAGCAG
New R- *APC*	ATTTGCCTGTGGTCCTCA
GSK-3B -F	GACTAAGGTCTTCCGACCCC
GSK-3B -R	GCTTGAATCCGAGCATGAGG

Table 2. The primer sequences for cloning *pre-miR-374* in pEGFP-c1 vector. Under the restriction sites of the enzymes have been drawn lines.

Primer ID	Sequence (5'-3')
F-CLONING-374	AGAT**CTCGAG**CCGAAAAGCATTTAACCT
R-CLONING-374	TAA**CCGCGG**GGCCTACTAATGTGCCAG

Cell culture and Transfection

Human Epithelial-like Colon adenocarcinoma (colorectal), SW742, NCBI code: C146 (Pasteur institute, Iran) cell line was cultured in Dulbecco's Modified Eagle Media (DMEM, Bioidea, Iran), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G sodium, and 100 µg/ml streptomycin sulfate (Bioidea, Iran) under a humidified condition at 37 °C with 5% CO2. The Calcium phosphate method was used for the transfection of constructs into cell lines.

MTT test

In order to the evaluation the cell survival after overexpression of *miR-374* was performed the MTT assays, and crystal violate analysis. In 96-well plate containing SW742 cells, MTT test were performed with transfection of several amounts of recombinant plasmid containing miR-374 precursor, 1E+02, 1E+01, 1E+00, 1E-01, 1E-02, 1E-03, 1E-04, 1E-05, 1E-06, 1E-07 and 1E-08 ng. Plates were classified into 3 modes (SW-pre-*miR374*, Sw742 empty vector and SW742-Scramble fragment,). The wavelength at 630 nm read using a Bio-Tek Instrument ELISA reader (USA) after passing the incubation time.

Statistical analysis

Relative RT-qPCR data were calculated by using excel 2007, Graphpad prism 8.2.0 tools. The difference between groups was compared by unpaired *t* test and two-way ANOVA GraphPad prism 8.2.0 (435) software. The efficiencies of gene expression were calculated with the LightCycler96 Roche software. P values of less than 0.05 were considered as statistically significant.

Results

The results of bioinformatics analysis with specific software provided a list including 255 miRNAs related to 7 components of the *WNT*-signaling pathway that were chosen based on some criteria such as the miRNA recognition element (MRE sites) and mirSVR scores (Table 3).

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Table 3. Number of miRNAs that target 3'-UTR of components of the WNT-pathway.

Row	Component of *WNT* pathway	Gene ID	Description	Number of miRNAs that target 3'-UTR
1	*APC*	324	*APC* regulator of *WNT* signaling pathway (*Homo sapiens* (human))	74
2	*CTNNB1*	1499	catenin beta 1 (*Homo sapiens* (human))	24
3	*GSK-3B*	2932	glycogen synthase kinase 3 beta (*Homo sapiens* (human))	53
4	*AXIN1*	8312	*axin 1* (*Homo sapiens* (human))	11
5	*HNF4A*	3172	hepatocyte nuclear factor 4 alpha (Homo sapiens (human))	25
6	*CCND1*	595	*cyclin D*1 (*Homo sapiens* (human))	49
7	*MYC*	4609	MYC proto-oncogene, bHLH transcription factor (Homo sapiens (human))	19

Here, we selected *hsa-miR-374*, which simultaneously target several key components of *WNT*-pathway and other components of the main pathways involved in CRC (Table 4). Binding site one of MRE of *hsa-miR-374* were in nucleotide 359-366 at 3'-UTR of *APC* (mirSVR score: -1.1305 and PhastCons score: 0.6718) and nucleotide 4525-4532 at 3'-UTR of *GSK-3B* (mirSVR score: -0.8140 and PhastCons score: 0.5930)**.** Table 5 shows the clinic-pathological data of 48 collected samples.

Table 4: Targeting of main components of CRC pathways by *Hsa-miR-374*a

	The main components of *WNT*/βcatenin pathway of CRC							**The main components of other KEGG pathways of CRC**									
miRNA	*APC*	*CTNNB1*	*GSK-3B*	*AXIN1*	*HNF4A*	*CCND1*	*MYC*	*Akt1*	*Akt3*	*Erbb2*	*Erbb3*	*Foxo1*	*Foxo3*	*CDKN1B*	*TP53*	*NFKB*	*Raf-1*
Hsa-miR-374a	*		*			*	*	*	*	*	*	*	*	*	*	*	*

Table 5: Characteristics of collected clinical samples

Characteristic	Detail	No. of patients	(%) of patients
Total number of patients	CRC	48	100
Gender	Male	24	50
	Female	24	50
Median age, range	≤35	4	8.33
	36-59	13	27.08
	>59	31	64.58
T stage	1	8	16.66
	2	5	10.41
	3	23	47.91
	4	5	10.41
	unknown	7	14.58
N stage	0 and 1	29	60.41
	2	4	8.33
	3 or undefined	15	31.25
Metastasis	Yes	32	66.66
	No	16	33.33
Type of cancer	Adenocarcinoma	48	100
Differentiation	poor	1	2.08
	moderately	13	27.08
	well	18	37.50
	unknown	16	33.33
Tumor site /configuration	colon	32	66.66
	rectum	7	14.58
	sigmoid	9	18.75
	polypoid	8	16.66
	ulcerated	11	22.91
Microscopic diagnosis	high N/C ratio hyper chromatics nucleus mitotic / malignant neoplasm/ atypical epithelial cells	37	77.083
	other	11	22.91

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Evaluation of gene expression in CRC samples using RT-qPCR revealed the down-regulation of *GSK-3β* (0.94 times, p= 0.0098**, t= 2.635, df= 96.00) and *APC* (0.96 times, p= 0.03*, t= 2.088, df= 95.90) and up-regulation of *miR-374* (1.22 times, p= 0.0071**, t= 2.802, df= 53.34) significantly in tumor samples of CRC samples in comparison to the non-tumor or marginal-tumor tissues (Unpaired t test with Welch's correction) (Fig. 1).

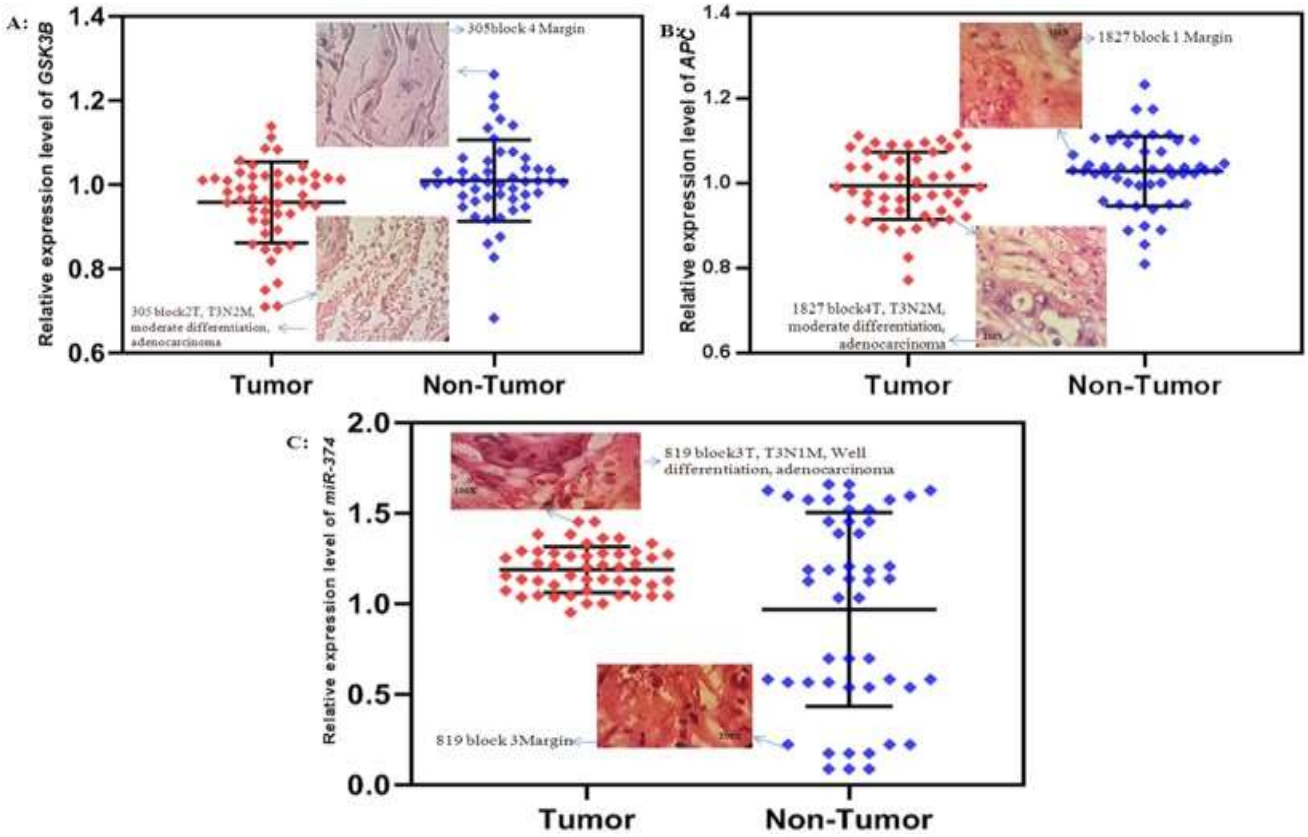

Fig. 1. Evaluation of *GSK-3β* (A), *APC* (B) and *hsa-miR-374* (C) expression levels in clinical samples of CRC by column and heat map graphs (t test data, p value< 0.05). The association between *GSK-3β* (A), *APC* (B) and *hsa-miR-374* (C) expression levels in clinical samples of CRC and clinic-pathological features of three samples (305, 1827 and 819). Microscopic figures of H-E staining in one of tumor and non-tumor sample (100X) have been showed by arrows.

Also, comparison of the expression level of aforementioned genes with clinic-pathological features revealed that down-regulation of *GSK-3β* and *APC* is related to high-grade tumors (T3) and age of patients (age< 51). Also, up-regulation of *miR-374* is related to poor differentiation and lower age of the patient (age> 35). In H-E staining of tumor tissue were observed high nucleus/ cytoplasm (N/C) ratio and infiltration of mononuclear cells compared with margins of tumor (Fig. 1). The results of the cloning process indicated the proper designing of the primers and the correct implementation of the PCR reaction (Fig. 2).

The cloned fragment with the length of 290bp associated with miR precursor was transfected into colorectal cell line. The results of over-expression (transfection with a recombinant vector) showed changes in the expression of the studied genes (Fig. 3). SW742 transfected cells showed up-regulation of *hsa-miR-374* (37.74 folds) and down-regulation of *GSK-3β* (0.68 folds) and *APC* (0.82 folds) than control cultures (Fig. 3).

The results of the MTT indicated that the cell survival by the transfection of the recombinant vector, containing the *miR-374* sequence, was increased. These results indicate a direct relationship between the formation of violet crystals and the decrease of *miR-374* levels (Fig. 4).

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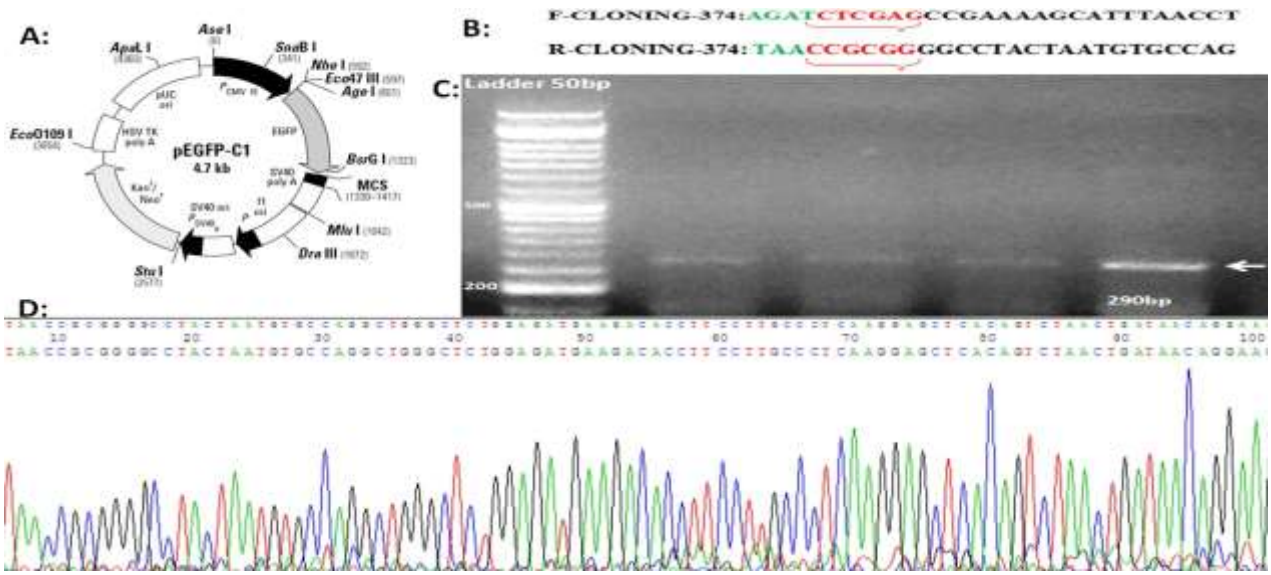

Fig. 2. A, Schematics view of pEGFP-C1 plasmid, B, primer sequences for cloning of precursor *miR-374* into pEGFP-C1. The restriction sites of *XhoI* and *SacII* enzymes have been shown at primer forward and reverse, respectively, C, Electrophoresis results of colony-PCR in positive recombinant colonies including pre *miR-374*, D, Sequencing results of amplicons.

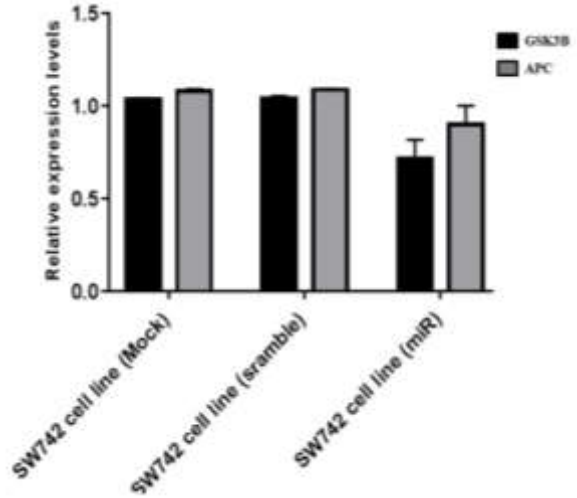

Fig. 3. The expression levels of two target genes down-regulated following *hsa-miR-374* over-expression in SW742 cell lines compared to control groups (p: 0.026**).

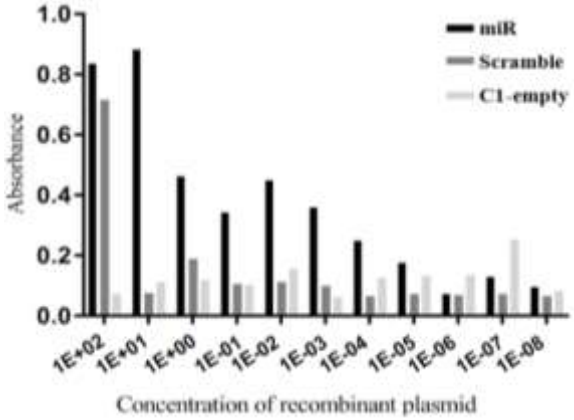

Fig. 4. Result of MTT test in SW742 cells indicated *miR-374* increased viability of cell. miR: transfected with recombinant pEGFP-C1 vector containing miR-374, Scramble: transfected with Scramble fragment, C1 empty: transfected with empty pEGFP-C1 (Two-way ANOVA).

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Discussion

More than 100,000 people annually are estimated to have died from CRC and more than 150,000 were affected by CRC worldwide. According to the Center for Disease Control and Prevention Statistics, CRC is the leading cause of death after lung cancer. The occurrence rate of this disease is increasing so that the incidence has doubled over the past 20 years (17, 18).

The *WNT*-signaling pathway plays a role in key biological processes and controls the stages of development and tissue homeostasis. Changes in the components of this pathway lead to developmental disorders and various cancers. During metastasis, the expression changes of mesenchymal markers and tumor microenvironment (TME) in epithelial cancer cells, leading to the ability to attack adjacent tissues and create high-grade tumors (19). In general, the *Wnt*-signaling pathway has two cononical and non- cononical pathways. The cononical process leads to the accumulation of *β-catenin*. In the absence of *Wnt*, the cytoplasmic stability of *β-catenin* is usually disabled with a complex consisting of scaffolds including *APC* and *GSK-3β* (20). In the presence of *Wnt*, components of these scaffolds were dissociated, *β-catenin* was released, thereinafter entering the nucleus, were expressed the proliferative genes such as *c-myc*, *c-jun* and *cyclineD1* (7). The defects in this pathway are associated with genetic mutations in cancer cells, including CRC, hepatocellular carcinoma and medulloblastoma. Previous studies revealed that *APC* containing mutation becomes unable to form a proper collection with *GSK-3β* and finally degradation of *β-catenin* (21). Aside from non-canonical *Wnt* pathways are classified into three other distinct pathways, *Wnt/JNK*, *Wnt/Ca2+* and *Wnt*/planar cell polarity (PCP). The mechanisms of downstream signal transduction through these three pathways are poorly understood (19). Here, we could detect down-regulation of two components of scaffold surrounding *β-catenin*, including *APC* in tumor tissues of CRC in comparison with non-tumor or marginal tumor tissues. The product of *APC* is a tumor suppressor protein that acts as an antagonist of the *Wnt*-signal transduction pathway. Also, *GSK-3* has two isoforms named *GSK-3α* and *GSK-3β* which expressed a serine/threonine kinase. *GSK-3* has multifaceted roles in several signal transduction pathways in cancer and autophagy-related diseases. Phosphorylation of *GSK-3* by *AKT* and other kinases leads to its inactivation and consequently increased glycogen and protein synthesis. In addition, *GSK-3* interaction with *axin* and *APC* regulated canonical *WNT*-signaling pathway (21). We revealed that *GSK-3β* was downregulated in CRC tumor tissues and probably after that, *β-catenin* was released and activated the proliferated genes in the nucleus.

In recent years, researches have uncovered that change in expression of many miRNAs and their target genes in CRC. For example, in CRC, *hsa-miR-143* specifically inhibits the phosphorylation of *KRAS* kinase. Valeri et al. in 2014 reported that the inhibition of *miR-135b* in mouse models of CRC reduced tumor growth by controlling germ cell proliferation, invasion and apoptosis (5). In their study showed that *miR-30b* was able to suppress rectal tumors through *KRAG*, *PIK3CD* and *BCL2* oncogenes. *miR-135b* affects apoptosis via the *APC*/ *Wnt*/ *β-catenin* signaling pathway. The silencing of *miR-135b* reduced the colony formation in mutated cells with *PI3KCA*.

In this study, *hsa-miR-374* was selected using target prediction tools according to the scoring system of the software, MRE and conservation (22), which simultaneously targets several key components of the *Wnt*-pathway. Several studies revealed that *miR-374* family members have the regulatory role in various cancers, cell growth, differentiation, reproductive disorders and epilepsy (23). The chromosomal locations of members of *MiR-374* family members in humans are at Xq13.2 the X-inactivation center region (Xic). *MiR-374b* was significantly up-regulated in hepatocellular carcinoma tissue (HCC) tissue and associated with grade and metastasis (24). In agreement with their findings, we revealed that *miR-374* was up-regulated in tumor clinical samples compared with non-tumor tissues. There are different conventional methods and new approaches to detect miRNAs

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(25, 26). We could optimize the cost-effective qRT-PCR as an efficient and precise quantitative method. Liao et al. conducted a study in 2014 to examine the relationship between miRNAs and tumor suppression (9). *miR-374a* activates *Wnt*-pathway to promote metastasis of breast cancer (27). it seems there is a logically contradiction between the expression of the *miR-374* and two related targets. These two targets, *APC* and *GSK-3β,* are two key components of the *Wnt* pathway.

It is possible that the down-regulation of these two genes may be due to the up-regulation of *miR-374* in clinical CRC tumor than non-tumor tissues. In addition, our data showed the up-regulation of *miR-374 and* down-regulation of two its target genes have a correlation with a higher grade of the tumor. As shown in our data, the highest point (Fig. 1C) and lowest points of the scatter graph (Fig. 1A) are related to T3 grade of tumor blocks compared with marginal blocks of CRC FFPE tissues. Furthermore, down-regulation of *APC* and *GSK-3β* following precursor of *miR-374* over-expression, indicated proper processing of the precursor molecules to mature miRNA in transfected cell lines and possibly targeting of *APC* and *GSK-3β* transcripts (Fig. 3). Although, several studies have been reported the expression alterations of different miRNAs related to CRC, but so far, the relationship between *miR-374* and the key elements of the *Wnt*-pathway in CRC has not been studied. Aside from, with decreasing the recombinant vector containing *pre-miR-374,* decreases cell viability (Fig. 4). Taken together, possibly *miR-374* conducted increasing in cell viability and proliferation. Therefore, the increase of the expression of this miRNA may be a reason to reduce the expression of these two genes in CRC tumor samples, and possibly the expression of this expression may be therapeutic application in CRC.

Bioinformatics analysis showed *miR-374* is targeting the key components of the *Wnt*-pathway. Performing bioinformatics analysis before implementing laboratory processes is a simple and inexpensive method to reduce the number of targets of miRNAs. Given contradictory expression of *miR-374* in tumor tissues relative to non-tumor samples, it might be considered as an onco-miR and a diagnostic biomarker in high-grade CRC.

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