Decreased expression of miR-874 and its tumor suppressive function in human colorectal cancer

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ABSTRACT. Dysregulation of miRNAs is associated with cancer development and progression. For example, aberrant expression of miR-874 has been found in some types of cancer. However, miR-874 expression and its clinical significance in colorectal cancer (CRC) have not yet been explored. The aim of the current study was to explore the effects of miR-874 in CRC tumorigenesis and development. Quantitative reverse-transcription PCR was performed to evaluate miR-874 levels in CRC cell lines and in 135 pairs of primary human CRC specimens and adjacent noncancerous tissues. The association of miR-874 expression with clinicopathological factors and prognosis was also analyzed. Furthermore, the effects of miR-874 on the biological behavior of CRC cells *in vitro* were investigated. Our results revealed that miR-874 expression was significantly downregulated in CRC cancer tissues and cell lines. Decreased miR-874 expression was significantly associated with larger tumor size, deeper invasion depth, and

advanced TNM stage *in vivo*. Additionally, low miR-874 expression in CRC was an independent predictor of poor survival. Moreover, overexpression of miR-874 inhibited cell proliferation, invasion, and migration, and promoted cell apoptosis of the SW620 CRC cell line *in vitro*. Taken together, these findings indicate that miR-874 may act as a tumor suppressor in CRC, and may serve as a novel therapeutic target for miR-based therapy.

Key words: miR-874; Colorectal cancer; Prognosis; Proliferation; Invasion; Apoptosis

INTRODUCTION

Colorectal cancer (CRC) is the third leading cause of deaths among those caused by all human malignancies (Siegel et al., 2012). Despite significant advances in the management of CRC, the survival of patients with advanced CRC has changed little over the past 10 years. Previous studies have demonstrated diverse genetic alterations in CRC, but the highly complex molecular mechanisms underlying CRC carcinogenesis and progression remain obscure. Therefore, it is necessary to identify novel markers for CRC, which can accurately define the biological characteristics of CRC tumors, improve therapeutic strategies, and predict clinical outcome.

MicroRNAs (miRs) are a class of naturally occurring, small (19-24 nucleotides in length), single-stranded, non-protein-coding RNAs, which suppress translation or promote the degradation of target messenger RNAs (mRNAs) through base pairing with the 3'-translated regions (Bartel, 2004; Bartel, 2009). It has been shown that miRs regulate many biological events including cancer development (Chen, 2005; Cech and Steitz, 2014). Some highly expressed miRs may function as oncogenes by repressing tumor suppressor genes, whereas lowly expressed miRs may function as tumor suppressors by negatively regulating oncogenes. In terms of CRC, abnormal expression of several miRs such as miR-17b, miR-133b, miR-124 have been reported (Ye et al., 2013; Zhang et al., 2013; Xiang and Li, 2014). Additionally, Zhang et al. (2013a) found that ectopic expression of miR-224 promoted CRC tumor cell proliferation, migration, and invasion *in vitro*. Zheng et al. (2014) indicated that downregulation of miR-132 in CRC was associated with tumor size, distant metastasis, and TNM stage. Furthermore, miR-218, miR-378, miR-378a-3p, and miR-378a-5p expressions were identified as independent prognostic factors for CRC (Yu et al., 2013; Li et al., 2014; Zhang et al., 2014a). miR-129 sensitized CRC cells to 5-FU both *in vitro* and *in vivo* (Karaayvaz et al., 2013), and miR-124 increased the radiosensitivity of CRC cells (Zhang et al. 2014b). Taken together, these previous findings suggest that miRs act not only as diagnostic and prognostic markers, but also as potential therapeutic targets of CRC.

miR-874 is located on chromosome 5q31.2, which is a well-known frequent fragile site in the human genome that is often deleted in cancers and genetic disorders, and this site is also specifically correlated with chromosomal rearrangements in cancer (Kesanakurti et al., 2013). miR-874 has been observed to be downregulated in maxillary sinus squamous cell carcinoma (Nohata et al., 2011), gastric cancer Jiang et al., 2014; Zhang et al., 2015), non-small cell lung cancer (Kesanakurti et al., 2013), and breast cancer (Wang et al., 2014), and acts as a candidate tumor suppressor in these tumors. However, the potential role of miR-874 in the regulation of CRC tumorigenesis is still unknown.

In the present study, we examined miR-874 expression in CRC tissues and cell lines. We also investigated the correlation between miR-874 levels and clinicopathological characteristics

and patient survival. Moreover, we explored the role of miR-874 in the regulation of biological behaviors of CRC cells.

MATERIAL AND METHODS

Patients and clinical specimens

This study was approved by the Research Ethics Committee of Wendeng Central Hospital of Weihai. Written informed consent was obtained from all included patients. All specimens were handled and made anonymous according to ethical and legal standards.

A total of 135 pairs of primary CRC and adjacent noncancerous tissues were obtained from patients who underwent surgery at Wendeng Central Hospital of Weihai between February 2009 and January 2011. The diagnoses of all samples were histopathologically confirmed by two pathologists. All specimens were frozen immediately in liquid nitrogen and stored at -80°C until analysis. Patients with two or more different malignancies were excluded. None of the patients had undergone chemotherapy or radiotherapy before surgery. Details of clinical and pathological characteristics of the patients are summarized in Table 1. Follow-up data were available for all patients. Overall survival was defined as the amount of time from the day of primary surgery to the date of death or the end of follow-up (for living patients).

Table 1. Correlation between miR-874 expression and different clinicopathological features in colorectal cancer patients.

Clinicopathological features	No. of cases	miR-874 expression		P value
		Low [N (%)]	High [N (%)]	
Age				
<60	61	33 (54.1%)	28 (45.9%)	0.270
≥60	74	35 (47.3%)	39 (52.7%)	
Gender				
Male	82	44 (53.7%)	38 (46.3%)	0.219
Female	53	24 (45.3%)	29 (54.7%)	
Tumor size				
<5cm	62	22 (35.5%)	40 (64.5%)	0.001
≥5cm	73	46 (63.0%)	27 (37.0%)	
Histology/differentiation				
Well + Moderate	66	30 (45.5%)	36 (54.5%)	0.172
Poor	69	38 (55.1%)	31 (44.9%)	
Depth of invasion				
T1 + T2	66	25 (37.9%)	41 (62.1%)	0.004
T3 + T4	69	43 (62.3%)	26 (37.7%)	
TNM stage				
I or II	58	15 (25.9%)	43 (74.1%)	<0.001
III	77	53 (68.8%)	24 (31.2%)	
Tumor site				
Colon	81	36 (44.4%)	45 (55.6%)	0.114
Rectum	54	32 (59.3%)	22 (40.7%)	

Cell lines and miR transfection

The human CRC cell lines HT29, HCT116, SW480, and SW620, as well as the NCM460 normal colonic epithelial cell line, were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin sulfate. All cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

miR-874 mimics and negative control (miR-NC) were designed and synthesized by GenePharma Co. (Shanghai, China). For RNA transfection, CRC cells were seeded into each well of 24-well plate and incubated overnight, then transfected with miR-874 mimics or miR-NC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. The cells were collected for further experiments 48 h post-transfection.

RNA extraction and quantitative reverse-transcription PCR

Total RNA was extracted from CRC cells and clinical specimens using TRIzol Reagent (Invitrogen). Complementary DNA (cDNA) was synthesized from isolated RNA using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed with a Taqman MicroRNA Assay Kit (Applied Biosystems) on the ABI 7500 real-time PCR detection system. U6 small nuclear RNA was used as an internal control. All reactions were run in triplicates, and the relative amount of miR-874 to U6 was calculated using the equation $2^{-\Delta Ct}$, where $\Delta CT = (CT_{miR-874} - CT_{U6})$.

Cell proliferation assay

The *in vitro* cell proliferation was measured using the MTT method. Briefly, CRC cells were seeded into 96-well plates (approximately 5 x 10³ cells/well) and incubated at 37°C after transfection. At different time points (24, 48, 72, and 96 h), 20 μL MTT solution (5 mg/mL; Sigma, St. Louis, MO, USA) was added into each well and the cells were incubated for another 4 hours. Then, 150 μL dimethyl sulfoxide was added to stop the reaction. The plates were gently shaken on a swing bed for 10 min and the absorbance value/optical density as measured at 490 nm on a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Detection of apoptosis by flow cytometry

The CRC cells were harvested, washed, and resuspended in ice-cold PBS after miR transfection. Then, the cells were treated with propidium iodide (10 mg/mL; Sigma) and an Annexin V-FITC antibody (50 mg/mL, BD Biosciences, San Jose, CA, USA) in the dark for 15 min at room temperature, and examined by flow cytometry (FACScan; BD Biosciences).

Cell invasion and migration assays

Cell invasion and migration assays were performed using 24-well transwell plates (8-mm pore size, Corning, New York, NY, USA). For the migration assay, approximately 1 x 10⁵ CRC cells suspended in serum-free media were added to the upper chamber. The lower chamber contained medium with 10% FBS as a chemoattractant. Following a 48 h-incubation, the cells located on the surface of the lower chamber were stained and counted using a microscope (Olympus Corp., Tokyo, Japan). The invasion assay protocol was similar to the migration assay except that the upper chambers were first covered with Matrigel.

Statistical analysis

All statistical analyses were performed using the SPSS 16.0 software package (SPSS,

Chicago, IL, USA). The differences between groups were assessed by Student *t*-tests and Chi-squared tests. Survival curves were constructed using the Kaplan-Meier method and compared by a log-rank test. The significance of survival variables was evaluated using a multivariate Cox proportional hazards regression analysis. P < 0.05 was considered statistically significant.

RESULTS

Decreased miR-874 expression in CRC tissues and cell lines

We performed a quantitative PCR analysis to detect the expression levels of miR-874 in CRC tissues and cell lines. Our results showed that miR-874 was significantly decreased in CRC tissues (mean ± SD: 7.15 ± 1.88) when compared to that in paired adjacent normal tissues (mean ± SD: 21.36 ± 4.51) (P < 0.01; Figure 1A). The miR-874 expression in four CRC cell lines (HT-29, HCT116, SW620, and SW480) was also significantly downregulated compared to that in the NCM460 normal colonic epithelial cell line (Figure 1B). The SW620 cell line, which possessed the lowest level of miR-874 expression among all tested cell lines, was selected for further studies.

Figure 1. Relative expression levels of miR-874 in colorectal cancer (CRC) tissues and cell lines. **A.** miR-874 expression was significantly lower in CRC tissues than that in the corresponding noncancerous tissues. miR-874 expression levels were calculated by the $2^{-\Delta Ct}$ method and normalized to U6 small nuclear RNA. **B.** miR-874 expression was downregulated in the CRC cell lines HT29, HCT116, SW620, and SW480 compared to that in the human normal colonic epithelial cell line NCM460. *P < 0.05; **P < 0.01.

Correlation between miR-874 expression and clinical features and prognosis of CRC patients

To evaluate the correlation between miR-874 expression and clinicopathological characteristics, the 135 CRC patients were classified into two groups (low or high) according to whether they had lower or higher expression than the median expression value of miR-874. We then compared the clinicopathological characteristics of the low and high miR-874 expression groups (Table 1) and found that low expression of miR-874 was significantly correlated with larger tumor size (P = 0.001), deeper invasion depth (P = 0.004), and advanced TNM stage (P < 0.001).

The Kaplan-Meier survival analysis showed that low miR-874 expression correlated with shorter overall survival (Figure 2). A univariate proportional hazard model also revealed a statistically significant correlation between overall survival and tumor size (P = 0.026), local

invasion (P = 0.035), and TNM stage (P < 0.001; Table 2). A multivariate Cox regression analysis including the aforementioned parameters revealed that miR-874 expression [relative risk (RR) = 3.658; P = 0.008] and TNM stage (RR = 5.344; P = 0.001) were independent prognostic markers for overall survival of CRC patients (Table 2).
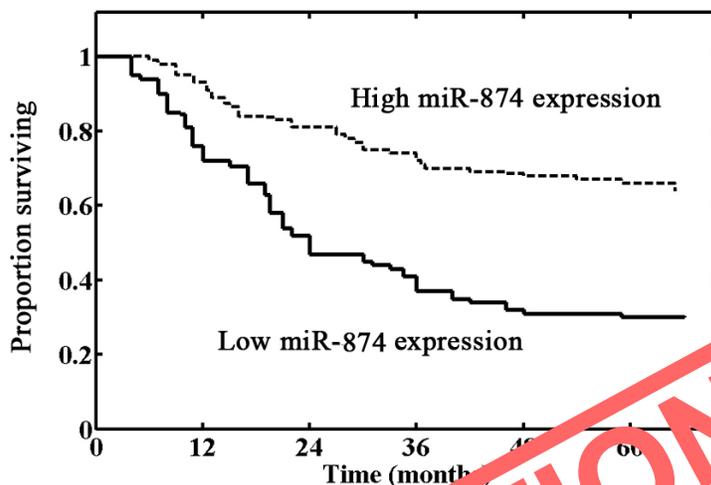

Figure 2. Kaplan-Meier survival curves for CRC patients based on miR-874 expression. Patients in the low expression group had significantly poorer prognosis than those in the high expression group (P < 0.001, log-rank test).

Table 2. Univariate and multivariate analyses of prognostic factors in colorectal cancer.

Variable	Univariate analysis		Multivariate analysis	
	HR	P value	HR	P value
Age (years)	1.743	0.132	-	-
Gender	1.246	0.745	-	-
Histological grading	1.811	0.186	-	-
Tumor size	2.732	0.026	1.825	0.082
Depth of invasion	2.318	0.035	1.413	0.225
Tumor site	1.321	0.612	-	-
Clinical stage	5.985	<0.001	5.344	0.001
MiR-874 expression	4.165	<0.001	3.658	0.008

Effects of miR-874 on CRC cell proliferation, apoptosis, invasion, and migration *in vitro*

Next, we assessed the biological role of miR-874 in SW620 cells. As shown in Figure 3A, the expression level of miR-874 in miR-874 mimic transfected cells was significantly higher compared with that of NC transfected cells (P < 0.001). The MTT assay showed that cell proliferation was significantly impaired after miR-874 mimic transfection compared to that of controls (Figure 3B).

We also observed an increase in cell apoptosis in miR-874 mimic transfected cells compared to that in controls (Figure 3C). Transwell invasion and migration assays were performed to investigate whether miR-874 had a direct influence on SW620 cell migration and invasion. As shown in Figure 3D and E, miR-874 mimic transfection impeded cell invasion and migration compared with those of controls.
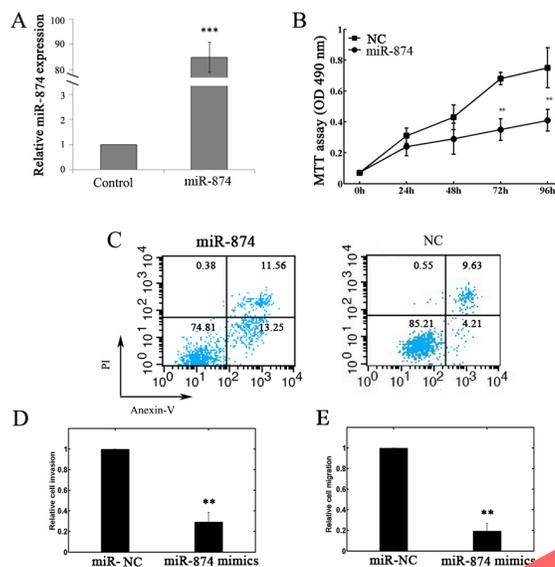
Figure 3. Effects of miR-874 on the biological behaviors of SW620 cells. **A.** The expression level of miR-874 in miR-874 mimic transfected cells was significantly higher compared with that of negative control (NC)-transfected cells. ***P < 0.001. **B.** Cell proliferation was measured by MTT assays in SW620 cells transfected with miR-874 mimics or NC. **P < 0.01. **C.** Apoptosis of SW620 cells was detected by flow cytometric analysis after transfection with miR-874 mimics or NC. **D. E.** miR-874 suppressed SW620 cell invasion and migration *in vitro*. The transwell invasion and migration assays showed that the number of invaded or migrated cells was significantly lower in the miR-874-transfected group than those in the NC-transfected group. **P < 0.01.

DISCUSSION

Dysregulation of miRs has been demonstrated to be involved in tumorigenesis and progression in various types of cancers; however, the elucidation of their potential roles in CRC remains in its early stages. In the present study, we found that miR-874 expression was significantly downregulated in CRC tissue samples and cell lines, and that the loss of miR-874 expression was associated with larger tumor size, deeper invasion depth, advanced clinical stage, and shorter overall survival, which indicates that miR-874 may be involved in CRC progression and could be used as a potential prognostic biomarker. Furthermore, the forced overexpression of miR-874 significantly inhibited CRC cell proliferation, invasion, and migration, as well as promoted cell apoptosis *in vitro*. To our knowledge, this is the first time that it has been shown that miR-874 may function as a tumor suppressor in CRC.

Previous research has revealed dysregulated miR-874 expression and its tumor suppressive function in many human malignancies. *In vitro*, ectopic miR-874 expression suppressed tumor cell proliferation and promoted cell apoptosis in head and neck squamous cell carcinoma (Nohata et al., 2013), breast cancer (Wang et al., 2014), and gastric cancer (Jiang et al., 2014). Upregulation of miR-874 also reduced the cell-invading ability of non-small cell lung cancer and maxillary sinus squamous cell carcinoma cells (Nohata et al., 2011; Kesanakurti et al., 2013). *In vivo*, Jiang et al. (2014) found that there was decreased miR-874 expression, which correlated with poor tumor differentiation, lymph node metastasis, and advanced tumor stage in patients

with gastric cancer. Furthermore, miR-874 overexpression significantly suppressed *in vivo* tumor growth and angiogenesis in nude mouse models (Kesanakurti et al., 2013; Jiang et al., 2014; Zhang et al., 2015). Taken together, these findings suggest that miR-874 may play an important role not only in tumor initiation and progression, but may also be a molecular target for therapies for human malignancies.

It is now understood that miRs execute their oncogenic or tumor suppressive functions by regulating the expression of target genes (Liu et al., 2014). With regard to miR-874, several targets have been reported including PPP1CA (Nohata et al., 2011), AQP3 (Jiang et al., 2014), HDAC1 (Nohata et al., 2013), MMP-2 (Kesanakurti et al., 2013), CDK9 (Wang et al., 2014), and STAT3 (Zhang et al., 2015). However, it is predicted that an average miR can have more than 100 targets (Brennecke et al., 2005), and more than one miR can converge on a single transcript target (Krek et al., 2005). Therefore, how miR-874 affects cancer progression accurately is still not well understood, and thus the corroboration of the molecular targets/characteristics of miR-874 remains an important facet in future investigations.

In summary, the present study showed that miR-874 was downregulated in CRC tissues and cell lines. Additionally, this low expression pattern was found to be significantly correlated with aggressive clinicopathological features and worse prognosis. Furthermore, *in vivo* functional assays demonstrated that miR-874 inhibited CRC cell growth and invasion. Together, our data implicate the potential application of miR-874 as a tumor suppressor in CRC therapy, and also as a tumor marker for predicting prognosis.

Conflicts of interest

The authors declare no conflict of interest.

REFERENCES

Bartel DP (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116: 281-297. http://dx.doi.org/10.1016/S0092-8674(04)00045-5

Bartel DP (2009). MicroRNAs: target recognition and regulatory functions. *Cell* 136: 215-233. http://dx.doi.org/10.1016/j.cell.2009.01.002

Brennecke J, Stark A, Russell RB and Cohen SM (2005). Principles of microRNA-target recognition. *PLoS Biol.* 3: e85. http://dx.doi.org/10.1371/journal.pbio.0030085

Cech TR and Steitz JA (2014). The noncoding RNA revolution-trashing old rules to forge new ones. *Cell* 157: 77-94. http://dx.doi.org/10.1016/j.cell.2014.03.008

Chen CZ (2005). MicroRNAs as oncogenes and tumor suppressors. *N. Engl. J. Med.* 353: 1768-1771. http://dx.doi.org/10.1056/NEJMp058190

Jiang B, Li Z, Zhang W, Wang H, et al. (2014). miR-874 Inhibits cell proliferation, migration and invasion through targeting aquaporin-3 in gastric cancer. *J. Gastroenterol.* 49: 1011-1025. http://dx.doi.org/10.1007/s00535-013-0851-9

Karaayvaz M, Zhai H and Ju J (2013). miR-129 promotes apoptosis and enhances chemosensitivity to 5-fluorouracil in colorectal cancer. *Cell Death Dis.* 4: e659. http://dx.doi.org/10.1038/cddis.2013.193

Kesanakurti D, Maddirela DR, Chittivelu S, Rao JS, et al. (2013). Suppression of tumor cell invasiveness and in vivo tumor growth by microRNA-874 in non-small cell lung cancer. *Biochem. Biophys. Res. Commun.* 434: 627-633. http://dx.doi.org/10.1016/j.bbrc.2013.03.132

Krek A, Grün D, Poy MN, Wolf R, et al. (2005). Combinatorial microRNA target predictions. *Nat. Genet.* 37: 495-500. http://dx.doi.org/10.1038/ng1536

Li H, Dai S, Zhen T, Shi H, et al. (2014). Clinical and biological significance of miR-378a-3p and miR-378a-5p in colorectal cancer. *Eur. J. Cancer* 50: 1207-1221. http://dx.doi.org/10.1016/j.ejca.2013.12.010

Liu GF, Tang D, Li P, Wang S, et al. (2014). S-1-based combination therapy vs S-1 monotherapy in advanced gastric cancer: a meta-analysis. *World J. Gastroenterol.* 20: 310-318. http://dx.doi.org/10.3748/wjg.v20.i1.310

Nohata N, Hanazawa T, Kikkawa N, Sakurai D, et al. (2011). Tumour suppressive microRNA-874 regulates novel cancer networks in maxillary sinus squamous cell carcinoma. *Br. J. Cancer* 105: 833-841. http://dx.doi.org/10.1038/bjc.2011.311

Nohata N, Hanazawa T, Kinoshita T, Inamine A, et al. (2013). Tumour-suppressive microRNA-874 contributes to cell proliferation through targeting of histone deacetylase 1 in head and neck squamous cell carcinoma. *Br. J. Cancer* 108: 1648-1658. http://dx.doi.org/10.1038/bjc.2013.122

Siegel R, Naishadham D and Jemal A (2012). Cancer statistics, 2012. *CA Cancer J. Clin.* 62: 10-29. http://dx.doi.org/10.3322/caac.20138

Wang L, Gao W, Hu F, Xu Z, et al. (2014). MicroRNA-874 inhibits cell proliferation and induces apoptosis in human breast cancer by targeting CDK9. *FEBS Lett.* 588: 4527-4535. http://dx.doi.org/10.1016/j.febslet.2014.09.035

Xiang KM and Li XR (2014). MiR-133b acts as a tumor suppressor and negatively regulates TBPL1 in colorectal cancer cells. *Asian Pac. J. Cancer Prev.* 15: 3767-3772. http://dx.doi.org/10.7314/APJCP.2014.15.8.3767

Ye J, Wu X, Wu D, Wu P, et al. (2013). miRNA-27b targets vascular endothelial growth factor C to inhibit tumor progression and angiogenesis in colorectal cancer. *PLoS One* 8: e60687. http://dx.doi.org/10.1371/journal.pone.0060687

Yu H, Gao G, Jiang L, Guo L, et al. (2013). Decreased expression of miR-218 is associated with poor prognosis in patients with colorectal cancer. *Int. J. Clin. Exp. Pathol.* 6: 2904-2911.

Zhang GJ, Zhou H, Xiao HX, Li Y, et al. (2013a). Up-regulation of miR-224 promotes cancer cell proliferation and invasion and predicts relapse of colorectal cancer. *Cancer Cell Int.* 13: 104. http://dx.doi.org/10.1186/1475-2867-13-104

Zhang GJ, Zhou H, Xiao HX, Li Y, et al. (2014a). MiR-378 is an independent prognostic factor and inhibits cell growth and invasion in colorectal cancer. *BMC Cancer* 14: 109. http://dx.doi.org/10.1186/1471-2407-14-109

Zhang J, Lu Y, Yue X, Li H, et al. (2013b). MiR-124 suppresses growth of human colorectal cancer by inhibiting STAT3. *PLoS One* 8: e70300. http://dx.doi.org/10.1371/journal.pone.0070300

Zhang X, Tang J, Zhi X, Xie K, et al. (2015). miR-874 functions as a tumor suppressor by inhibiting angiogenesis through STAT3/VEGF-A pathway in gastric cancer. *Oncotarget* 6: 1605-1617. http://dx.doi.org/10.18632/oncotarget.2748

Zhang Y, Zheng L, Huang J, Gao F, et al. (2014b). MiR-124 Radiosensitizes human colorectal cancer cells by targeting PRRX1. *PLoS One* 9: e93917. http://dx.doi.org/10.1371/journal.pone.0093917

Zheng YB, Luo HP, Shi Q, Hao ZN, et al. (2014). miR-132 inhibits colorectal cancer invasion and metastasis via directly targeting ZEB2. *World J. Gastroenterol.* 20: 6515-6522. http://dx.doi.org/10.3748/wjg.v20.i21.6515