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Asia-Pacific Journal of Blood Types and Genes

2017, 1(3):33-37

APJBG

miR–34a is associated with clinical outcome of colorectal cancer patients

Zhidan Tian[1], Yongya Ren[2], Xiaohong Qi[2*]

[1]*Deptartmen of Pathology, Nanjing Hospital Affiliated to Nanjing Medical University, Nanjing, Jiangsu, 210006, China;*

[2]*Deptartment of Pathology and Pathophysiology, Nanjing Medical University, Nanjing, Jiangsu, 211166, China*

ABSTRACT

Aberrant expression of microRNA–34a (miR–34a) has been reported to be involved in the tumorigenesis and progression of various classes of malignancies. However, its role in colorectal cancer (CRC) has not been completely clarified. In the current study, we have investigated the clinical significance of miR–34a. MiR–34a expression in forty–three cases of colorectal cancer tissues decreased significantly compared to that in the adjacent non–tumorous colorectal tissues ($P < 0.05$), as detected by real–time quantitative RT–PCR (qRT–PCR). Significantly, the expression of miR–34a was correlated with infiltration depth and clinical TNM stage ($P < 0.05$). The miR–34a however had no correlation with other features, such as age, gender, site, tumor sizes, lymph node metastasis, serous membrane infiltration (all $P > 0.05$). MiR–34a is a tumor suppressor miRNA that plays a vital role in the oncogenesis and progression of colorectal cancer. This study suggests that miR–34a may be a new tumor marker or prognostic factor in colorectal cancer. The strategies to increase miR–34a level might be a critical targeted therapy for CRC in the future.

Keywords: miR–34a, colorectal cancer, TNM stage, qRT–PCR

INTRODUCTION

MicroRNAs (miRNAs), a new class of 21–25 nucleotides noncoding and single–stranded RNAs, were recently discovered in both animals and plants. They trigger translational repression and/or mRNA degradation mostly through complementary binding to the 30–untranslated regions of target mRNAs. Studies have shown that miRNAs can regulate a wide array of biological processes such as cell proliferation, differentiation, and apoptosis. Accumulating evidence suggests that alterations of miRNAs expression may play various roles in the pathogenesis of many human cancer[1–4].

Recently, miR–34a has been demonstrated to be a direct transcriptional target of p53 and it is commonly deleted in various types of cancers. Decreased expression of miR–34a is partly due to the inactivating mutations of p53 in tumors. MiR–34a was found to be deregulated in numerous human tumors, including pancreatic cancer, breast cancer, and non–small cell lung cancer. However, the precise, critical roles of miR–34a in colorectal cancers largely remain to be elucidated. We investigated the expression levels of miR–34a in human colorectal cancers and have sought to clarify whether miR–34a are truly associated with clinicopathological features of colorectal cancer. Their possible role in human colorectal cancer development is discussed below.

MATERIALS AND METHODS

Patients and tissue samples

Colorectal cancer tissues and adjacent non–tumorous colorectal tissues were collected from 43 color–

*Correspondence to: Xiaohong Qi, Deptartment of Pathology and Pathophysiology, Nanjing Medical University, Nanjing, Jiangsu, 211166, China. TEL: +86–25–86869331; E–mail: qxh@126.com.

The authors declared no conflict of interests.

ectal cancer patients who underwent hepatectomy between 2006 and 2009 at the Nanjing Drum Tower Hospital and Shanghai Meishan Hospital after informed consent and verification by a pathologist. The hard and firm tumor tissues were trimmed free of normal tissue and snap frozen in liquid nitrogen immediately after resection. No patient in the current study received chemotherapy or radiation therapy before the surgery. The group consisted of 19 females and 24 males, with a median age of 54 years (range 38–93 years). The distribution according to the clinical TNM stage was as follows: stage Ⅰ in 4 cases, stage Ⅱ in 15 cases, stage Ⅲ and stage Ⅳ in 24 cases. There were 22 cases of lymph node metastasis, 21 cases without lymph node metastasis.

All reagents were purchased from commercial biomedical suppliers and were used without further purification. RNA isolation reagents, such as isopropanol, ethanol, diethyl pyrocarbonate (DEPC) water, RNase–free plastic reaction vials, RNase inhibitors and disposable mini–homogenizers were purchased from Invitrogen(USA) or Ambion(USA) as previously described. pMD–18T vector and rTaq were purchased from TaKaRa(Japan). *E.coli* TG1 host was from preservation of laboratory.

RNA extraction and qRT–PCR

Total RNA was extracted from the patients´ colorectal samples using TRIzol (Invitrogen, USA) according to the manufacturer´s protocol. RT–PCR was used to confirm the expression levels of mRNAs and miRNAs. For mRNAs detection, RT–PCR was performed according to the protocol of ImProm–Ⅱ™ Reverse Transcriptase System (Promega, USA). MiR–34a primers ***(Table 1)*** were designed by Primer3 software and synthesized by Invitrogen (Shanghai, China). The optimal annealing temperature of Bio–Rad Gradient PCR for miR–34a and U6 was 60℃.

Table 1 **Primers for miR–34a and U6**

miRNA	Primers
U6	Forward: 5´–CTCGCTTCGGCAGCACA–3´
	Reverse: 5´–AACGCTTCACGAATTTGCGT–3´
miR–34a	Forward: 5´–CTCAACTGGTGTCGTGGAGTCGGCAA TTCAGTTGAGAACAACCA–3´
	Reverse: 5´–ACACTCCAGCTGGGTGGCAGTGTCTT AGCTG–3´
URP	5´–TGGTGTCGTGGAGTCG–3´

qRT–PCR was performed on the ABI 7500 fluorescent sequence detection system (Perkin–Elmer, USA). Comparative qRT–PCR was done in triplicate, including no–template controls. Briefly, after reverse transcription of 50 ng of total–RNA, cDNA was generated. The PCR reaction consisted of 22 cycles (95℃ for 15 sec, 60℃ for 30 sec) after an initial denaturation step (95℃ for 3 min). U6 was used as an internal control. Fold changes were determined using the equation $2^{-\Delta\Delta C_T}$ relative to matched reference sample. The data were then transformed to $\log_2$ values.

Statistical analysis

Data are expressed as mean±standard error (SE). Student´s *t* test was applied to analyze the differences between the groups. Statistical analysis was performed with SPSS software (version 13). Only $P < 0.05$ were considered statistically significant.

RESULTS

miR–34a are expressed in colorectal cancer tissues

The expression levels of miR–34a and U6 in tumors and the paired adjacent non–tumorous colorectal tissues by RT–PCR are showed in ***Fig.1***. The expression levels of miR–34a in tumors were significantly decreased compared with those in the paired adjacent non–tumorous colorectal tissues in 43 cases of colorectal cancer patients. The expression levels of miR–34a in adjacent non–tumorous colorectal tissues is 0.38 times as in tumors ($P < 0.05$). The expression levels of miR–34a, the amplification curve and melting curve of qRT–PCR are showed in ***Fig.2–4***.

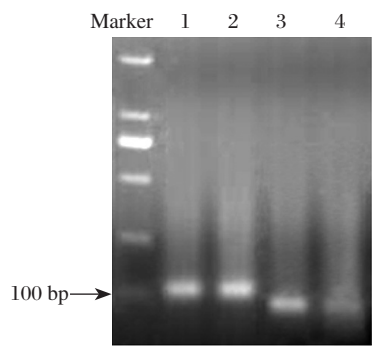

Fig.1. **The relative expression of miR–34a in colorectal cancer tissues and adjacent non–tumorous colorectal tissues determined by RT–PCR.** Lane 1 and lane 2 are expression of U6 in adjacent non–tumorous colorectal tissues and colorectal cancer tissues; lane 3 and lane 4 are expression of miR–34a in adjacent non–tumorous colorectal tissues and colorectal cancer tissues.

The relationship between the expression of miR–34a and the clinical characteristics

In tumors which did not show infiltrated serosa compared with those in the paired adjacent non–tumorous colorectal tissues, there were 4 cases (25.0%) with low expression. In infiltrated serosa cases, there

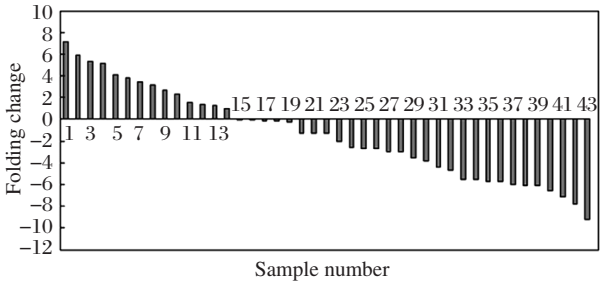

Fig. 2. **The expression of miR−34a detected by qRT−PCR.** The folding changes of colorectal cancer tissues and adjacent non−tumorous colorectal tissues in 43 cases.

were 25 cases (92.6%) with low expression. There were no colorectal tissues in TNM phase Ⅰ in which the expression of miR−34a was low expression, 4 cases (33.3%) in TNM phase Ⅱ showed low expression, 25 cases (92.6%) in TNM phase Ⅲ showed low expres−sion. Additionally, as shown in ***Table 2***, the expression of miR−34a was significantly correlated with infiltra−tion depth and clinical TNM stage (P <0.05). The miR−34a however had no correlation with other features, such as age, gender, site, tumor sizes, lymph node me−tastasis, serous membrane infiltration (P > 0.05).

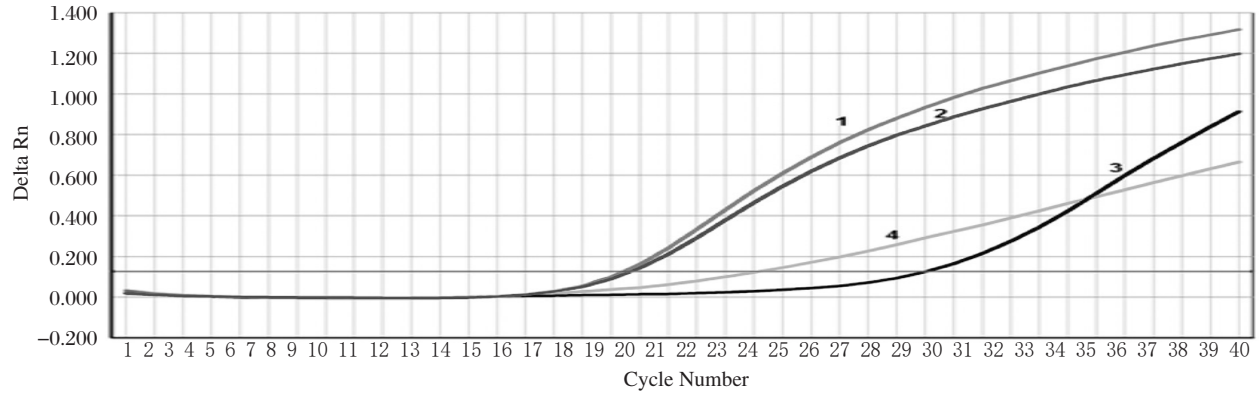

Fig. 3. **Amplification curve of qRT−PCR.** 1 and 2 are amplification curves of U6 in adjacent non−tumorous colorectal tissues and colorectal cancer tissues; 3 and 4 are amplification curves of miR−34a in adjacent non−tumorous colorectal tissues and colorectal cancer tissues, respectively.

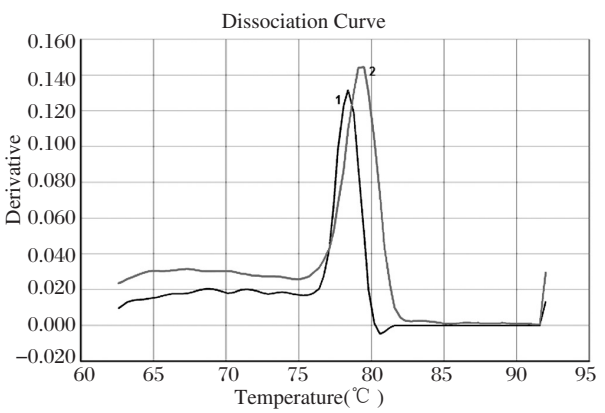

Fig. 4. **Melting curve of qRT−PCR.** 1: melting curve of U6 in adjacent non−tumorous colorectal tissue and colorec−tal cancer tissue; 2: melting curve of miR−34a in adjacent non−tumorous colorectal tissue and colorectal cancer tissue.

Table 2 **Sequences of single target−siRNA and dual−siRNA**

Clinicopathological Pa−rameters	Cases(n)	miR−34a relavant ex−pression (mean±SE)	P
Gender			0.29
Male	24	2.01±4.27	
Female	19	0.65±4.03	
Age (years)			0.96
＜60	16	1.36±3.69	
≥60	27	1.43±4.50	
Site			0.45
Colorectal	24	1.82±4.42	
Rectum	19	0.84±3.85	
Tumor diameter(cm)			0.10
≤3	16	0.03±3.97	
＞3	27	2.22±4.14	
Infiltration depth			0.01
Not infiltrated serosa	16	−0.58±4.43	
Infiltrate to serosa	27	2.58±3.50	
Clinical TNM stage			0.03
Ⅰ	4	−3.64±3.60	
Ⅱ	12	1.29±3.22	
Ⅲ、Ⅳ	27	2.32±4.29	
Lymph node metastasis			0.07
No	21	0.24±4.09	
Yes	22	2.52±4.03	

DISCUSSION

MicroRNAs (miRNAs), a new class of 21−25 nu−cleotides noncoding and single−stranded RNAs, were recently discovered in both animals and plants. They trigger translational repression and/or mRNA degra−dation mostly through complementary binding to the 30−untranslated regions of target mRNAs. Studies have shown that miRNAs can widely regulate biolog−ical processes such as cell proliferation, differentia−tion, and apoptosis[1−4]. Recently, abnormal expressed miRNAs have been suggested to be associated with various human disorders, including cancer, such as

leukemias, lung and breast cancers. Furthermore, it has been shown that miRNAs can function as tumor suppressors or oncogenes, and repress the expression of important cancer−related genes, and therefore might prove useful biomarkers in the diagnosis and treatment of cancer [5−8].

Colorectal cancer is one of the most common forms of cancer in digestive tract, and the incidence has been an increasing trend in recent years. The occurrence and development of colorectal cancer are closely correlated with oncogene activation, anti−oncogene mutation and cell cycle regulation. But the pathogenesis of colorectal cancer is not clear[9−11]. Recently, aberrant up− and down−regulation of miRNA species in human colorectal cancers has been reported, some miRNAs in tumors were significantly decreased, such as miR−143, miR−145, let−7[12−14]; some miRNAs in colorectal cancers were significantly increased, such as miR−31, miR−21[15,16].

MiR−34a is located on human chromosome 1p36.23, and hemizygous deletion of chromosome 1p36.23 occurs in 30% of advanced stage tumors. Recently, miR−34a has been demonstrated to be a direct transcriptional target of p53 and it is commonly deleted in various types of cancers. Decreased expression of miR−34a is partly due to the inactivating mutations of p53 in tumors[17]. The biological targets of miR−34a have been recently identified and miR−34a induces G1 arrest, apoptosis, and senescence by regulation of critical cell cycle motors or apoptosis inhibitors including CDK4/6, cyclin E2, cyclin D1, E2F3, Bcl−2, and MYCN. Studies have shown that miR−34a can regulate cell cycle and inhibit cell growth [18−20]. MiR−34a has been found to be deregulated in numerous human tumors, including pancreatic cancer, breast cancer, non−small cell lung cancer, but it is controversial in colorectal cancer[21−22]. In our study, we analyzed 43 samples of colorectal cancer, including 24 cases of colon cancer and 19 of rectal cancer, and their adjacent non−tumorous colorectal tissues to identify the expression of miR−34a. The results showed that miR−34a was down−regulated in the majority of colorectal cancer compared with adjacent non−tumorous colorectal tissues. The expression of miR−34a was significantly correlated with infiltration depth and clinical TNM stage ($P < 0.05$). The miR−34a however had no correlation with other features, such as age, gender, site, tumor sizes, lymph node metastasis, and serous membrane infiltration ($P > 0.05$). These observations imply that the miR−34a may play a certain role in the development of colorectal cancer and may promote invasion of colorectal cancer cells.

MiR−34a was also studied functionally *in vitro* in human colon cancer cell lines. Tazawa *et al*. transfected seven miRNAs (miR−16, −34a, −34b, −34c, −146, −147, and −205) into HCT116 and RKO cells, and found that the expression of miR−34a was very low, while a marked induction of miR−34a was detected after p53 activation. The introduction of miR−34a caused a remarkable inhibition of cell proliferation in both HCT116 and RKO cells compared with that of control miRNA[21]. MiR−34a is a direct transcriptional target of p53, which is a transcription factor that coordinates cellular responses to stresses such as DNA damage and oncogene activation. Additionally, the p53 binding site is located within the gene encoding miR−34a. Recent studies have reported that miR−34a can be activated by p53 and inhibit the expression of E2F, c−myc, CDK4, cyclin D1, cyclin E2, to make the tumor cells stagnate in G1 phase, thereby inhibiting tumor cell growth and promoting tumor cell apoptosis [21]. Survivin is the strongest inhibitor of apoptosis, mainly expressed in the cell cycle G2/M phase, which regulates the cell cycle and promotes cell proliferation[22]. Survivin negatively interferes with apoptosis, supposedly due to their ability to inhibit a limited number of caspases and interfere the function of p53 [23,24]. Cyclin D1 is an important member in cyclin family involved in cell cycle regulation, specifically regulating the G1−S transition by pRb. Cyclin D1 plays an important role in the cell proliferation process[25]. A study of Sun *et al*. [18] has shown that miR−34a can regulate cyclin D1 genes. Recently, researches have reported that survivin and cyclin D1 are up−regulated in colorectal cancer and have a synergistic relationship[26]. Our previous study reported that miR−34a is down−regulated in colorectal cancer, together with previous reports show that miR−34a may regulate survivin and cyclin D1 by p53 in colorectal cancer. Survivin may interfere with the function of p53, so as p53 can not decrease the activation of expression of miR−34a. Therefore, the expression of cyclin D1 is up−regulated, and cyclin D1 promoted tumor cell growth and proliferation.

In conclusion, the current observations strongly proved that miR−34a is a tumor suppressor miRNA that plays a vital role in the oncogenesis and progression of colorectal cancer by targeting multiple pathways. The study suggests that miR−34a may be a new tumor marker or prognostic factor in colorectal cancer. The strategies to increase miR−34a level might be a critical targeted therapy for colorectal cancer in the future.

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(Received 09 August 2017, Revised 28 August 2017, Accepted 11 September 2017)