

## RESEARCH ARTICLE

# Expression and Clinical Significance of miRNA-34a in Colorectal Cancer

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### Abstract

**Background:** The aim of this study was to investigate differences of miRNA-34a expression in benign and malignant colorectal lesions. **Materials and Methods:** Samples of cancer, paraneoplastic tissues and polyps were selected and total RNA was extracted by conventional methods for real-time PCR to detect the miRNA-34a expression. In addition, the LOVO colorectal cancer cell line was cultured, treated with the demethylating agent 5-azacytidine and screened for differentially expressed miRNA-34a. **Results:** After the drug treatment, the miRNA-34a expression of colorectal cancer cell line LOVO was increased and real-time PCR showed that levels of expression in both cell line and colorectal cancer tissues were low, as compared to paraneoplastic tissue ( $p < 0.05$ ). Polyps tissues had significantly higher expression than paraneoplastic and colorectal cancer samples ( $p < 0.05$ ). **Conclusions:** miRNA-34a-5p may play a role as a tumor suppressor gene in colorectal cancer, with involvement of DNA methylation.

**Keywords:** miRNA-34a-5p - colorectal cancer - DNA methylation

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### Introduction

In recent years, with the accelerated pace of life, increased pressure, and westernized diet habit, the prevalence of colorectal cancer had increased year by year, and the trend was increasing every year. With large increase rate, there would be about 50 million patients died of colorectal cancer each year, which was the 3rd highest reason of cancer death, thus it had become a malignancy that seriously threatened the people's lives and health. The occurrence of colorectal cancer was a polygenic disease, when the genes were changed, it would lead to the tumor growth. The DNA methylation was considered to be an important cause of colorectal cancer (Kumar et al., 2009); while the roles of miRNA regulation were also increasingly subject to the researchers' attention (Schimasnski et al., 2009), in which the mutual regulation of DNA methylation and miRNA had become a hot research spot of colorectal cancer (Bao et al., 2004; Weber et al., 2007; Grady et al., 2008). Research suggests that relying solely on miRNA detection and can not be used as basis for determining the diagnosis of colorectal cancer, but it can provide a theoretical basis for the incidence of colorectal cancer, provide targets for future therapy (Corte et al., 2012; Giraldez et al., 2013; Zhou et al., 2013). This study started from the mutual relationships between DNA methylation and miRNA, aiming to find

the new mechanism towards the occurrence of colorectal cancer from the complex gene regulatory networks, thus providing new theoretical and experimental data for the diagnosis and treatment of colorectal cancer (Vire et al., 2006; Kim et al., 2008; Vilkin et al., 2009). The studies had shown that the imbalance of miRNA-34a-5p expression could lead to the occurrence of a variety of tumors in vivo, such as prostate cancer and breast cancer, etc (Guessous et al., 2010; Liu et al., 2011; Vinall et al., 2012), while there was few report about the roles of miRNA-34a-5p in colorectal cancer, so how exactly were the roles of miRNA-34a-5p in colorectal cancer? And whether it was closely related to DNA methylation, this study was designed to explore their roles in the development of colorectal cancer.

### Materials and Methods

#### Cell

The colorectal cancer cell line LOVO was purchased from Cell Bank of China Academy of Medical Sciences, Shanghai. After removed from the dry ice reserving box, the cells were rapidly recovered and seeded, with the density as  $5 \times 10^5$ /ml, into the RPMI1640 medium (containing 10% fetal bovine serum (FBS), 100 U/ml streptomycin and 100 U/ml penicillin), the cells were then cultured with the wall-adherence culture method,

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and placed in an incubator with 37°C humidity and 5% CO<sub>2</sub>, the medium was changed every 1-2 d to keep the cell density maintained within 1×10<sup>5</sup>/ml-1×10<sup>6</sup>/ml, and the cells in the logarithmic growing phase were taken for the experiment.

#### Tissue samples

The patients, treated in the department of digestive endoscopy, First Affiliated Hospital of Harbin Medical University, from August 2013 to November 2013, were selected, among whom 15 cases were colorectal polyps (11 males, with the average age as 52 years old, and 4 females, with the average age as 64 years old). Among these 15 cases, 8 cases were of inflammatory polyps, and 7 cases were of adenomatous polyps; 15 cases were colorectal cancer (12 males, with the mean age as 61 years old, and 3 females, with the average age as 67 years old). This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Harbin Medical University. Written informed consent was obtained from all participants. The pathological evidence confirmed all these cases were the adenocarcinoma, the paraneoplastic tissues were taken from the visually normal intestinal wall tissues that were 5 cm away from the tumors, and each sample was taken 2 specimens, about 0.7 cm size. The taken specimens were immediately cut into pieces, then put into the lyophilized tube and stored at -80°C.

#### Gene chips

The colorectal cancer cell line LOVO was performed the microarray analysis of miRNA and mRNA determination before and after the drug treatment, the miRNA chips were used to extract 1×10<sup>6</sup>-1×10<sup>7</sup> cells from the LOVO cell line before and after the treatment of demethylated agent 5-azacytidine, then added 1 ml RNA extraction reagent Trizol to completely extract RNA when the cells were completely split. The absorbance of RNA at 260 nm, 280 nm and 230 nm was then measured by the Nanodrop spectrophotometer, respectively, which could be used to accurately estimate the RNA concentrations. Then the denaturing agarose gel electrophoresis was performed (formaldehyde reagent was droplet-added) to test the purity and integrity of RNA (Figure 1). After the completion of sample RNA extraction, the miRCURY™ Hy3™/Hy5™ Power labeling kit (Exiqon, Vedbaek,

Denmark) was used to label miRNA. After the labeling reaction was terminated, the Hy3™-labeled samples and miRCURY™ LNA Array (v.18.0) (Exiqon) were hybridized. The total 25µl labeled sample mixture was added with 25 µl hybridized buffer, and denatured at 95°C for 2min, which was then placed on ice for 2 min, and hybridized with the 12-Bay Hybridization Systems (Hybridization System-Nimblegen Systems, Inc, Madison, WI, USA) at 56°C for 16-20 hours, which could maintain the constant temperature and mix evenly to ensure the homogeneity of hybridization and enhance the signals. After the hybridization, Washbuffer kit (Exiqon) was used for the several times' washing; finally, the samples were dried and centrifuged at 400 rpm for 5 minutes. The chips were then scanned by the Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA). The scanned signals were introduced into GenePix Pro 6.0 software (Axon) for the data extraction. The miRNAs was repeated for the mean value, and the Median normalization method was used for the standardization. After the standardization, the fold change method was used to screen the differentially expressed miRNAs, Table 2. The MEV software (v.4.6, TIGR) was used to perform the hierarchical cluster analysis (Figure 2). The RNA integrity was detected by the denaturing gel electrophoresis.

#### Real-time PCR

The LOVO cell lines before and after the treatment of 5-azacytidine, as well as the above tissue samples, were

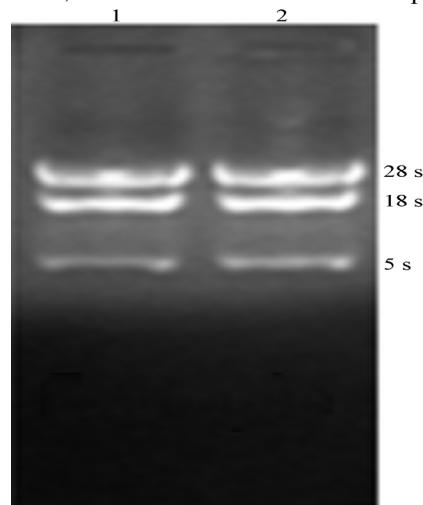


Figure 1. Integrity of RNA samples

Table 1. Real-Time Quantitative PCR Using Primers List

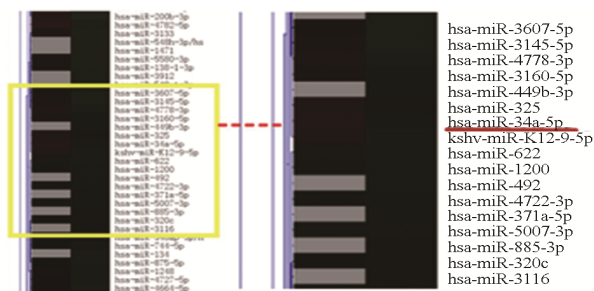
Gene name	5' and 3' bilateral primer sequences	Annealing temperature (°C)	Product length (bp)
U6	F:5'GCTTCGGCAGCACATATACTAAAAT3' R:5'CGCTTCACGAATTTGCGTGTCA3'	60	89
hsa-miR-34a-5p	GSP:5'GGGGTGGCAGTGTCTTAGC3' R:5'CAGTGCGTGTCTGGAGT3'	60	64

\*The corresponding miRNA-34a-5p specific primer was GSP, and the primer that matched the RT primer was R

Table 2. Lists the Difference miRNA-34a-5p

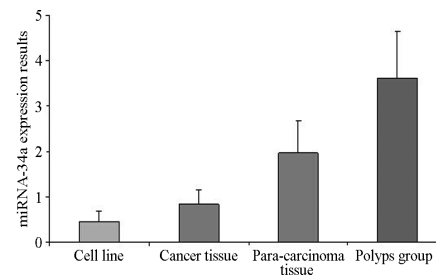
Sample No	U6ct (1)	miRNA-34a-5p ct (2)	(2)- (1)	(2-1) sample- (2-1)J10	2 <sup>-ΔΔCT</sup> CT
J10	12.571	18.996	6.425	0	1
W1	12.809	19.943	7.135	0.079	0.61

added 1 ml TRIZOL reagent and pipetted repeatedly for the homogenization. The homogenized samples were then re-incubated, which exhibited upper and lower layers after the incubation, the upper were colorless aqueous layer, while the lower layer was red phenol-chloroform. RNA was dissolved in the upper layer, accounting for 40% of the tube volume. The above aqueous layer was then poured into a new centrifuge tube and 0.5 ml isopropanol was added to precipitate RNA, after 10 min high-speed centrifugation, the precipitated RNA was washed and redissolved. The above samples were placed into a centrifuge tube, with 800  $\mu$ l TRIZOL reagent for the sufficient lysis and a small amount of chloroform for the RNA extraction, the ultraviolet absorption method was used to test the RNA quality, the computer would automatically generate and save the results according to the settings. After measuring the concentration and purity, the denaturing agarose gel electrophoresis was performed, and the RT-PCR method was used to synthesize cDNA for the real-time quantitative PCR. A brief centrifugation at 5000 rpm was performed. After the sampling loading, the above 384-PCR plates were placed on the Realtime PCR instrument for the PCR reaction: shown in Table 1.



**Figure 2. Clustering of miRNA Hierarchical Clustering for Normalized Data of Expressed miRNA in all Samples.** Red indicates high relative expression, and green indicates low relative expression

95°C, 10min; 40 PCR cycles (among which 95°C, 10 sec; 60°C, 60 sec (the fluorescence was separately collected). Firstly, the PCR product melting curve was created, and after the amplification reaction completed, the temperature was raised gradually from 60°C to 99°C with (95°C, 10 sec; 60°C, 60 sec; 95°C, 15 sec), the instrument performed the detection according to the pre-designed procedures, with Ramp Rate as 2%). When performed the quality testing with the real-time quantitative PCR, although the amounts of each tissue and cell samples added were the same, due to the inevitable measuring error of RNA concentration quantitation and calculation error of reverse transcription efficiency during the experiment. The real cDNA contents of samples with the same volume were in fact not identical, in order to correct such errors; U6 (with substantially constant expression level among different samples) was used as the internal reference. U6 was set as the internal control, and the CT value was used to calculate the relative expression amount of each group.  $\Delta\Delta CT = \Delta CT$  (experimental sample) -  $\Delta CT$  (standard sample),  $\Delta CT = CT$  (relative gene) -  $CT$  (reference gene), the folds of gene expression difference were the relative quantification  $2^{-\Delta\Delta CT}$ , the experiments were repeated three times, shown in Table 3, Figure 3.



**Figure 3. miRNA-34a-5p Expression Results in Colorectal Cancer, Paraneoplastic Tissues and Polypus Tissues**

**Table 3. miRNA-34a-5p Expression Results in Colorectal Cancer, Paraneoplastic Tissues and Polypus Tissues**

Pathological results	Colorectal cancer tissues	Paraneoplastic tissues	Polypus tissues	Pathological results
LOVO (after the medication)	1			
LOVO (before htemedication)	0.6			
SW480	0.24			
SW620	0.19			
LS-174T	0.67			
HCT-8	0.37			
HCT-116	0.7			
Adenocarcinoma	0.38	0.88	1.83	Inflammatory polyps
Adenocarcinoma	0.43	1	2.51	Inflammatory polyps
Adenocarcinoma	0.49	1.14	2.67	Adenomatous polyps
Adenocarcinoma	0.51	1.19	2.69	Tubular adenoma
Adenocarcinoma	0.59	1.38	2.91	Tubular adenoma
Adenocarcinoma	0.68	1.58	3.08	Inflammatory polyps
Adenocarcinoma	0.8	1.87	3.2	Serrated adenoma
Adenocarcinoma	0.88	2.06	3.45	Tubular adenoma
Adenocarcinoma	0.91	2.12	3.94	Inflammatory polyps
Adenocarcinoma	0.98	2.29	4.2	Inflammatory polyps
Adenocarcinoma	1.09	2.53	4.51	Tubular adenoma
Adenocarcinoma	1.12	2.6	4.9	Chronic mucousal inflammation
Adenocarcinoma	1.15	2.68	4.77	Inflammatory polyps
Adenocarcinoma	1.23	2.87	4.8	Inflammatory polyps
Adenocarcinoma	1.35	3.14	4.9	Tubular adenoma

### Statistical analysis

The relative quantification method was used to analyze the differences of miRNA-34a relative expression levels in colorectal cancer, paraneoplastic tissues and polypus tissues. The results were processed with SPSS19.0, the intergroup difference comparison was performed the data ranking firstly, then the ranked sequences were set as the measurement variables, the non-parametric paired sample comparison used the Wilcoxon signed rank test, with  $p < 0.05$  considered as the statistical significance.

## Results

### RNA extraction

As it could be seen from the above figure, the 28S, 18S, 5S bands of RNA samples were clear, indicating that the RNA purification was complete and reliable, and met the chip quality requirements.

### miRNA-34a-5p PCR

W1 in the above figure was before the drug treatment, J10 was after the drug treatment, and the error correction method revealed that after the drug treatment, the cell line LOVO exhibited high miRNA-34a-5p expression, while the expression before the treatment was low.

### Cluster analysis

As shown in the miRNA clustering figure, before and after the treatment of demethylated agent 5-azacytidine: miRNA-34a-5p showed the differential expression, exhibiting red after the treatment red, 0.857, while green before the treatment, 0.373, with the difference as about 2.3 times.

### miRNA-34a-5p expressions

The comparison between the colorectal cancer and polypus tissues revealed that: the statistical analysis result:  $0.01 < p < 0.02$ , with the statistical significance; namely that the miRNA-34a-5p expression in polypus tissue was higher than that in colorectal cancer. Comparison between the paraneoplastic tissues and polypus tissues: statistical analysis:  $p < 0.05$ , with statistical significance; namely that the miRNA-34a-5p expression in polypus tissue was higher than that in paraneoplastic tissues. Comparison between the colorectal cancer and paraneoplastic tissues: statistical analysis:  $p < 0.05$ , with the statistical significance; namely that the miRNA-34a-5p expression in paraneoplastic tissues was higher than that in colorectal cancer.

## Discussion

miRNA-34a-5p was the miRNA with relatively plenty studies, it was once reported that its expression in the urinary system tumors and glial tumors exhibited the downward trend (Toyota et al., 2008; Yamakuchi and Lowenstein, 2009). As one member in the miRNA family, it could produce the anticancer effects through acting on several target genes. Yao once reported that miRNA-34a-5p could reduce the invasive ability of bladder cancer cells,

thus exhibited the role of tumor suppressor gene, and it could also increase the sensitivity of bladder cancer cells towards the chemotherapeutic drugs. Yang reported that miRNA-34a-5p could affect the DNA damage repairing through target-regulating SIRT1, and speed up the aging process of cells (Aranha et al., 2011). Although, there have been many studies about miRNAs reported in colorectal cancer (Kanaan et al., 2012; Wu et al., 2012; Zhou et al., 2013) but in the large intestine, the miRNA-34a-5p expression was still controversial, and the relative research was rare, we came to the conclusions through the microarray analysis that in the non-demethylated drug-treated LOVO cells. The miRNA-34a-5p expression was reduced, while in the 5-azacytidine-treated cells, the miRNA-34a-5p expression was increased up to two-fold than the former cells, thus it prompted that miRNA-34a-5p might play the role of tumor suppressor gene in colorectal cancer cell line LOVO, and its expression was closely related to the DNA methylation.

Among the literatures, miRNA-34a-5p played different roles in the tumors of different organs. In order to further confirm the roles of miRNA-34a-5p in colorectal cancer, we selected and cultured six kinds of colorectal cancer cell lines (LOVO, SW480, SW620, LS-174T, HCT-8, HCT-116, which were all the colorectal cancer cells with the characteristics of high invasion and lymph node metastasis). And it was found in these cell lines that the miRNA-34a-5p expression values were in a low level, thus it could be initially determined that miRNA-34a-5p played the role of tumor suppressor gene in colorectal cancer cell lines. Toyota once found that miRNA-34b and miRNA-34c, members of miRNA-34 family, acted as the tumor suppressor gene in the primary colorectal cancer and was associated with the methylation of CpG island, the subsequent experiments proved that miRNA-34b/cCpG Island was the important target of silence appearance of primary colorectal cancer (He et al., 2007c), which was consistent with our results.

In order to confirm this inference, miRNA-34a-5p, from the cancer tissues and paraneoplastic tissues (more than 5 cm away from the cancer tissues) of colorectal cancer patients found by the colonoscopy (who were all confirmed by pathology after the surgery), as well as from the colonoscopy-found colorectal polyps patients (also confirmed pathologically), were performed the Realtime PCR testing. The results showed that: miRNA-34a-5p was downregulated in colorectal cancer tissues, and the expression in paraneoplastic tissues was higher than the cancer tissues, the comparison between the above two had the statistical difference,  $p < 0.05$ , indicating that the miRNA-34a-5p expression in colorectal cancer tissues belonged to the category of tumor suppressor gene, while it was found from the values that the miRNA-34a-5p expression in colorectal cancer tissues was lower than those in colorectal cancer cell lines. The possible reason might be that the colorectal cancer tissues were all sampled from the endoscopic pathological specimens; the sampling volume was small, and all from the tumor tissue surface. There might be mixed with the inflammatory cells or necrotic cells, leading to the inadequate purity of tumor cells. Certain study had shown that, the absence

of miR-34a gene or the promoter methylation resulted in the downregulation of miR-34a, thus the cell growth was accelerated, the cycle was shortened and the apoptosis process was disturbed, followed by the promoted tumorigenesis (He et al., 2007b), and this result was consistent with our experiments that miRNA-34a-5p played the role of tumor suppressor gene.

Tazawa detected miRNA of 25 colon cancer samples, and found that miR-34a of 36% samples exhibited the downregulation than paraneoplastic tissues, although this ratio was lower than our experimental results, some scholars suggested in the results of colorectal cancer cell lines that the proliferation of colon cancer cell line that over-expressed miR-34a plasmid was declined, indicating miRNA-34a-5p could inhibit the proliferation of colon cancer cells; when established the tumor inside the nude mice, then intravenously injected miR-34a into the nude mice for the observation. About two weeks later, the tumor size shrank, suggesting that miR-34a might promote the apoptosis of colon cancer cells, and might become a very important therapeutic gene.

Similarly, the comparison of miRNA-34a-5p expression between colorectal cancer and polypus tissues exhibited the significant difference,  $p < 0.05$ . The results showed that in the benign diseases, the miRNA-34a-5p expression was upregulated, which further proved that the downregulated miRNA-34a-5p expression could really induce the occurrence of cancer. Meanwhile, the difference analysis of miRNA-34a-5p expression in polypus tissues and paraneoplastic tissues suggested that they exhibited the statistical significance,  $p < 0.05$ , while the expression in polypus tissue was higher than that in paraneoplastic tissues. The possible reason might be that although the pathological sampling was more than 5 cm away from the paraneoplastic tissues, and the naked eye observation suggested the samples as the normal tissues. It could not rule out the possibility of potential migration of cancer cells; although the polyps were the neoplasm, it was also the benign tissue, so the expression of miRNA-34a-5p was higher than paraneoplastic tissues. The research ideas and results of this experiment were consistent with Tian (Bartel, 2009), and also showed the necessity of the expanded surgical resection towards the diseased segments in the treatment of colorectal cancer.

The recent study indicated that, miR-34a exhibited the potential of antiproliferation and could regulate the cell cycle transfer from G1 phase to S phase (Tarasov et al., 2007). The subsequent studies found that miR-34a was a very important small RNA molecule that was directly regulated by p53. The exogenous miR-34a could induce the apoptosis, inhibit the cell reproductive cycle, thus controlling the aggregation and metastasis of tumor cells, indicating that miR-34a might be a very important mediator in the tumor inhibition signaling network by p53 (Calin et al., 2004; Hermeking, 2010). p53 could activate the expression of miR-34a, and the upregulated miR-34a could regulate the cascades of a series of genes and promote the p53-mediated apoptosis (Chang et al., 2007; Raver-Shapira et al., 2007). The miR-34a-mediated cell cycle regulation could be realized through regulating multiple target genes, including CCNE2, CDK4 and MET

(He et al., 2007a). In fact, there had been studies that paid attention to the correlation of abnormally expressed miR-34a and the incidence of some tumors. For example, the precursor of miR-34a was expressed abnormally in the malignant lymphoma; Chang found that the miR-34a expression was absent in the pancreatic cancer (Zanette et al., 2007). Tazawa found that the downregulated miR-34a might be associated with the occurrence of colon cancer (Tazawa et al., 2007). In addition, the expression deficiency of miR-34a, that located on p36 of chromosome as a tumor suppressor gene, in the neurofibroma (Welch et al., 2007) caused the occurrence of this tumor. In summary, miRNA-34a-5p had an important role in the occurrence and development of tumors, could mark the various periods and invasion of tumors. Some studies had suggested that the miRNA-34a-5p expression in the peripheral blood was quite stable, even under relatively severe environmental conditions, the serum miRNA-34a-5p value was able to be consistent with the actual value, the serum miRNA-34a-5p had become the molecular marker towards the diagnosis and prognosis determination of liver cancer, prostate cancer, lung cancer and gastric cancer, but because of the high cost, and lack of a large clinical sample for the confirmation, it was not widely carried out clinically.

The above experiment comprehensively confirmed that miRNA-34a-5p could inhibit the excessive cell growth and proliferation, thereby suppressed the formation of colorectal cancer gene, namely acting as a tumor suppressor gene. It could be speculated that only when the normal colorectal cancer cells lost miRNA-34a-5p for some reason, it would lead to the loss of tumor suppressor function, thus causing the formation of colorectal cancer.

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