RESEARCH ARTICLE

Expression and Clinical Significance of MicroRNA-376a in Colorectal Cancer

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Abstract

    The incidence of colorectal cancer (CRC) is increasing in many Asian countries and microRNAs have already been proven to be associated with tumorigenesis. Currently, microRNA-376a (miR-376a) expression and association with clinical factors in CRC remains unclear. In this study, real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was carried out on 53 matched pairs of CRC and adjacent normal mucosa to investigate the expression levels of miR-376a. According to the high or low expression of miR-376a, patients were divided into two groups. The relationship between miR-376a expression and clinicopathological factors of 53 patients was evaluated. Survival analysis of 53 CRC patients was performed with clinical follow-up information and survival curves were assessed by the Kaplan-Meier method. Immunohistochemistry (IHC) staining was performed on sections of paraffin-embedded tissue to investigate the vascular endothelial growth factor (VEGF) expression. MiR-376a showed low expression in cancer tissues compared to the adjacent normal tissues and altered high miR-376a expression tended to be positively correlated with advanced lymph node metastasis and shorter patient survival. VEGF IHC positivity was significantly more common in patients with high expression levels of miR-376a. Those results demonstrated that miR-376a may be a meaningful prognostic biomarker and potential therapeutic target in colorectal cancer.

Keywords: microRNA - colorectal cancer - clinicopathology - vascular endothelial growth factor

Introduction

    Colorectal cancer (CRC) is one of the most common cause of cancer-related death worldwide and its incidence has increased by a factor of two to four in many Asian countries over the past few decades (Sung et al., 2005). Although the burden of CRC have increased rapidly in some developed countries (Ng and Wong, 2013), the death rates have continued to rise. CRC carcinogenesis is a multi-step process and malignant transformation requires a series of genetic and epigenetic changes. Thus, focusing on the molecular and genetic features of tumors and their hosts may be a viable way to discover and develop new targeted therapies (Kelley et al., 2011).

    MicroRNAs (miRNAs) are a class of small, non-coding, regulatory RNAs, which can decrease the expression levels of target miRNAs or regulate translation (Brennecke et al., 2003; van den Berg et al., 2008). MiRNAs are known as important factors in gene regulation, apoptosis, cell proliferation, and differentiation (Bartel, 2004). Functional studies performed in cancer ce ll lines or animal models of various cancers suggest that, miRNAs can function as tumor suppressors, oncogenes, and regulate cancer pathways (Farazi et al., 2011). Aberrant expression of specific miRNAs has been reported in many tumor types such as CRC, breast cancer, cervical cancer, non-small cell lung cancer, ovarian cancer and bladder cancer as well as being associated with patients' survival data, metastasis, and other clinicopathological factors (Toyama et al., 2012; Wan et al., 2012; Shen et al., 2013; Wang et al., 2013; Xiao et al., 2013; Liu et al., 2014; Xu et al., 2014). A previous study showed that miR-32 expression was upregulated in CRC tissues, and high expression was significantly correlated with the tumor stage, distal metastases and poor prognoses (Wu et al., 2013b). In functional studies, overexpression of miR-32 can promote SW480 cell proliferation, migration, and invasion, reduce apoptosis, and decrease the expression of phosphatase and tensin homologue (PTEN) at the posttranscriptional level (Wu et al., 2013a). Lou et al. found that miR-625 expression was significantly downregulated *in vitro* and *in vivo*, and was positively correlated with poor survival (Lou et al., 2013). Therefore, altered miRNA expression may be a potential diagnostic biomarker for cancer (Luo et al., 2011).

    MicroRNA-376a (miR-376a) has been reported in several human cancers, including upregulation in esophageal cancer tissues, plasma of breast cancer, and downregulation in melanoma, and hepatocellular carcinoma (Zehavi et al., 2012; Zheng et al., 2012; Cuk et al., 2013; Zhao et al., 2013). In particular, Kunte

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et al. found that levels of miR-376a expression were significantly increased with CRC tumor development, using the azoxymethane (AOM)-treated rat model (Kunte et al., 2012). The relevance of miR-376a expression in human CRC tissues is still unknown.

This study measured the differential expression of miR-376a between CRC tissues and paired adjacent normal tissues in 53 CRC patients. Association of miR-376a expression with clinicopathological factors and prognosis was also investigated. Vascular endothelial growth factor (VEGF) is an important biomarker for CRC. We investigated the VEGF expression by Immunohistochemistry (IHC).

Materials and Methods

Patients and tissue samples

Surgical specimens of cancer tissue and paired adjacent normal mucosa were obtained from 53 patients with colorectal cancer who underwent surgery without preoperative treatment at the China-Japan Union Hospital of Jilin University, from 2010 to 2012. All tissue samples were immediately frozen in liquid nitrogen and stored at -80°C in the hospital tissue bank until the extraction of total RNAs. Sections of paraffin-embedded tissue from surgically resected specimens were stored and used for IHC staining. Clinicopathological information, including age, gender, tumor size, location, histological grade, clinical stage, lymph node metastasis, venous invasion, and liver metastasis, was available for all patients. This study was approved by the medical ethics committee of the China-Japan Union Hospital.

Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNAs of colorectal tissues were isolated using TriPure RNA Isolation Reagent (Roche Diagnostics, Shanghai, China) accrding to the manufacturer instructions. The cDNAs were reverse-transcribed from 1.0 μg of total RNAs and qRT-PCR was performed using the All-in-OneTM miRNA qRT-PCR Detection Kit (GeneCopoeia, Guangzhou, China) by ABI fast 7500 real-time PCR System (Applied Biosystems). U6 small nuclear RNA (snRNA) was selected to be the endogenous control. The miRNA qPCR Primers were ordered from GeneCopoeia, Inc. The catalog numbers of the All-in-OneTM miRNA qPCR Primers were as follows: hsa-miR-376a, HmiRQP0467; snRNA U6, HmiRQP9001. The following temperature profile was used: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 10 sec, and extension at 65°C for 29 sec. All qRT-PCR reactions were performed in triplicate. The relative amount of miR-376a to snRNA U6 was calculated using the equation $2^{-\Delta C}$, where $\Delta Ct = (Ct_{miR-376a} - Ct_{U6})$.

Immunohistochemistry (IHC)

IHC staining was carried out on 4μm thick sections of paraffin-embedded tissue from surgically resected specimens. After deparaffinization and rehydration, tissues were treated by citrate buffer (pH 6.0) with microwave heating for 20 min for antigen retrieval. VEGF IHC was performed using mouse anti-human VEGF monoclonal antibody (Maixim.bio, Fuzhou, China) and ultrasensitiveTM S-P kit (Maixim.bio, China), according to manufacturer instructions. Brown staining of the cytoplasm was considered to be a positive expression of VEGF.

Statistical analysis

All statistical analyses were performed using SPSS 13.0 software (SPSS). Statistical differences between miRNA expression levels in colorectal cancer and paired normal mucosa were evaluated using the Wilcoxon matched pairs tests. The relationships between the miRNA expression levels and clinicopathologic parameters were evaluated with the χ^2 test. Kaplan-Meier curves were used to analyze the survival data and differences in survival rates were assessed with the log-rank test. A Cox proportional hazards model was used for multivariate analysis of survival data. All tests were two-sided and differences were considered to be significant when $p<0.05$.

Results

Expression levels of miR-376a in CRC and normal colorectal tissue.

MiR-376a expression in 53 pairs of CRC and normal colorectal tissues were detected by qRT-PCR. As shown in Figure 1, after normalization to U6 expression levels, the relative levels of miR-376a expression in human CRC tissues were significantly lower than in the adjacent normal tissues ($p=0.0294$).

Clinicopathological significance of miR-376a expression in CRC.

To assess the clinical value of altered miR-376a expresson in CRC, the relationship between miR-376a expression and clinicopathological factors of 53 patients was evaluated by the χ^2 test (Table 1). Of the 53 CRC patients, 33 showed a decrease in miR-376a expression and 20 had an increase expression relative to the matched adjacent normal tissue. As shown in Table 1, a positive correlation was observed between high miR-376a
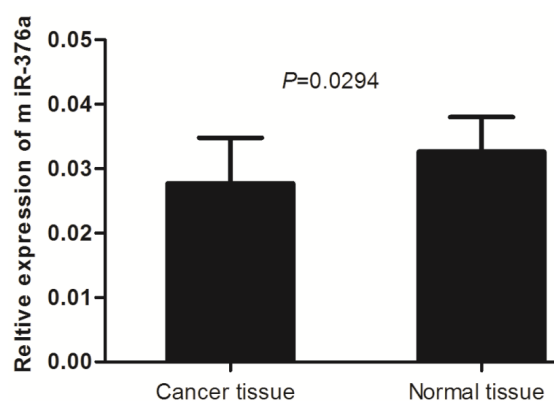

Figure 1. MiR-376a Expression Levels were Lower in Cancer Tissue Than those in Normal Adjacent Mucosa ($p=0.0294$)

expression and lymph node metastasis (p=0.0248). There was no significant association between miR-376a and other clinicopathological parameters, such as age, gender, tumor size, location, histological grade, clinical stage, venous invasion and liver metastasis. These results suggest that altered expression of miR-376a might be associated with colorectal cancer progression.

Association of miR-376a expression with prognosis in patients with CRC.

Survival analysis of 53 CRC patients was performed with clinical follow-up information and survival curves were assessed using the Kaplan-Meier method. As shown in Figure 2, overall survival was lower in patients with high miR-376a expression than in those patients with low miR-376a expression (p=0.0314). The results of the multivariate Cox proportional hazards regression analyses

Table 1. Relationship between miR-376a Expression and Clinicopathological Characteristics of Patients with CRC (n=53)

factor	expression of miR-376a		P value
	high expression (n=20)	low expression (n=33)	
Age (year)			
<60	4	10	0.41
≥60	16	23	
Sex			
Male	12	22	0.624
Female	8	11	
Size			
>50mm	12	17	0.582
<50mm	8	16	
Location			
Righ colon	5	7	0.449
Left colon	4	12	
Rectum	11	14	
Histological grade			
Well, Moderately	18	22	0.056
Poorly, other grades	2	11	
Stage			
T1-T2	1	6	0.17
T3-T4	19	27	
Lymph node metastasis			
Positive	13	11	0.0248*
Negative	7	22	
Venous invasion			
Positive	9	10	0.28
Negative	11	23	
Liver metastasis			
Positive	5	7	0.749
Negative	15	26	

*p<0.05

for overall survival are shown in Table 2. Multivariate analysis indicated that the high expression levels of miR-376a were independent and significant prognostic factors for survival (HR, 2.98; 95%CI, 1.00-9.93; p, 0.048,). Thus, miR-376a expression might relate to patients survival and be a potential biomarker for CRC.

Relationship between VEGF expression and miR-376a expression

IHC was used to explore the relationship between levels of miR-376a expression and expression of VEGF in the 53 CRC samples. IHC staining was performed on all colorectal specimens (Figure 3). The results are shown in Table 3. VEGF positive was significantly more common in patients with high levels of miR-376a expression (p<0.01).
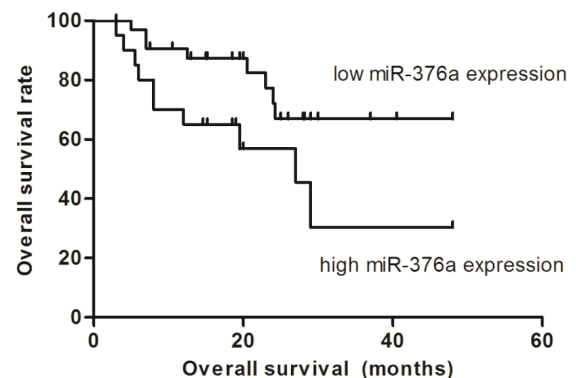

Figure 2. The Overall Survival was Lower in Patients with High miR-376a Expression than in those Patients with Low miR-376a Expression (p=0.0314)
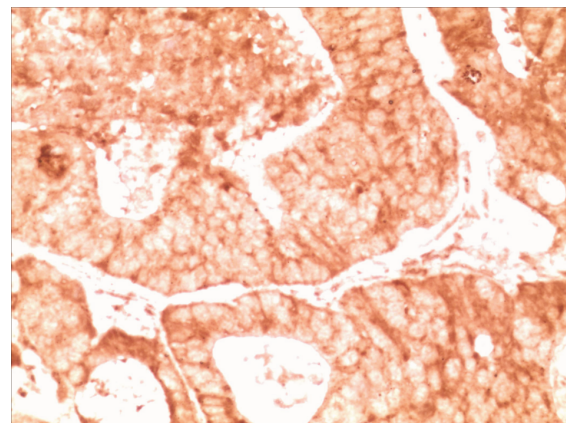

Figure 3. IHC Staining for VEGF in CRC Tissue. VEGF immunoreactivity in cytoplasm of tumor cells(200×)

Table 2. Univariate Analysis and Multivariate Analyses for Overall Survival in 53 CRC Patients.

Clinicopathological Characteristics	Univariate analysis			Multivariate analysis		
	HR	95 %CI	P	HR	95 %CI	P
Age (<60/≥60years)	2.23	0.78-6.41	0.136	-	-	-
Sex (Male/Female)	1.33	0.49-3.60	0.573	-	-	-
Size (>50mm/<50mm)	1.724	0.67-4.46	0.262	-	-	-
Histological grade (Well, Moderate- ly/Poorly, other grades)	2.17	0.69-6.81	0.186	0.25	0.08-0.76	0.015*
Stage (T1-T2/T3-T4)	1.98	0.50-7.79	0.33	-	-	-
Lymph node metastasis (Positive/ Negative)	0.32	0.12-0.81	0.016*	2.45	0.67-8.93	0.173
Venous invasion (Positive/ Negative)	0.35	0.13-0.95	0.040*	1.82	0.51-6.49	0.355
Liver metastasis (Positive/Negative)	0.38	0.13-1.16	0.09	1.02	0.32-3.25	0.968
MiR-376a expression (high/low)	2.99	1.10-8.11	0.031*	2.98	1.00-8.83	0.048*

HR; Hazard ratio; 95% CI; 95% confidence interval; *p<0.05

Table 3. Association between Expression of VEGF and Expression of miR-376a

| | | Expression of miR-376a | | *P* value |
		High Expression (n=20)	Low Expression (n=33)	
VEGF	Positive	8	3	0.0072*
	Negative	12	30	

*$p<0.05$

Discussion

This study found a significant low expression of miR376a in CRC tissues as compared to corresponding adjacent normal tissues, which has never been reported before. Although in most CRC patients the expression level of miR-376a is low, altered high miR-376a expression tends to be associated with lymph node metastasis ($p=0.0248$) and have a shorter survival ($p=0.0314$). Moreover, multivariate analysis indicates that high expression of miR-376a is an independent, significant prognostic factor for CRC patients' survival. Similar results with other miRNAs have been reported in relation to CRC, gastric cancer, primary gallbladder carcinoma, and glioma (Liu et al., 2012; Nishimura et al., 2012; Lu et al., 2013; Peng et al., 2013). Those findings show that miRNAs may be related to cancer patients' clinicopathological factors and prognoses. As a result, miR-376a may be a potential biomarker for diagnoses and prognoses of CRC.

As shown in Table 3, VEGF IHC positive status was significantly more common in patients with high expression levels of miR-376a. There may be a direct or indirect relation between miR-376a and VEGF. Analysis by the public programs, TargetScan (http://www.targetscan.org) and miRnada (http://www.microrna.org), indicated that insulin-like growth factor 1 receptor (IGF1-R) and vascular endothelial zinc finger-1 (VEZF1) are theoretically the targets of miR-376a. VEGF has been demonstrated to be induced by IGF1-R and IGF1-R is a target of miR-376a (Warren et al., 1996; Zehavi et al., 2012). At the same time, VEZF1 also has the ability to enhance the expression of VEGF in tumor angiogenesis (Bruderer et al., 2013). No matter which is the target of miR-376a or what mechanism induces the expression of VEGF, it may be an important process in oncogenesis and needs to be investigated further both *in vivo* and *in vitro*. Despite the number of clinical samples used in this study, more clinical and biological research is needed for further understanding of miR-376a and its target genes. In particular, more mechanistic studies are needed to investigate the method through which miR-376a is downregulated in CRC and how altered high levels of miR-376a contribute to lymph node metastasis of cancer cells and poor survival rates.

In conclusion, our results showed the altered expression of miR-376a in colorectal cancer (CRC) for the first time. The results showed that miR-376a had a low expression in the tested CRC tissues and the high levels of miR-376a may be associated with lymph node metastasis. High miR-376a expression was proven to be a significant, independent factor for CRC prognosis.

MiR-376a may be a meaningful prognostic biomarker and potential therapeutic target in colorectal cancer.

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