Genetics and Molecular Research

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Decreased miR-452 expression in human colorectal cancer and its tumor suppressive function

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Genet. Mol. Res. 15 (2): gmr.15027730
Received September 2[illegible], 201[illegible]
Accepted January 15, 2016
Published May 23, 2016
DOI: http://dx.doi.org/10.4238/gmr.15027730

ABSTRACT. MicroRNA-452 (miR-452) is dysregulated in some human malignancies, and is correlated with tumor progression. However, its expression and function in human colorectal cancer (CRC) remain unclear. The aim of our study was to explore the effects of miR-452 in CRC tumorigenesis and development. Using reverse transcription quantitative real-time polymerase chain reaction, we detected miR-452 expression in CRC cell lines and primary tumor tissues. We also examined the association between miR-452 expression and clinicopathological factors. We then investigated the effects of miR-452 on the biological behavior of CRC cells. miR-452 expression was significantly downregulated in CRC compared with the adjacent noncancerous tissues. A low level of miR-452 was associated with larger tumor size, deeper invasion depth, and advanced TNM stage. Multivariate Cox regression analysis identified decreased miR-452 expression as an independent factor predicting poor prognosis for CRC patients. In addition, *in vitro* functional analysis showed that overexpression of miR-452 in HCT116 cells reduced cell proliferation, promoted cell apoptosis, and inhibited cell invasion and migration.

These findings indicate that miR-452acts as a tumor suppressor in CRC, and would serve as a novel molecular therapeutic agent for the treatment of the disease.

Key words: miR-452; Colorectal cancer; Prognosis; Proliferation; Invasion

INTRODUCTION

Colorectal cancer (CRC) is the most frequently diagnosed malignancy of the gastrointestinal tract, and ranks as the third most common cancer worldwide (Jemal et al., 2011). Despite recent advances in surgical techniques, new chemotherapy regimens, radiotherapy, and targeted molecular therapy, the long-term prognosis for patients with advanced disease and metastasis remains poor. Previous studies have reported many oncogenes and tumor-suppressor genes that are closely associated with CRC (Olsen et al., 2015; Ramireddy et al., 2015; Rogers et al., 2015; Sun et al., 2015), but the highly complex molecular mechanisms underlying its carcinogenesis and progression remain obscure. Therefore, it is urgently necessary to identify reliable biomarkers of CRC for its early diagnosis, effective therapy, and prognosis evaluation.

MicroRNAs (miRs) are a class of naturally occurring, short (about 22 nucleotides in length), single-stranded, non-protein coding RNAs that negatively regulate gene expression. It is estimated that about 60% of genes can be regulated by miRs. They suppress translation or promote the degradation of target messenger RNAs (mRNAs) through base pairing with the 3'-untranslated regions (3'-UTRs) (Bartel, 2004, 2009). Previous research has shown that miRs play a critical role in various biological processes, such as development, differentiation, cell growth, inflammation, stress response, and endocrine homeostasis. Emerging evidence demonstrates that aberrant miR expression is strongly associated with cancer initiation and progression, which may provide a new and promising means of detecting and treating cancer (Zhang et al., 2007; Dieckmann et al., 2012; Takahashi et al., 2013). miRs can function as either oncogenes or tumor suppressors according to the roles of their target genes. Deregulation or dysfunction of miRs are involved in many processes of tumor progression including cell proliferation, apoptosis, invasion, metastasis, angiogenesis, and epithelial-to-mesenchymal transition (Wu et al., 2014; Shi et al., 2015; Yin et al., 2015). Functional miRs may be applied to cancer diagnosis and prognosis, and can also act as potential novel therapeutic targets.

miR-452 is a recently identified cancer-related miR. It is downregulated in non-small cell lung cancer (NSCLC) (He et al., 2015), glioma (Liu et al., 2013), and prostate cancer (Kristensen et al., 2014), and acts as a potential tumor suppressor in these tumors. In contrast, the level of miR-452 is significantly increased in bladder cancer and hepatocellular carcinoma, and acts as a candidate oncogene (Veerla et al., 2009; Puerta-Gil et al., 2012; Zheng et al., 2014a). In addition, decreased miR-452 expression is associated with Adriamycin resistance in breast cancer cells (Hu et al., 2014). However, to date there has been no report on the use of miR-452 in CRC. In the present study, we examined miR-452 expression in CRC tissues and cell lines. We also investigated the correlation between miR-452 levels and clinicopathological characteristics and patient survival. Moreover, we explored the role of miR-452 in the regulation of the biological behavior of CRC cells.

MATERIAL AND METHODS

Patients and clinical specimens

This study was approved by the Research Ethics Committee of the China-Japan Friendship Hospital. Written informed consent was obtained from all patients. All specimens were handled and made anonymous according to the ethical and legal standards.

A total of 165 pairs of primary CRC samples and adjacent noncancerous tissues were obtained from patients who underwent surgery at the China-Japan Friendship Hospital between January 2008 and March 2010. The diagnosis of all samples was histopathologically confirmed by two pathologists. All specimens were frozen immediately in liquid nitrogen and stored at -80°C until required. Patients with two or more different malignancies were excluded. None of the patients had undergone chemotherapy or radiotherapy before surgery. The details of the 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 time from the day of primary surgery to death or the end of follow-up (for living patients).

Table 1. Correlation between miR-452 expression and clinicopathological features of colorectal cancer.

Clinicopathological features	No. of cases	miR-452 expression		P value
		Low (N=)	High (=%)	
Age				
<60	75	40 (53.3%)	35 (46.7%)	0.290
≥60	90	43 (47.8%)	47 (52.2%)	
Gender				
Male	96	51 (53.1%)	45 (46.9%)	0.243
Female	69	32 (46.4%)	37 (53.6%)	
Tumor size				
<5cm	94	39 (41.5%)	55 (58.5%)	0.007
≥5cm	71	44 (62.0%)	27 (38.0%)	
Histology/differentiation				
Well + Moderate	83	37 (44.6%)	46 (55.4%)	0.162
Poor	82	46 (56.1%)	36 (43.9%)	
Depth of invasion				
T1 + T2	60	20 (33.3%)	40 (66.7%)	0.001
T3 + T4	105	63 (60.0%)	42 (40.0%)	
TNM stage				
I, II	68	24 (35.3%)	44 (64.7%)	0.001
III	97	59 (60.8%)	38 (39.2%)	
Tumor site				
Colon	92	42 (45.7%)	50 (54.3%)	0.118
Rectum	73	41 (56.2%)	32 (43.8%)	

Cell lines and miR transfection

The human CRC cell lines (HT29, HCT116, SW480, and SW620) and 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 Roswell Park Memorial Institute (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-452 mimics and a negative control (miR-NC) were designed and synthesized by GenePharma Co. (Shanghai, China). CRC cells were plated on a six-well plate at a density

of 3 x 10^5 cells/well for approximately 24 h prior to transfection. Transient transfections of miR-452 mimics or miR-NC (20 nM) were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer instructions. The cells were collected for further analysis 24 h later.

RNA extraction and reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted from clinical specimens using TRIzol reagent (Invitrogen Corp, Carlsbad, CA, USA) according to the manufacturer instructions. RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Complementary DNA (cDNA) was synthesized from isolated RNA using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed with a TaqMan MicroRNA Assay Kit (Applied Biosystems) on an ABI7500 real-time PCR detection system. Quantitative PCR was conducted at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. U6 small nuclear RNA was used as an internal control. All reactions were run in triplicate. The cycle threshold (Ct) values were recorded, and the relative amount of miR-452 to U6 was calculated using the equation $2^{-\Delta Ct}$, where $\Delta CT = (CT_{miR-452} - CT_{U6})$.

Cell proliferation assay

Cell proliferation was investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, approximately 1 x 10^3 cells were seeded on a 96-well plate and incubated for 1, 2, 3, and 4 days. At the indicated time-point, 20 µL MTT (5 mg/mL) (Sigma, USA) was added to each well and incubated for another 4 h. The supernatants were then removed and 150 µL dimethyl sulfoxide (Sigma, USA) was added to terminate the reaction. The absorbance value (optical density (OD) was measured at 490 nm on a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Detection of apoptosis by flow cytometry

Forty-eight hours after transfection, the CRC cells were harvested, washed, and resuspended in ice-cold phosphate-buffered saline. The cells were then treated with propidium iodide (10 mg/mL; Sigma) and Annexin V-fluorescein isothiocyanate (FITC) (50 mg/mL, BD Biosciences) in the dark for 15 min at room temperature, and examined by flow cytometry (FACScan; BD Biosciences).

Cell invasion and migration assays

Six-well Transwell chambers (8-mm pore size, Corning, NY, USA) were used to investigate cell invasion and migration. For the migration assay, approximately 1 x 10^5 CRC cells in serum-free media were seeded into the upper chambers after miR-452 mimic or miR-NC transfection. The lower chamber contained medium with 20% FBS to stimulate migration. Following a 48-h incubation, the cells located on the lower surface of the chamber were stained and counted using a microscope (Olympus Corp., Tokyo, Japan). For the invasion assay, the

upper chambers were first covered with 5 mg/mL Matrigel, and the other steps were the same as for the migration assay.

Statistical analysis

All statistical analyses were performed using the SPSS 16.0 software package (SPSS, Chicago, IL, USA). The significance of differences between groups was estimated by the Student's *t*-test and the chi-square test. Survival curves were constructed using the Kaplan-Meier method and compared using the 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-452 expression in CRC tissues and cell lines

miR-452 expression in CRC tissues and cell lines was measured by RT-qPCR. Figure 1A shows that the level of miR-452 was significantly reduced in the CRC tissues (mean ± SD: 8.25 ± 2.13) compared with the paired adjacent noncancerous tissues (mean ± SD: 23.73 ± 5.67) ($P < 0.01$). miR-452 expression in the four CRC cell lines was also clearly downregulated (Figure 1B, $P < 0.05$). The HCT116 cell line, which possessed the lowest miR-452 expression among all the tested cell lines, was chosen for the subsequent *in vitro* experiments.

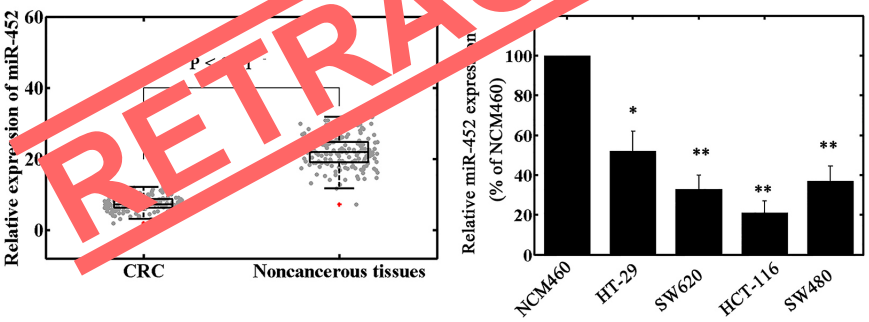

Figure 1. Relative expression levels of miR-452 in colorectal cancer (CRC) tissues and cell lines. **A.** miR-452 expression was signiðcantly lower in CRC tissues than in the corresponding noncancerous tissues. miR-452 expression levels were calculated by the $2^{-\Delta Ct}$ method and normalized to U6 small nuclear RNA. **B.** miR-452 expression was downregulated in CRC cell lines HT29, HCT116, SW480, and SW620, compared with human normal colonic epithelial cell line NCM460. *$P < 0.05$; **$P < 0.01$.

Association between clinicopathological characteristics and miR-452 expression in CRC patients

We further investigated the association between miR-452 expression and the clinicopathological characteristics of CRC. CRC samples were classified into a low miR-452 expression group (N = 83), and a high miR-452 expression group (N = 82) according to the median miR-452 expression level of all the CRC samples. The association between

clinicopathological characteristics and miR-452 expression is summarized in Table 1. We found that low miR-452 level was closely correlated with tumor size (P = 0.007), tumor depth (P = 0.001), and TNM stage (P = 0.001). No significant difference was observed between miR-452 expression and other clinical features such as age, gender, tumor site, and differentiation.

Prognostic values of miR-452 expression in CRC patients

We then determined whether miR-452 expression had prognostic potential for CRC patients. Using the Kaplan-Meier method and the log-rank test, we found the overall survival of patients with low miR-452 expression was significantly shorter than those with high miR-452 expression (P < 0.001; Figure 2). Moreover, the univariate proportional hazard model also revealed a statistically significant correlation between overall survival and tumor size (P = 0.025), local invasion (P = 0.032), and TNM stage (P < 0.001, Table 2). Multivariate Cox regression analysis enrolling the significant parameters mentioned above revealed that miR-452 expression (relative risk (RR) 3.825; P = 0.015) and TNM stage (RR 5.232; P = 0.001) were independent prognostic markers for CRC patients (Table 2).

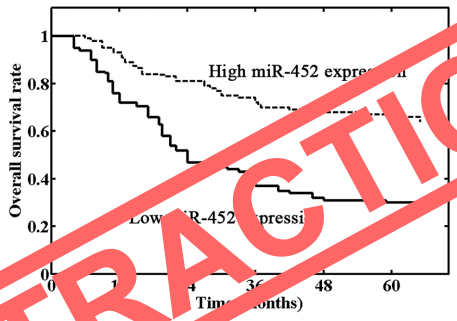

Figure 2. Kaplan-Meier survival curves of patients with colorectal cancer based on miR-452 expression status. Patients in the low expression group had significantly poorer prognosis than those in the high expression group (P < 0.001, log-rank test).

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

Variable	Univariate analysis		Multivariate analysis	
	HR	P value	HR	P value
Age (years)	1.632	0.215	-	-
Gender	1.134	0.759	-	-
Histological grading	1.704	0.173	-	-
Tumor size	2.629	0.025	2.116	0.078
Depth of invasion	2.388	0.032	1.524	0.283
Tumor site	1.226	0.634	-	-
Clinical stage	4.973	< 0.001	5.232	0.001
miR-452 level	4.178	< 0.001	3.825	0.015

Effects of miR-452 overexpression on the biological behavior of HCT116 cells

Finally, we explored the role of miR-452 in regulating the biological behavior of HCT116 cells. miR-452 expression in HCT116 cells was evidently upregulated after miR-452 mimic transfection (Figure 3A). As shown in Figure 3B and 3C, miR-452 overexpression

impaired HCT116 cell proliferation and promoted cell apoptosis compared with the miR-NC group. In addition, the invasion and migration capability of HCT116 cells was significantly reduced after miR-452 mimic transfection (Figure 3D and 3E).

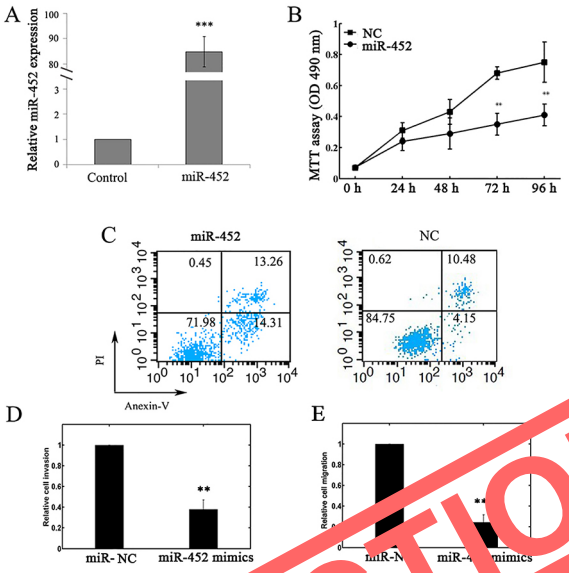

Figure 3. Effects of miR-452 on the biologic behavior of HCT 6 cells **A.** The expression level of miR-452 in miR-452 mimic-transfected cells was signi cantly higher than in the negative control (NC)-transfected cells. ***P < 0.001. **B.** Cell proli fation was easured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in HCT 6 cells transfected with miR-452 mimics or the negative control. **P < 0.01. **C.** Apoptosis of HCT116 cells was detected by flow cytometric analysis after transfection with miR-452 mimics or NC. **D.** miR-452 suppressed HCT116 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-452-transfected group than in the NC-transfected group. **P < 0.01.

DISCUSSION

Dysregulation of miR expression plays an important role in the initiation and development of human cancers. In terms of CRC, abnormal expression of several miRs such as miR-27b, miR-133b, and miR-124 has been reported (Ye et al., 2013; Zhang et al., 2013b; Xiang and Li, 2014). Zhang et al. (2013a) found that ectopic expression of miR-224 promotes CRC tumor cell proliferation, migration, and invasion *in vitro*. Zheng et al. (2014b) indicated that downregulation of miR-132 in CRC is associated with tumor size, distant metastasis, and TNM stage. Furthermore, miR-218, miR-378, miR-378a-3p, and miR-378a-5p expression levels are independent prognostic factors for CRC patients (Yu et al., 2013; Li et al., 2014; Zhang et al., 2014a). miR-129 sensitizes CRC cells to 5-fluorouracil both *in vitro* and *in vivo* (Karaayvaz et al., 2013), and miR-124 can increase the radiosensitivity of CRC cells (Zhang et al., 2014b). These ðndings suggest that miRs might play an important role in CRC initiation and development, and have great potential for clinical application.

In the present study, we observed low miR-452 expression in CRC specimens and cell lines, providing the first evidence that miR-452 downregulation is closely associated with CRC

carcinogenesis. We then investigated the correlation between decreased miR-452 levels and the aggressive clinicopathological features of CRC tissues. Overexpression of miR-452 in HCT116 cells reduced cell proliferation, enhanced cell apoptosis, and impaired cell invasion and migration. These findings reveal that miR-452 might be involved in CRC progression and might contribute to molecular-targeted therapy. In addition, our research showed that CRC patients with low miR-452 levels tended to have shorter overall survival than those with high miR-452 levels. Multivariate Cox hazard regression analysis identified low miR-452 expression as an independent indicator of unfavorable prognosis. To our knowledge, this is the first study to investigate the expression and clinical significance of miR-452 in CRC.

Previous research has described miR-452 downregulation in many human malignancies, as well as its function as a tumor suppressor by targeting a number of oncogenes. In NSCLC, low miR-452 expression is associated with advanced tumor stage and lymph node metastasis (He et al., 2015). *In vitro*, upregulated miR-452 inhibits the invasive capability of NSCLC cells by targeting oncogene *BMI1*. In prostate cancer, miR-452 inhibited proliferation, migration, and invasion of tumor cells by direct or indirect regulation of pathways related to the cell cycle and cellular adhesion (Kristensen et al., 2014). High miR-452 methylation correlated with high PSA level, high T-stage, high Gleason score, and short recurrence-free survival time in prostate cancer patients treated by radical prostatectomy. In addition, miR-452 was downregulated in human gliomas, especially, high-grade, undifferentiated gliomas (Liu et al., 2013). Upregulation of miR-452 suppressed glioma stem-like traits and tumorigenesis, both *in vitro* and *in vivo*. Further, overexpression of miR-452 significantly increased the sensitivity of MCF-7 breast cancer cells to Adriamycin and enhanced cell apoptotic via targeting insulin-like growth factor-1 receptor (IGF1R) (Xu et al., 2014).

In contrast to the antitumor properties mentioned above, miR-452 may act as a potential oncogene in bladder cancer and hepatocellular carcinoma (Veerla et al., 2009; Puerta-Gil et al., 2012; Zheng et al., 2014a). Veerla et al. (2009) confirmed miR-452 upregulation in bladder urothelial carcinomas, and its correlation with lymph node metastasis and shorter overall survival. Zheng et al. (2014a) reported that miR-452 expression levels increased in hepatocellular carcinoma tissues and cell lines. Overexpression of miR-452 can promote cell proliferation and invasion, and inhibit apoptosis in hepatocellular carcinoma cell lines, through targeting cyclin-dependent kinase inhibitor 1B (CDKN1B). Therefore, miR-452 plays dual functions in cancer pathogenesis and progression, and the role of miR-452 could be tumor-specific and possibly dependent on its targets in different cancer types.

In conclusion, our results revealed that miR-452 was downregulated in CRC cell lines and clinical samples. Decreased miR-452 expression was associated with aggressive clinicopathological features and poor prognosis. Regulation of miR-452 expression influenced the biological behavior of the CRC cells. These findings suggest that miR-452 may act as a tumor suppressor in CRC initiation and development, and would be a novel prognostic marker and a potential therapeutic target for the disease.

Conflicts of interest

The authors declare no conflict of interest.

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