

## Original Article

# Prognostic value of miR-221-3p, miR-342-3p and miR-491-5p expression in colon cancer

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**Abstract:** Increasing evidence has demonstrated that microRNAs (miRNAs) are involved in colon cancer initiation and progression, and may serve as diagnostic and prognostic biomarkers for colon cancer. Here, we investigated the levels of miR-9-1, miR-203-3p, miR-221-3p, miR-342-3p, miR-491-5p and miR-503-5p in 90 pairs of colon cancer and adjacent normal tissues, and explored the relationship between their expression and clinical outcome of colon cancer. Five miRNAs (miR-203-3p, miR-221-3p, miR-342-3p, miR-491-5p and miR-503-5p) were dysregulated in colon cancer tissue ( $P < 0.05$ ). The levels of miR-503-5p in larger tumors ( $\geq 6$  cm) were higher than those in smaller ones ( $< 6$  cm) ( $P = 0.031$ ), while the levels of miR-203-3p and miR-491-5p in patients aged 70 years and older were higher than those in patients aged younger than 70 years ( $P = 0.019$  and  $0.049$ , respectively). The high levels of miR-221-3p (HR = 2.416, 95% CI 1.314-4.445,  $P = 0.005$ ), miR-342-3p (HR = 1.807, 95% CI 1.003-3.253,  $P = 0.049$ ) and miR-491-5p (HR = 1.868, 95% CI 1.032-3.384,  $P = 0.039$ ) were significantly associated with worse survival time. Moreover, combination analysis of miR-221-3p, miR-342-3p and miR-491-5p expression revealed that patients with 3 highly expressed miRNAs had lower survival rates compared with those with zero-to-two highly expressed miRNAs (HR = 2.100, 95% CI 1.157-3.813,  $P = 0.015$ ), especially those with TNM stages I and II (HR = 4.204, 95% CI 1.762-10.030,  $P = 0.001$ ). Our results suggest that the three-miRNA signature may help doctors better predict prognosis and guide treatment decisions for colon cancer.

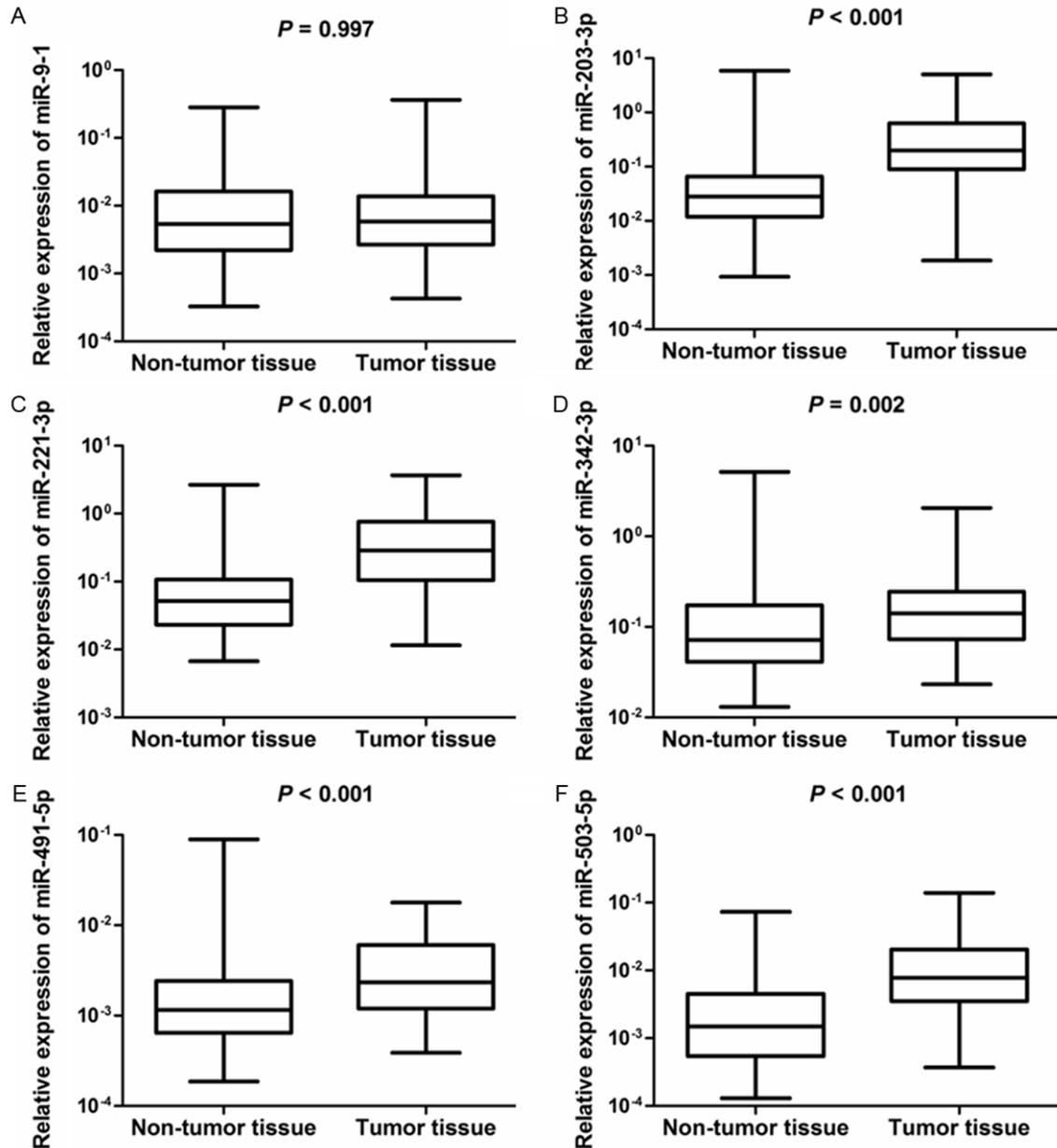
**Keywords:** miR-221-3p, miR-342-3p, miR-491-5p, prognosis, colon cancer

## Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide, accounting for 10% of all new cancer cases and over 1.23 million deaths per year [1]. China is one of countries with high incidence of CRC, in which CRC ranks third among all cancer sites in cancer incidence and fifth in cancer mortality [2]. In addition, CRC incidence and mortality rates are continuing to rise in China. For example, in Shanghai, CRC incidence rate increases annually by 4.2% since 1973 [3]. The prognosis of early-stage CRC is usually favorable, but nearly two-thirds of CRC cases have spread to adjacent or distant organs when diagnosed [4]. In recent years, significant progress has been made in

the molecular pathogenesis and clinical treatment of CRC, but advanced CRC is still extremely difficult to treat. There is no substantial improvement in the prognosis of the majority of CRC patients, especially those in the later stages of the disease. CRC is rapidly becoming a main public health problem due to its high incidence and mortality rates in China.

MicroRNAs (miRNAs) are a class of endogenous small non-coding RNA molecules that regulate gene expression at the post-transcriptional level. To date, more than 2500 miRNAs have been identified in human (miRBase database 20.0) [5]. It is assumed that a given miRNA can target hundreds of target genes and a gene can be targeted by multiple miRNAs [6]. miRNAs are



**Figure 1.** The expression levels of miR-9-1 (A), miR-203-3p (B), miR-221-3p (C), miR-342-3p (D), miR-491-5p (E) and miR-503-5p (F) in 90 pairs of colon cancer and adjacent normal tissues.

involved in almost every biological process, such as cell proliferation, apoptosis and tumorigenesis. Dysregulation of miRNAs contributes to diverse diseases, including cancer [7]. miRNAs act as oncogenes and tumor suppressors in cancer initiation, progression and metastasis [8-12], and affect response to treatment and prognosis of cancer patients [11-18]. For example, Zhang et al. [13] identified six-miRNA signature in 178 patients with stage II colon cancer that can be used to predict the risk of

the recurrence in patients and determine whether or not patients can benefit from chemotherapy. Chen et al. [12] found that miR-214 was downregulated in CRC with liver metastasis, leading to the upregulation of FGFR1 and the promotion of liver metastasis. Furthermore, FGFR1 is one of hot targets for anticancer drug development [19]. As a crucial factor in tumor occurrence and development, miRNA is likely to be new breakthroughs in cancer diagnosis and therapy.

miR-221-3p, miR-342-3p and miR-491-5p and colon cancer

**Table 1.** Association between miRNAs expression and the clinicopathological features

Characteristics	miR-9-1			miR-203-3p			miR-221-3p		
	Low	High	P value	Low	High	P value	Low	High	P value
Age (years)									
≥ 70	21 (46.7)	25 (58.1)	0.296	18 (40.9)	28 (63.6)	0.054	19 (43.2)	27 (61.4)	0.135
< 70	24 (53.3)	18 (41.9)		26 (59.1)	16 (36.4)		25 (56.8)	17 (38.6)	
Sex									
Male	23 (51.1)	24 (53.3)	1.000	27 (60.0)	20 (44.4)	0.205	25 (55.6)	22 (48.9)	0.673
Female	22 (48.9)	21 (46.7)		18 (40.0)	25 (55.6)		20 (44.4)	23 (51.1)	
Tumor size (cm)									
≥ 6	18 (40.9)	16 (36.4)	0.827	14 (31.8)	20 (45.5)	0.280	14 (31.8)	20 (45.5)	0.274
< 6	26 (59.1)	28 (63.6)		29 (67.4)	25 (55.6)		30 (68.2)	24 (54.5)	
Histology grade									
well	1 (2.2)	4 (8.9)	0.371	0 (0)	5 (11.1)	0.016	3 (6.7)	2 (4.4)	0.781
moderate	26 (57.8)	23 (51.1)		22 (48.9)	27 (60.0)		23 (51.1)	26 (57.8)	
poor	18 (40.0)	18 (40.0)		23 (51.1)	13 (28.9)		19 (42.2)	17 (37.8)	
LNM									
no	19 (42.2)	15 (33.3)	0.515	15 (33.3)	19 (42.2)	0.515	13 (28.9)	21 (46.7)	0.127
yes	26 (57.8)	30 (66.7)		30 (66.7)	26 (57.8)		32 (71.1)	24 (53.3)	
TNM stage									
I	6 (13.3)	2 (4.5)	0.317	3 (6.8)	5 (11.4)	0.566	5 (11.1)	3 (6.8)	0.317
II	20 (44.4)	27 (61.4)		27 (60.0)	20 (45.5)		27 (60.0)	20 (45.5)	
III	18 (40.0)	14 (31.8)		14 (31.1)	18 (40.9)		12 (26.7)	20 (45.5)	
IV	1 (2.2)	1 (2.3)		1 (2.2)	1 (2.3)		1 (2.2)	1 (2.3)	
Characteristics	miR-342-3p			miR-491-5p			miR-503-5p		
	Low	High	P value	Low	High	P value	Low	High	P value
Age (years)									
≥ 70	20 (45.5)	26 (59.1)	0.286	19 (43.2)	27 (61.4)	0.135	19 (44.2)	27 (60.0)	0.200
< 70	24 (54.5)	18 (40.9)		25 (56.8)	17 (38.6)		24 (55.8)	18 (40.0)	
Sex									
Male	21 (46.7)	26 (57.8)	0.399	22 (48.9)	25 (55.6)	0.673	23 (51.1)	24 (53.3)	1.000
Female	24 (53.3)	19 (42.2)		23 (51.1)	20 (44.4)		22 (48.9)	21 (46.7)	
Tumor size (cm)									
≥ 6	16 (36.4)	18 (40.9)	0.829	13 (30.2)	21 (46.7)	0.280	13 (30.2)	21 (46.7)	0.130
< 6	27 (62.8)	27 (62.8)		30 (69.8)	24 (53.3)		20 (69.8)	24 (53.3)	
Histology grade									
well	2 (4.4)	3 (6.7)	0.896	3 (6.7)	2 (4.4)	0.896	2 (6.7)	2 (4.4)	0.847
moderate	25 (55.6)	24 (53.3)		24 (53.3)	25 (55.6)		25 (55.6)	24 (53.3)	
poor	18 (40.0)	18 (40.0)		18 (40.0)	18 (40.0)		17 (37.8)	19 (42.2)	
LNM									
no	16 (35.6)	18 (40.0)	0.828	16 (35.6)	18 (40.0)	0.828	16 (35.6)	18 (40.0)	0.828
yes	29 (64.4)	27 (60.0)		29 (64.4)	27 (60.0)		29 (64.4)	27 (60.0)	
TNM stage									
I	5 (11.1)	3 (6.8)	0.888	6 (13.3)	2 (4.5)	0.545	5 (11.1)	3 (6.8)	0.888
II	24 (53.3)	23 (52.2)		23 (51.1)	24 (54.5)		24 (53.3)	23 (52.2)	
III	15 (33.3)	17 (38.6)		15 (33.3)	17 (38.6)		15 (33.3)	17 (38.6)	
IV	1 (2.2)	1 (2.3)		1 (2.2)	1 (2.3)		1 (2.2)	1 (2.3)	

Gene expression analysis can help improve the current understanding of miRNA function in

cancer and may be used to develop predictive markers for patient classification. In this study,

we investigated the levels of six miRNAs (miR-9-1, miR-203-3p, miR-221-3p, miR-342-3p, miR-491-5p and miR-503-5p) in 90 pairs of colon cancer and adjacent normal tissues, and evaluated the relationship between miRNA expression and clinical features and prognosis of patients with colon cancer.

## Materials and methods

### *Patients and samples*

A total of 90 pairs of colon cancer and paracancerous tissues were selected from the Biobank of National Engineer Center for Biochip at Shanghai. Patients were pathologically diagnosed with confirmed primary colon cancer during July 2006 and May 2007. Furthermore, all patients did not receive anti-cancer treatment, including chemotherapy and radiotherapy, before surgery. Ninety matched adjacent normal tissues were collected at sites more than 5 cm away from the tumor margin. The end point of the study was overall survival. All patients gave written informed consent according to the protocol approved by the Committees of National Engineer Center for Biochip at Shanghai and Tongren Hospital.

### *RNA isolation*

Total RNA was isolated from the FFPE tissues by using the RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The concentration and quality of the isolated RNA were assessed on a NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE). All RNA samples were stored at -80°C until subsequent use.

### *Quantitative reverse transcription polymerase chain reaction (qRT-PCR)*

Reverse transcription was performed with the miScript Reverse Transcription Kit (Qiagen, Hilden, Germany) in a final volume of 20 µl containing 6 µl of total RNA, 4 µl of 5× miScript RT buffer and 1 µl of miScript Reverse Transcriptase Mix. The reaction mixtures were incubated at 37°C for 60 min, at 95°C for 5 min, and then held at 4°C. Quantitative RT-PCR was carried out using the miScript SYBR Green PCR kit (Qiagen, Hilden, Germany) on an ABI 7900 Real-time PCR system (Applied Biosystems, CA, USA). Each qRT-PCR reaction was performed in

a final volume of 20 µl containing 1× QuantiTect SYBR Green PCR Master mix (Qiagen, Hilden, Germany), 2 µl of the cDNA, 0.5 mM of each primer. The reaction mixtures were incubated at 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s. All reactions were carried out in triplicate. The expression levels of miRNAs were normalized to the endogenous control RNU6B, and were calculated with the formula  $2^{-\Delta\Delta Ct}$ .

### *Statistical analyses*

Comparison of miRNA levels between colon cancer and adjacent normal tissues was using a Student's t test. The nonparametric test Mann-Whitney-Wilcoxon was used to assess the relationship between miRNA levels and other characteristics. The associations between miRNA levels and continuous variables were tested with the Spearman rank correlation. The expression levels of miRNA were divided into 2 groups (high and low expression groups) according to the median levels, respectively. The chi-square test was used to assess the association of miRNA expression with clinicopathologic characteristics. Survival analysis was performed using the Kaplan-Meier method, and differences between the groups were tested using the log rank test. Hazard ratio (HR) and 95% confidence intervals (CIs) were calculated using the Cox proportional hazard model. All analyses were two-sided, and a *P* value of less than 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS software package (version 19.0, SPSS Inc., Chicago, IL, USA).

## Results

### *Expression levels of miRNAs in colon cancer*

There was no difference in miR-9-1 expression between cancer and normal tissues. Five miRNAs (miR-203-3p, miR-221-3p, miR-342-3p, miR-491-5p and miR-503-5p) were upregulated in colon cancer tissues compared with paracancerous tissues (*P* < 0.05) (**Figure 1**). In addition, there was positive correlation between miR-503-5p level and tumor size (*P* = 0.017). The levels of miR-503-5p in larger tumors (≥ 6 cm) were higher than those in smaller ones (< 6 cm) (*P* = 0.031) (**Figure S1**). Although miR-203-3p and miR-491-5p expression were not correlated with age, the levels of miR-203-3p and

**Table 2.** Univariate and multivariate Cox regression analysis of overall survival in patients with colon cancer

Features	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
age (years), $\geq 70$ vs. $< 70$	1.755 (0.941-3.273)	0.077		
sex, male v female	1.203 (0.672-2.156)	0.534		
tumor size (cm), $\geq 6$ vs. $< 6$	0.761 (0.423-1.370)	0.363		
tumor differentiation, poor vs well, moderate	0.640 (0.358-1.144)	0.132		
TNM stage, III+IV vs. I+II	2.925 (1.628-5.258)	$< 0.001$	4.684 (1.292-16.981)	0.019
LNM, yes vs. no	2.990 (1.664-5.374)	$< 0.001$	2.170 (0.473-9.960)	0.319
miR-9-1, high vs. low	1.481 (0.826-2.653)	0.187		
miR-203-3p high vs. low	1.384 (0.774-2.475)	0.273		
miR-221-3p, high vs. low	2.416 (1.314-4.445)	0.005	2.043 (1.095-3.812)	0.025
miR-342-3p, high vs. low	1.807 (1.003-3.253)	0.049	1.625 (0.895-2.950)	0.110
miR-491-5p, high vs. low	1.868 (1.032-3.384)	0.039	1.605 (0.878-2.934)	0.125

miR-491-5p in patients aged 70 years and older were higher than those in patients aged younger than 70 years ( $P = 0.019$  and  $0.049$ , respectively) (Figure S2).

#### Associations of miRNA expression with clinicopathologic characteristics

Ninety patients were divided into two groups based on the expression levels of miRNAs (i.e., low and high expression groups). The expression level of miR-203-3p was significantly associated with histology grade ( $P = 0.016$ ) (Table 1). No other difference was observed between miRNAs expression and clinicopathologic characteristics.

#### Survival analyses

During follow-up (median: 27 months; range 3 to 85 months), 46 (51.1%) of 90 patients died. The median survival time was 65.5 months. The 3-, 5- and 7-year survival rates were 61.1%, 48.5% and 48.5%, respectively. Survival analyses showed that high levels of miR-221-3p (HR = 2.416, 95% CI 1.314-4.445,  $P = 0.005$ ), miR-342-3p (HR = 1.807, 95% CI 1.003-3.253,  $P = 0.049$ ) and miR-491-5p (HR = 1.868, 95% CI 1.032-3.384,  $P = 0.039$ ) were significantly associated with worse survival time (Table 2) (Figure 2). However, only miR-221-3p expression remain significantly after adjusted for TNM and LNM (HR = 2.043, 95% CI 1.095-3.812,  $P = 0.025$ ). Moreover, combination analysis of miR-221-3p, miR-342-3p and miR-491-5p expression revealed that patients with zero-to-

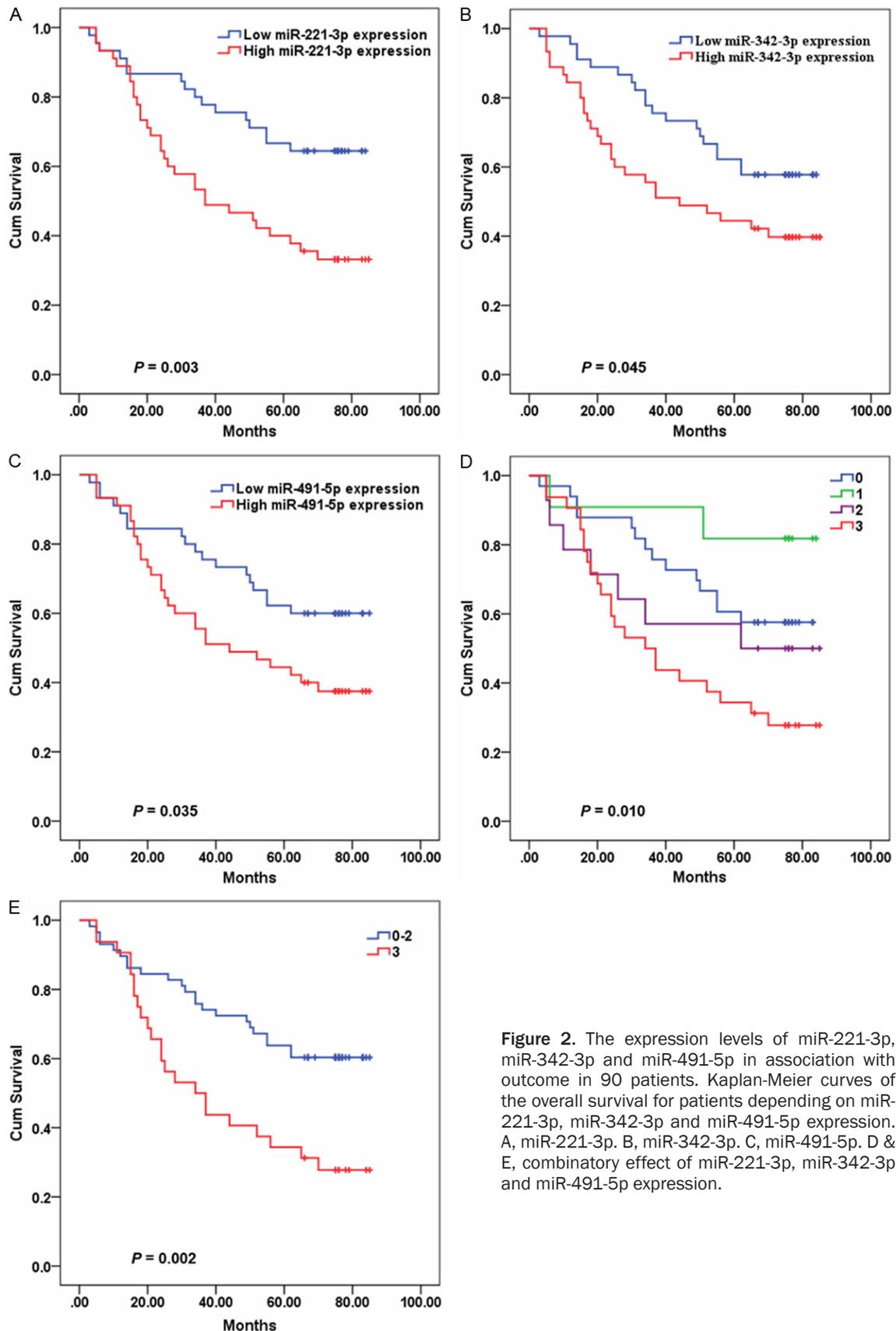
two highly expressed miRNAs had a 60 percent 7-year overall survival rate, whereas patients with 3 highly expressed miRNAs had a 28 percent 7-year overall survival rate (adjusted HR = 2.100, 95% CI 1.157-3.813,  $P = 0.015$ ) (Table 3).

We further performed stratification analyses on clinicopathologic characteristics. Significant associations between miRNA expression and overall survival were found in patients with TNM stages I and II. The high levels of miR-221-3p (HR = 2.796, 95% CI 1.157-6.760,  $P = 0.022$ ), miR-342-3p (HR = 2.929, 95% CI 1.180-7.266,  $P = 0.020$ ) and miR-491-5p (HR = 2.836, 95% CI 1.143-7.038,  $P = 0.025$ ) were inversely associated with overall survival (Figure 3). In addition, patients with 3 highly expressed miRNAs displayed a poorer survival compared with those with zero-to-two highly expressed miRNAs (HR = 4.204, 95% CI 1.762-10.030,  $P = 0.001$ ).

#### Discussion

It is widely accepted that CRC is a group of diverse heterogeneous diseases arising through various molecular pathways. This heterogeneity determines tumor prognosis and response to therapy and brings great challenges not only in studying the molecular basis of the disease, but also in clinical patient management. To date, there are some molecular markers, such as microsatellite instability, the mutation status of BRAF, KRAS and PICK3CA, that are in use for treatment decisions and patient stratifica-

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**Figure 2.** The expression levels of miR-221-3p, miR-342-3p and miR-491-5p in association with outcome in 90 patients. Kaplan-Meier curves of the overall survival for patients depending on miR-221-3p, miR-342-3p and miR-491-5p expression. A, miR-221-3p. B, miR-342-3p. C, miR-491-5p. D & E, combinatory effect of miR-221-3p, miR-342-3p and miR-491-5p expression.

**Table 3.** Combined Effects of miR-221-3p, miR-342-3p and miR-491-5p expression on survival time of patients with colon cancer

No. of highly expressed genes	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
0	1		1	
1	0.377 (0.086-1.660)	0.197	0.363 (0.082-1.602)	0.181
2	1.385 (0.559-3.433)	0.482	1.186 (0.478-2.942)	0.714
3	2.267 (1.163-4.420)	0.016	1.907 (0.964-3.772)	0.064
0-2	1		1	
3	2.396 (1.339-4.288)	0.003	2.100 (1.157-3.813)	0.015

tion. However, patient groups classified by these markers differ remarkably in their response to treatment. There is a strong need to identify novel biomarkers that better define CRC patients whose cancer is aggressive and lethal. In this study, we found that 5 miRNAs were overexpressed in human colon cancer tissues, and the expression levels of miR-221-3p, miR-342-3p and miR-491-5p affected the prognosis of patients with colon cancer.

Dysregulation of miR-221 has been reported in various types of cancer such as CRC [20, 21], prostate [22] and pancreatic cancers [10]. Overexpression of miR-221 promotes many types of cancer cell proliferation, migration and invasion [10, 20, 21, 23, 24], but inhibits prostate cancer cell growth and invasion and induces apoptosis [22]. Accumulating evidence has indicated that dysregulation of miR-221 plays an important role in the development and progression of CRC. miR-221 demonstrated an oncogenic function in CRC [20, 21, 25]. Overexpression of miR-221 promoted CRC cell invasion and metastasis by targeting CDKN1C and RECK [20, 21, 25]. Anti-miR-221 improves the sensitivity of CRC cells to radiation in vivo by upregulation of PTEN, indicating that miR-221 is an important regulator of the radiation response [17]. In this study, we also demonstrated that miR-221-3p was upregulated in colon cancer, and had potential as a prognostic biomarker. However, in contradiction to the above mentioned results, Yuan et al. [26] found that the passenger strand of miR-221 was down-regulated in CRC and exhibit tumor suppressor-like properties.

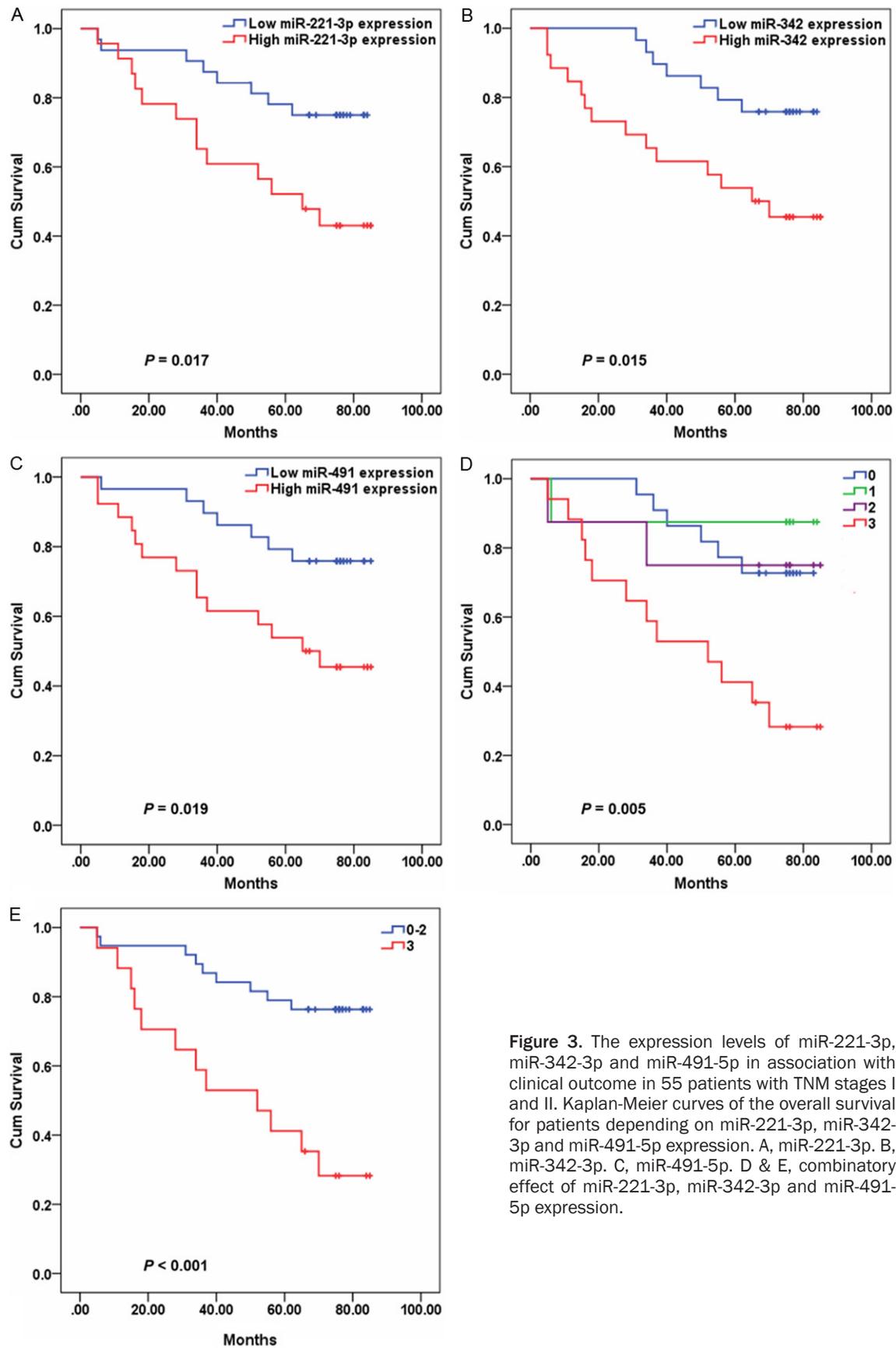
miR-491 functions as a tumor suppressor gene in vitro, and is downregulated in several cancers, including oral squamous cell carcinoma

(OSCC), pancreatic cancer, glioblastoma, breast cancer and hepatocellular carcinoma (HCC) [27-31]. Zhou et al. [31] found that miR-491 suppressed the metastasis of HCC by inhibiting the expression of matrix metalloproteinase and epithelial to mesenchymal transition. A new study

revealed that arsenic trioxide restored the expression of miR-491 via demethylation, resulting in inhibition of the migration/invasion potential of liver cancer cells [18]. In colon cancer cell line DLD-1, miR-491 inhibits Bcl-XL expression and induces apoptosis [9]. Furthermore, miR-491-5p, one mature form of miR-491, suppresses the growth of OSCC, pancreatic cancer and glioblastoma by targeting GIT1, TP53, EGFR, CDK6 and Bcl-XL genes [27-29]. However, in the present study, we found that the level of miR-495-5p was increased in colon cancer, especially in patients aged 70 years and older. High miR-491-5p expression correlated with poor overall survival of patients with colon cancer, which was not in agreement with previous report that OSCC patients with low miR-491-5p expression had poor prognosis [27]. Furthermore, we also found that there was no difference in miR-491-5p expression between colon cancer and adjacent normal tissues in patients aged younger than 70 years (data not shown). These result indicated that there may exist different mechanism of miR-495-5p in older patients with colon cancer. It has been demonstrated that miRNAs act as oncogenes or tumor suppressors in a context dependent manner [32], even in the same type of cancer [33]. Moreover, Li et al. [29] found that miR-491-5p and miR-491-3p targeted different genes and may have different roles in glioblastoma. Further studies are necessary to shed light on the complex role of miR-491-5p in colon cancer.

Downregulation of miR-342 is not only observed in leukemia [34], lung adenocarcinoma [35], CRC [8, 36] and breast cancer [37, 38], but also affects the sensitivity of cancer cell to anti-cancer drugs [34, 39]. In CRC, overexpression of miR-342 leads to a dramatic reduction of DNMT1 expression and thus reactivates

miR-221-3p, miR-342-3p and miR-491-5p and colon cancer



**Figure 3.** The expression levels of miR-221-3p, miR-342-3p and miR-491-5p in association with clinical outcome in 55 patients with TNM stages I and II. Kaplan-Meier curves of the overall survival for patients depending on miR-221-3p, miR-342-3p and miR-491-5p expression. A, miR-221-3p. B, miR-342-3p. C, miR-491-5p. D & E, combinatory effect of miR-221-3p, miR-342-3p and miR-491-5p expression.

ADAM23, Hint1, RASSF1A and RECK, which inhibit cell proliferation and invasion [8]. Furthermore, miR-342 expression has been reported to be associated with the prognosis of breast cancer [37]. However, contrary to the above mentioned results, we found that the level of miR-342-3p was weak but significantly increased in colon cancer, and was inversely associated with the prognosis of patients with colon cancer, which was similar to miR-491-5p. Grady et al. [36] reported that hypermethylation of miR-342 was found in 86% of CRC and in 67% of adenomas, while a recent study reported that only 32.6% of CRC were hypermethylated [40]. These results indicate that inter-tumor heterogeneity exist in CRC and miR-342-3p may play distinct role in CRC cells with different driver genes.

In conclusion, we showed for the first time in clinical specimens that patients with high miR-221-3p, miR-342-3p and miR-491-5p expression had higher risk of death, especially those with TNM stages I and II. Since most patients were 70 years or older in our study, further comprehensive studies are needed to validate these results and to establish them as markers for use in the diagnosis and prognosis of patients with colon cancer.

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#### Disclosure of conflict of interest

None.

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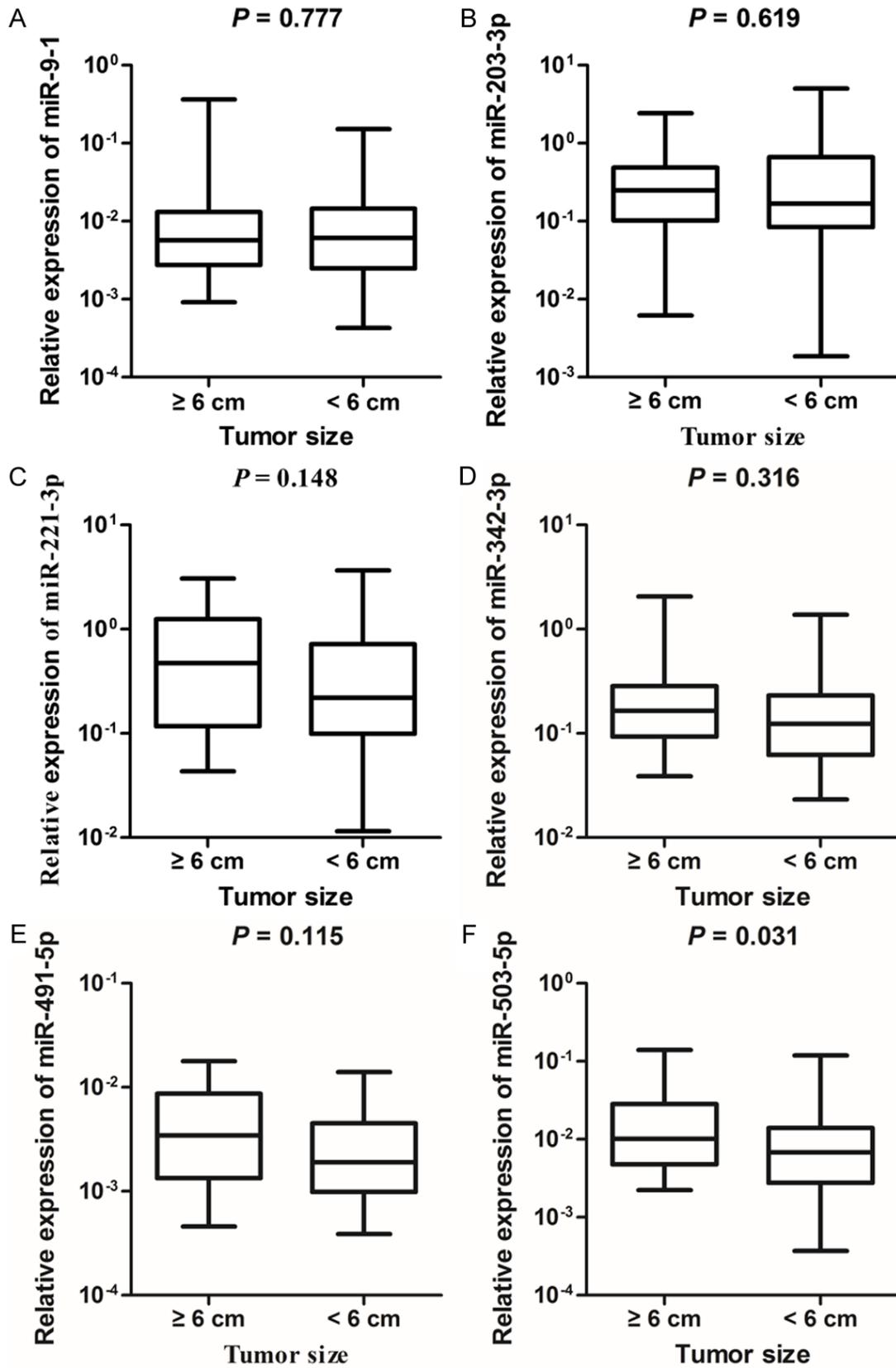


Figure S1. The expression levels of miR-9-1 (A), miR-203-3p (B), miR-221-3p (C), miR-342-3p (D), miR-491-5p (E) and miR-503-5p (F) in relation to tumor size.

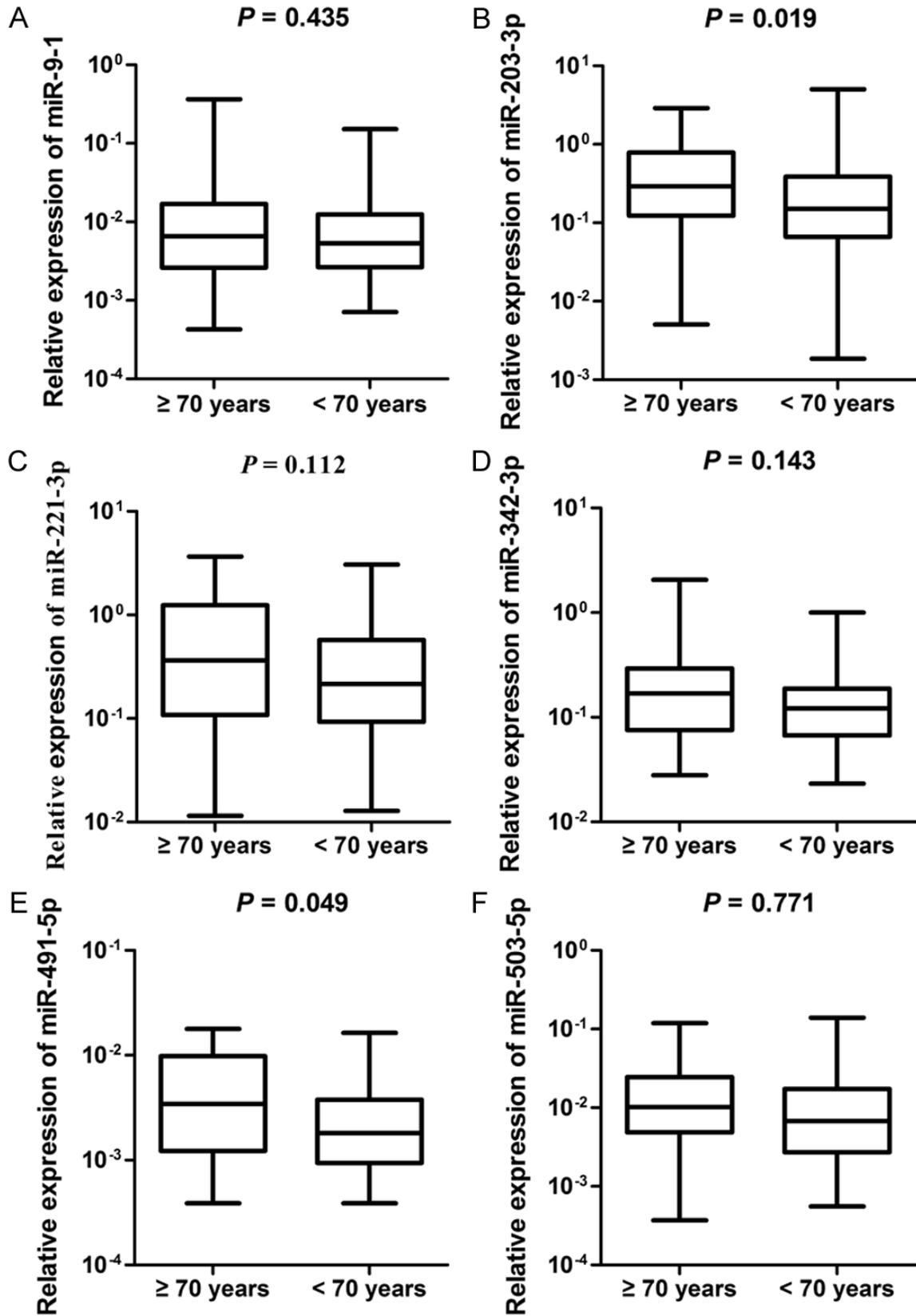


Figure S2. Age-related changes of the expression levels of miR-9-1 (A), miR-203-3p (B), miR-221-3p (C), miR-342-3p (D), miR-491-5p (E) and miR-503-5p (F).