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Original Article

In situ hybridization analysis of the expression of miR-106b in colonic cancer

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Abstract: Background: MicroRNA-106b (miR-106b) is thought to be an oncogenic microRNA that promotes tumor growth and metastasis. The potential predictive value of miR-106b was studied in colonic cancer patients. Methods: The expression of miR-106b was examined in 180 colonic cancer cases using in situ hybridization (ISH) technique and was evaluated semi-quantitatively by examining the staining index. The Correlation of miR-106b expression and clinic-pathological features was analyzed by Spearman Rank Correlation. Wilcoxon signed rank test was used for assessing the expression difference of miRNA-106b between colonic cancerous and para-cancerous ones, and their effects on patient survival were analyzed by a log-rank test and the Kaplan-Meier method. Results: MiR-106b was higher expressed in para-cancerous tissues, compared with colonic cancerous ones ($P < 0.001$). A positive correlation of miR-106b levels between colonic and para-cancerous tissues was also observed (CC = 0.523, $P < 0.001$). Furthermore, the expression of miR-106b was not significantly correlated with clinic-pathological parameters, including gender, age, histological grade, tumor size, pT stage, pN stage, pM stage and pTNM stage of the patients. Histological grade was positively correlated with pT stage ($P = 0.011$), pN stage ($P = 0.036$) and pTNM stage ($P = 0.009$). Patients expressing high levels of miR-106b both in colonic cancer tissues and para-cancerous ones have a relatively longer survival time but the difference is not statistically significant ($P = 0.16$). Conclusions: The expression difference of miR-106b levels between colonic tissues and para-cancerous tissues is statistically significant, but the miR-106b levels were not quite correlated with clinic-pathological characteristics and overall survival times of patients with colonic cancer. Lower levels of miR-106b may be connected with neoplastic effects due to interference with TGF-β signaling, providing evidence that down-regulation of miR-106b might also play an important role in the progression of the disease. The study results are consistent with the literature and support the notion that miR-106b is an oncogenic microRNA.

Keywords: MicroRNA-106b, in situ hybridization, colonic cancer

Introduction

MicroRNAs (miRNAs) are small, non-coding single-stranded RNA molecules. They are transcribed as long primary transcripts, processed to the precursor miRNAs (pre-miRNA), and further processed to yield approximately 22-nucleotide duplexes of which one arm gives rise to the mature miRNA [1]. MiRNAs were discovered in 1993, when a small RNA encoded by the *C. elegans* gene lin-4 was found to have an antisense transcript complementary to lin-14 [2]. MiRNAs regulate gene expression by binding to the 3'-untranslated regions (3'UTRs) of specific mRNAs.

Increasing evidence supported that miRNAs played an essential role in the multiple processes of carcinogenesis, including cell growth, apoptosis, differentiation, invasion and angiogenesis of tumor blood vessels [3, 4]. MiRNAs could function as oncogenic miRNAs or tumor suppressor miRNAs [5, 6]. Dysregulation of miRNA expression has been found in various types of human cancers, including cancers occurred in the breast, colon, and lung, chronic lymphocytic leukemia and malignant glioma [7-11].

Over-expression of miR-106b has been observed in a variety of human tumors, including colorectal cancer [12], gastric cancer [13], hepatocellular carcinoma [14] and head and neck squamous cell carcinomas [15]. Phenotypic analyses revealed that miR-106b family promoted exit from G1 and entry into S phase.

Table 1. Association between the miR-106b expression and clinic-pathological features in patients with colorectal cancer

Characteristics	Cases	miR-106b expression level in colonic cancer tissues		*P* value	Cases	miR-106b expression level in para-cancerous tissues		*P* value
		No of low expression	No of high expression			No of low expression	No of high expression	
Gender				0.593				0.757
Male	94	55	28		91	41	42	
Female	89	46	28		86	39	44	
Age (years)				0.118				0.8
≤ 60	41	18	16		41	19	19	
> 60	142	83	40		136	61	67	
Tumor size (cm)				0.993				0.075
≤ 5	106	59	31		101	40	53	
> 5	73	42	22		73	40	30	
Histological grade				0.668				0.895
I	16	7	6		16	8	7	
II	115	63	35		110	51	54	
III	52	31	15		51	21	25	
pT stage				0.372				0.278
T1	3	2	1		3	1	1	
T2	12	4	4		12	7	4	
T3	137	76	45		135	56	71	
T4	28	19	5		24	15	9	
pN stage				0.372				0.777
N0	112	60	35		111	46	54	
N1	51	30	16		48	25	23	
N2	20	11	5		18	9	9	
pM stage				0.665				0.526
M0	178	98	54		173	78	84	
M1	4	3	1		3	2	1	
pTNM stage				0.602				0.799
I	13	4	5		13	2	5	
II	97	56	29		96	6	48	
III	67	38	20		63	40	31	
IV	4	3	1		3	2	1	

The oncogenic properties of the miR-106b family of microRNAs may stem from combined positive regulation of the cell cycle and additional functions [16].

In recent years, the in situ hybridization (ISH) technique was found to be widespread applicated in diagnostic clinical practice. Detection of miRNAs in formalin-fixed paraffin embedded (FFPE) tissues sections has been demonstrated by ISH using locked nucleic acid (LNA) probes [17], which impled that miRNA analysis of FFPE tissue could offer a very promising technique for discovering biomarkers and novel targets in cancer research.

In our previous study, we used microarray technology to analyze the different miRNA expression profiles between colonic cancer and para-cancerous tissues, and found that 14 miRNAs were associated with colonic cancer in which the expression of miR-106b was up-regulated in colonic cancer tissues, compared with the para-cancerous control. These miRNAs could play an important role in the carcinogenesis of colon [18].

In this study, the expression of miRNA-106b was evaluated by the ISH technique using FFPE tissue sections and an LNA modified probe. MiRNA-106b expression patterns were obser-

ved in colonic cancer and para-cancerous tissues.

Materials and methods

Patients and tissue specimens

Paraffin-embedded colonic tumor tissues and para-cancerous tissues were selected from 180 colonic cancer patients that were diagnosed and treated in the Department of General Surgery, Tongji Hospital, Tongji University. Histological confirmation of the colonic diagnosis and scoring of all the cases were performed at least by two independent pathologists according to the WHO Histological Classification. The tissues were acquired from the archival collections of the Department of Pathology, and used for subsequent in situ hybridization and immunohistochemistry. The clinic-pathological data is illustrated in **Table 1**. Tumors were staged according to the TNM staging system. All the tissue samples were from untreated patients undergoing surgery. The survival rate of the patients was calculated from the date of resection to the date of death.

In situ hybridization (ISH)

Two tissue microarrays including 116 pairs of CRC and corresponding non-tumor tissues are purchased from BioChip (Shanghai, China) and placed in a Tecan Freedom Evo automated hybridization instrument (Tecan, Ma·nnedorf, Switzerland) in which the following steps were performed: proteinase-K treatment 15 μg/ml at 37°C for 15 min, pre-hybridization in Exiqon hybridization buffer (Exiqon, Vedbæk, Denmark) at 50°C for 60 min,hybridization with 200 nM miR-106b probe, stringent washes with 5×SSC, 1×SSC and 0.2×SSC buffers at 50°C over 20 min, DIG blocking reagent (Roche, Mannheim, Germany) in maleic acid buffer containing 2% sheep serum, alkaline phosphatase-conjugated anti-digoxigenin (diluted 1:500 in blocking reagent, Roche) at 4°C for 24 h, enzymatic development using 4-nitroblue tetrazolium (NBT) and 5-brom-4-chloro-30-Indolyl-phosphate (BCIP) substrate (Roche) forming dark-blue NBT-formazan precipitate at 25°C for 60 min, nuclear fast red counterstain (Vector Laboratories, Burlingname, CA), at 25°C for 1 min. The slides were then dismantled in water, dehydrated in alcohol solutions and mounted with eukitt mounting medium (VWR, Herlev, Denmark). For each patient, two slides were hybridized with the full length miR-106b probe.

Scoring of ISH

The expression of miR-106b in 180 paraffin-embedded colonic cancer specimens was examined and scored separately by two independent investigators blinded to the histopathological features and patient data for the samples. In each section, 5 × 1000 tumor cells were counted randomly, and the scores were determined by combining the proportion of positively stained tumor cells and the intensity of staining. The proportion of positively stained tumor cells was graded as follows: 0, less than or equal to 10% positive tumor cells; 1, greater than 10% positive tumor cells. The cells at each intensity of staining were recorded on a scale of 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). For tumors that showed heterogeneous staining, the predominant pattern was taken into account for scoring. The staining index (SI) was calculated as follows: staining index = proportion of positively stained tumor cells × staining intensity.

Statistical analysis

Statistical analysis was performed using SPSS software (v.19.0, SPSS, Chicago, III). Wilcoxon signed rank test was used for assessing the expression difference of miRNA-106b between colonic cancerous and para-cancerous ones. Bivariate correlations between study variables were calculated via Spearman's rank correlation coefficients. Survival curves were plotted by the Kaplan-Meier method and compared by the log-rank test. A difference of $P < 0.05$ between groups was considered significant.

Results

Expression levels of miR-106b in colonic cancerous and para-cancerous tissues

We identified gene expression levels of the miR-106b in 180 colonic tumor tissues and para-cancerous ones. For the specific identification of miR-106b in tissue sections using ISH, we employed high-affinity LNA-containing DNA oligomers labeled at the 5'-end with digoxigenin. The clinic-pathological parameters of the 180 colonic cancer patients used in this study are described in **Table 1**. Differences between the two groups were evaluated using the

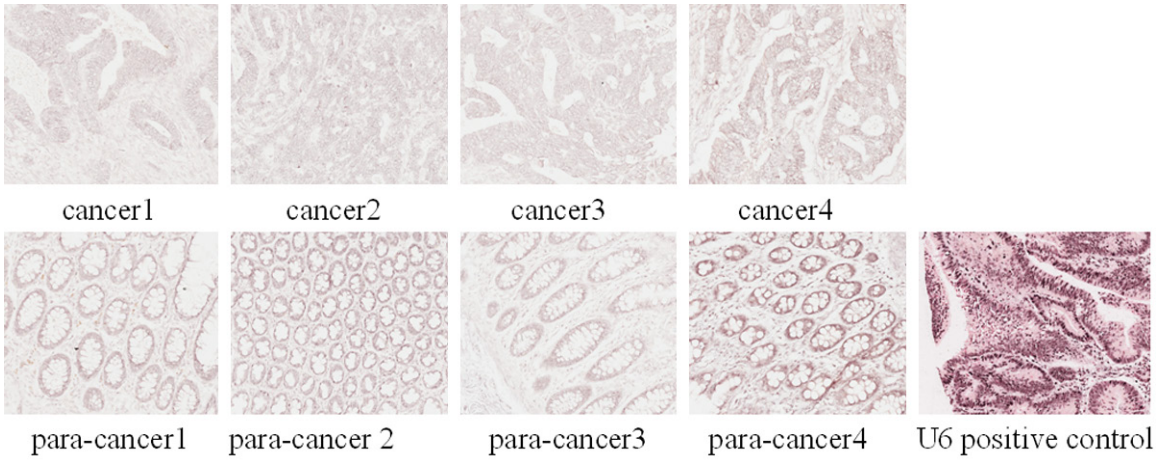

Figure 1. In situ hybridization (ISH) analysis of microRNA (miRNA)-106b with locked nuclei acid probes. A. ISH with a positive control probe (U6 small nuclear RNA [snRNA]) shows positive staining in the nuclei of all cellular compartments.

Wilcoxon test for 180 paired samples. We found that miR-106b was higher expressed in para-cancerous tissues, compared with colonic cancerous ($P < 0.001$) (**Figure 1**). In addition, Spearman order correlation analysis showed that expression level of miR-106b in colonic tumor tissues was positively correlated with the miR-106b level in para-cancerous ones (CC = 0.533, $P < 0.001$).

Correlation of miR-106b with clinic-pathological characteristics of colonic cancer

To further evaluate whether miR-106b high-expression was linked to the clinical progression of colonic cancer, we analyzed the association of miR-106b with the clinic-pathological status of colonic cancer patients (**Table 1**). Tumors expressed lower levels of miR-106b, suggesting that miR-106b up-regulation was somewhat associated with tumor suppression. However, no significant correlation was observed between miR-106b expression and gender, age, histological grade, tumor size, pT stage, pN stage, pM stage and pTNM stage of the patients. Histological grade was positively correlated with pT stage ($P = 0.011$), pN stage ($P = 0.036$) and pTNM stage ($P = 0.009$).

MiR-106b expression is not related to poor survival of colonic cancer patients

A log-rank test and Kaplan-Meier analysis were used to calculate the effect of miR-106b expression on patient survival (**Figure 2**). Kaplan-Meier curves showed no relationship between miR-106b and cumulative survival in patients with colonic cancer. Patients expressing high levels of miR-106b both in colonic cancer tissues and para-cancerous ones have a relatively longer survival but the difference is not statistically significant ($P = 0.16$). Specifically, the median cumulative survival percent of patients whose tumors expressed high levels of miR-106b was 60.7, whereas the median survival percent of those with low levels of miR-106b expression was 51.5. The median cumulative survival percent of patients whose para-cancerous tissues expressed high levels of miR-106b was 59.3, whereas the median survival percent of those with low levels of miR-106b expression was 55.0.

Discussion

Previous studies have revealed that miRNA alterations may play a fundamental role in different steps of tumor formation and progression [19]. MiR-106b family was overexpressed in multiple tumor types and was correlated with the expression of genes that regulate the cell cycle. Thus, miR-106b family members contributed to tumor cell proliferation in part by regulating cell cycle progression and by modulating checkpoint functions [16].

It has been proved that the miR-106b-25 cluster, up-regulated in a subset of human gastric tumors, is activated by E2F1 in parallel with its host gene, Mcm7. In turn, miR-106b and miR-

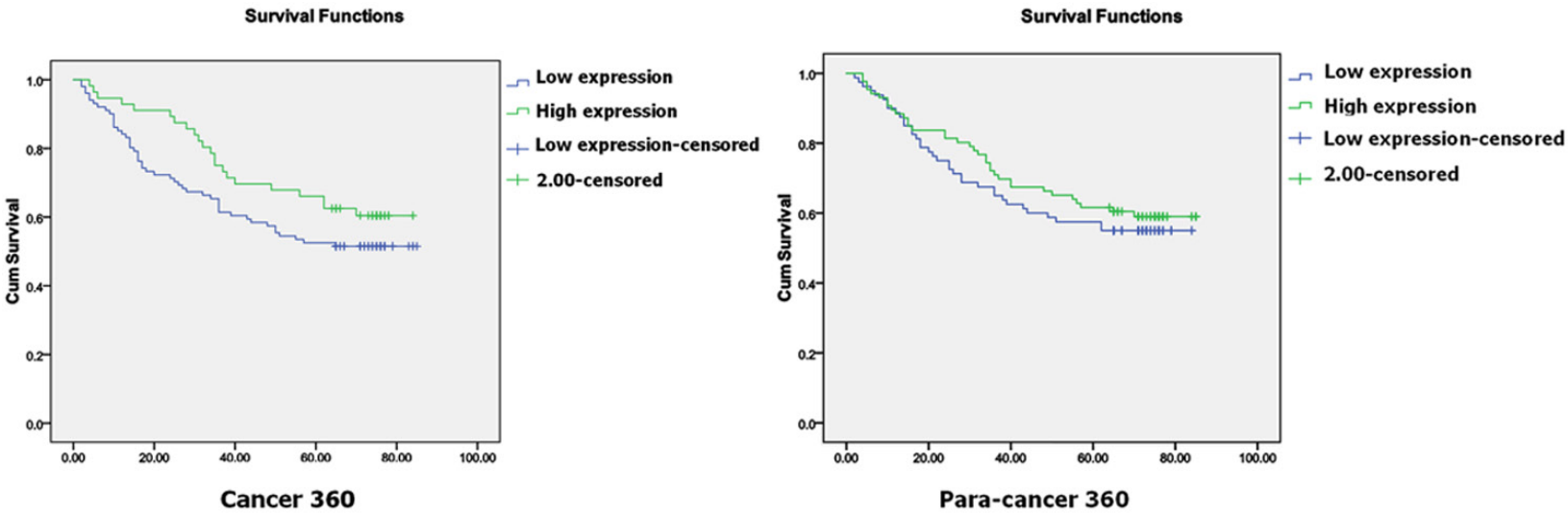

Figure 2. Kaplan-Meier curves showing no relationship between miR-106b and cumulative survival in patients with colonic cancer. Patients expressing high levels of miR-106b both in colonic cancer tissues and para-cancerous ones have a relatively longer survival but the difference is not statistically significant ($P = 0.16$).

93 regulate E2F1 expression, establishing a miRNA directed negative feedback loop. Furthermore, up-regulation of these miRNAs impairs the TGFb tumor suppressor pathway, interfering with the expression of CDKN1A (p21Waf1/Cip1) and BCL2L11 (Bim) [20].

It is also demonstrated a significant correlation between miR-106b, Six1, and activated TGF-β signaling in human breast cancers, and further showed that high levels of miR-106b and miR-93 in breast tumors significantly predicted shortened time to relapse. These findings expand the spectrum of oncogenic functions of miR-106b-25, and may provide a novel molecular explanation, through the Six1 regulated miR-106b-25 cluster, by which TGF-β signaling shifts from tumor suppressive to tumor promoting [24].

Although published data on microRNA-106b have identified it as overexpressed in several types of cancers and functionally linked to proliferation and anti-apoptotic pathways [21], there was also evidence that this microRNA is invariant in many tissues [22, 23].

The expression level of miR-106b was significantly upregulated in the tumor compared to the non-tumoral renal parenchyma. However, metastatic patients tended to have lower level of miR-106b in renal cell carcinoma (RCC) compared to the patients in remission. Moreover, miR-106b indicated significant potential to predict early metastasis after nephrectomy. Surprisingly, miR-106b, considered to be oncogenic [25], has significantly higher expression levels in RCC of patients with better prognosis. A possible explanation for this contradiction lies involvement of the miR-106b family (miR-106b, miR-93, and miR-25) in TGF-β signaling [26]. The role of TGF-β signaling in cancer pathogenesis was characteristically ambiguous [27]. In the early events of carcinogenesis, TGF-β levels are lower and indicated features of a tumor suppressor, but in the late phase, within the development of metastatic disease, the degree of TGF-β activation increases and lead to the promotion of immunosuppression, neoangiogenesis and progression of the disease. This was the first report that the expression of miR-106b had a correlation with the development of metastasis and relapse-free survival in RCC patients after nephrectomy.

In our previous study, all tissues samples used for microarray were from patients confirmed as showing no evidence of lymph node metastasis. Metastatic patients may have lower miR-106b levels in colonic tissues compared to that of paracancerous ones. In addition considering the TNM stage of colonic caner, it is possible due to a general tendency for miR-106b levels to decrease from earlier stages towards advanced. Higher levels of miR-106b in selected colonic tissues may be connected with antineoplastic effects due to interference with TGF-β signaling. When lower levels of miR-106b occurred within the development of metastatic disease, the degree of TGF-β activation increased and lead to the promotion of immunosuppression, neoangiogenesis and progression of the disease.

In summary, the results of this study indicated that the expression difference of miR-106b levels between colonic tissues and para-cancerous tissues was statistically significant. The miR-106b levels were not quite correlated with clinic-pathological characteristics and overall survival times of patients with colonic cancer. Lower levels of miR-106b in selected colonic tissues may be connected with neoplastic effects due to interference with TGF-β signaling, providing evidence that down-regulation of miR-106b might also played a pivotal role in the progression of the disease. The results of this study were consistent with the literature and support the notion that miR-106b was an oncogenic microRNA.

Disclosure of conflict of interest

None.

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