Decreased serum miR-181a is a potential new tool for breast cancer screening

LI-JUAN GUO and QING-YUN ZHANG

Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Clinical Laboratory, Peking University Cancer Hospital and Institute, 100142 Beijing, P.R. China

Received March 9, 2012; Accepted April 18, 2012

DOI: 10.3892/ijmm.2012.1021

Abstract. Breast cancer (BC) screening is important for early detection, but conventional tumor markers lack the desired sensitivity. Aberrant microRNA (miRNA) expression plays an important role in tumor formation and development. Thus, serum miRNAs represent potential BC biomarkers. microRNA-181a (miR-181a) is deregulated in many types of human cancer and is a key oncogenic regulator, but the relationship between serum miR-181a and BC diagnosis has not been investigated. This study investigated serum miR-181a levels in BC patients and healthy controls and compared the diagnostic value of serum miR-181a as a BC tumor marker with the conventional tumor markers CA153 and CEA. Serum miR-181a and miR-16 (as a control) were quantified by real-time quantitative RT-PCR in 20 plasma samples. The promising results prompted analysis of 227 additional samples. The levels of CA153 and CEA were measured using electrochemiluminescence assays. Median miR-181a levels were significantly lower in patients with BC compared to healthy controls (P=0.001). ROC analysis demonstrated the sensitivity and specificity of miR-181a for BC diagnosis at 70.7 and 59.9%, respectively, whereas the sensitivities of CA153 and CEA were 10.53 and 9.21%. As a tumor marker, serum miR-181a expressed a higher level of sensitivity [55.28% (68/123)] in the early stage of BC diagnosis (ductal carcinoma *in situ*, TNM I and II) than the CA153 and CEA markers (8.13 and 7.32%, respectively). There were no significant associations between miR-181a levels and other clinicopathological parameters. These results suggest that serum miR-181a may represent a novel biomarker for primary BC as well as for early stage BC diagnosis. In combination with other markers, serum miR-181a may improve the sensitivity of BC screening.

Correspondence to: Professor Qing-Yun Zhang, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Clinical Laboratory, Peking University Cancer Hospital and Institute, 52 Fucheng Road, 100142 Beijing, P.R. China
E-mail: zhqy_208@163.com

Key words: miR-181a, breast cancer, real-time PCR, diagnosis

Introduction

Breast cancer (BC) is the most frequently diagnosed cancer and the leading cause of cancer mortality in women, accounting for 23% of all cancer cases and 14% of cancer-related deaths (1). For 2011, an estimated 230,480 new cases of invasive BC and 39,520 BC deaths were expected among US women (2,3). Reliable markers are still lacking, and the most promising serum markers, CEA and CA153, are not sensitive or specific enough for screening asymptomatic women. Currently, the most efficacious clinical screening tool for BC is mammography (1). This approach is too expensive to be feasible in most developing countries, where screening rates continue to be lower for poor women (1,2). Serum-based screening is easier, less invasive and more affordable than mammography. Thus, leading to an increased interest in identifying and validating serum-based biomarkers for the diagnosis of BC.

microRNAs (miRNAs) are a class of 20-25 nucleotide nonprotein-coding small RNAs that negatively regulate gene expressions by cleaving, inhibiting or degrading mRNA translation. Negative regulation by miRNAs controls crucial physiological processes, such as cell differentiation, proliferation, apoptosis, development and cell metabolism (4-8). Numerous studies have shown that aberrant miRNA expression is associated with the development and progression of various types of human cancer (9-13). Although their biological function remains largely unknown, some miRNAs have functions similar to those of oncogenes or tumor suppressors (14-16). Bioinformatic data indicate that miRNAs have the potential to regulate at least 20-30% of all human genes. A single miRNA can control the expression of hundreds of miRNA gene targets (17,18). More than 50% of the annotated human miRNA genes are located in cancer-associated genomic regions or in fragile sites and may thus play important roles in tumorigenesis (19).

Recently, miRNAs in serum and plasma have been investigated as promising novel biomarkers for cancer diagnosis and prognosis (20). Many studies have emerged recently showing that miR-181a is expressed at low levels in a variety of tumors including lung (21), oral (22), hepatocellular (23), ovarian (24) and glioblastoma (25). However, published studies regarding serum miR-181a in BC do not exist. On the basis of findings in other types of cancer, we hypothesized that deregulated miR-181a may also be present in the serum of patients with BC and might even serve as a diagnostic marker.

In the present study, we first investigated circulating miR-181a levels in the sera from a small sample of BC and healthy subjects. Based on promising results in the small sample, we also analyzed a larger sample and compared the sensitivity of miR-181a in diagnosing early stage BC to the sensitivity of CA153 and CEA. Further analysis explored the relationship of serum miR-181a levels with BC clinical and pathological features.

Materials and methods

Study subjects. In this study, 152 women were recruited who had a histologically confirmed diagnosis of primary BC (median age, 50.38 years; range, 25-83 years). Serum samples were collected from 75 age-matched healthy women who underwent medical examinations at the Beijing Cancer Hospital between March and October 2010 (median age, 48.4 years; range, 22-60 years).

Indicators. The level of CEA and CA153 were determined by electrochemiluminescence immunoassays using the Modular E170 automated analyzer (Roche, Basel, Switzerland). The normal upper limits were 5.0 mg/l for CEA and 25 kU/l for CA153. Hormone receptor (ER, PR) status was determined by routine immunohistochemical methods. Tumors were staged according to the AJCC tumor-node-metastasis (TNM) staging system, and histopathological grading was also performed (lymph node metastasis/no lymph node metastasis and ER and PR status). Clinicopathological characteristics of the BC patients/samples are shown in Table I. This study was approved by the Clinical Research Ethics Committee of the Peking University Cancer Hospital.

Sample processing. We collected 6 ml of venous blood from the antecubital fossa into an EDTA tube. The blood was centrifuged at 1600 rpm at 4˚C (Sorvall Biofuge Stratos, Heraeus, Germany) for 5 min to spin down the blood cells. The supernatant was removed to a 1.5 ml Eppendorf tube followed by a second centrifugation at 12,000 rpm for 15 min at 4˚C (Heraeus Fresco 21 microcentrifuge, Thermo Electron Corporation, Langenselbold, Germany) to completely remove cell debris. Serum samples were frozen within 4 h of blood collection and stored at -70˚C until analysis.

Total-RNA extraction. Total-RNA was extracted from 500 μl serum using TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. Total-RNA was suspended in a fixed volume of 100 μl diethyl pyrocarbonate (DEPC)-treated water for use in the reverse transcription (RT) reaction.

Stem-loop primer based RT reaction. Mature miRNA sequences were obtained from the miRBase database (http://microrna.sanger.ac.uk/). Stem-loop primers, specific primers and universal primers were designed using Primer 5.0 (Table II). cDNA was synthesized from total-RNA via RT. First, 10 μl of purified total RNA plus 3 μl stem-loop RT primer was denatured at 70˚C for 5 min before quenching on ice for 5 min. We then added 0.5 μl dNTP (Tiangen Biotech Co., Ltd., Beijing, China), 2 μl MDTT, 0.2 μl RNase inhibitor, 1 μl

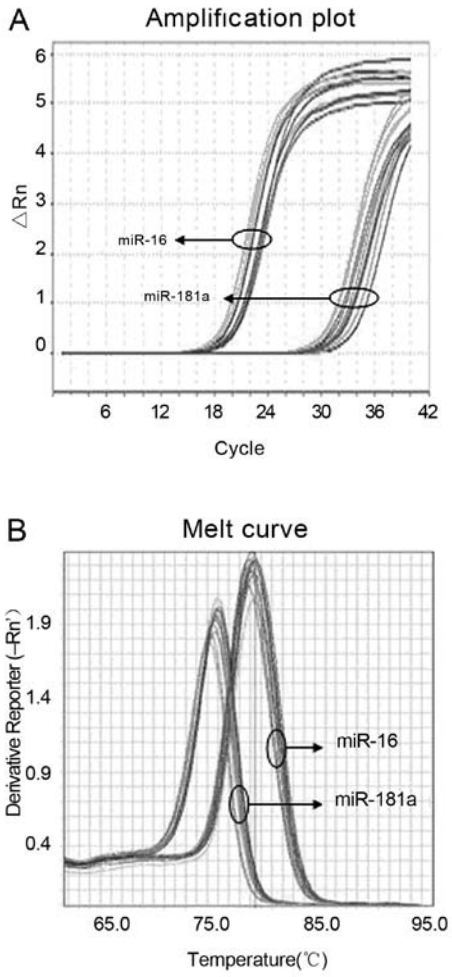

Figure 1. miR-16 and miR-181a (A) amplification plots and (B) melt curves were used to examine miRNA purity. The appearance of single peaks for the miRNAs during melting curve analysis indicated that miR-16 and miR-181a could be efficiently extracted and specifically amplified from serum samples.

M-MLV reverse transcriptase (Promega, Madison, WI, USA) and 3.3 μl 1X M-MLV RT buffer (Promega) for a final reaction volume of 20 μl (Table III). Each reverse transcription reaction was performed in a 96-well plate using a Bio-Rad MyCycler™ Thermal Cycler (Bio-Rad, USA) for 30 min at 16˚C, 30 min at 37˚C and 10 min at 70˚C. Reactions were held at 4˚C.

Real-time PCR. The SYBR-Green qRT-PCR assay was used for miRNA quantification and was performed on an ABI 7500 Real-Time PCR instrument (Applied Biosystems). Each reaction was performed in a final volume of 20 μl that contained 1 μl of cDNA, 0.5 mmol/l of each primer and 1X SYBR-Green PCR Master Mix (Roche). The amplification profile was as follows: denaturing at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. Melting curve analysis was performed to validate the specificity of the expected PCR product (Fig. 1). All samples were run in duplicate, including blank controls without cDNA. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold in qPCR. Serial dilution of cDNA was used to generate the standard curves. There was excellent linearity between the log of the miRNA concentration and the cycle threshold (Ct) value (Fig. 2).

Table I. Clinical characteristics and serum miR-181a levels in the breast cancer patients.

Characteristic	n	miR-181a levels (mean ± SD)	Mann-Whitney U/ Kruskal-Wallis H/t-test[a]
Group			P<0.001
Healthy group	75	1.0908±0.50821	
Breast cancer group	152	0.8385±0.69453	
TNM stage			P=0.285
DCIS	4	1.0546±0.60466	
I	40	0.9220±0.71448	
II	79	0.8837±0.75118	
III	13	0.5700±0.37882	
IV	16	0.57103±0.47530	
Invasive ductal carcinoma histological grade	137		P=0.269
I	5	1.20271±0.87914	
II	104	0.79574±0.68236	
III	19	0.94171±0.82403	
Lymph node metastasis			P=0.742
Positive	72	0.8425±0.72189	
Negative	72	0.8201±0.66547	
ER status			P=0.213
Positive	104	0.8425±0.74682	
Negative	45	0.8632±0.57218	
PR status			P=0.944
Positive	92	0.7723±0.71820	
Negative	57	0.9425±0.64144	

Data not known for 8 cases of lymph node metastasis and 3 cases each for ER and PR status; [a]Significance was determined by the appropriate test.

Table II. Sequences of the miR-16 and miR-181a stem-loop reverse transcriptase (RT) primers and PCR primers.

Primer	Sequence
RT primers	
miR-16	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGCCAAT-3'
miR-181a	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTCACCGA-3'
Specific primers	
miR-16	5'-GGCGTAGCAGCACGTAAATAT-3'
miR-181a	5'-GAACATTCAACGCTGTCGGTG-3'
Universal primer	5'-ATCCAGTGCAGGGTCCGAGGTA-3'

miR-16: y = -1.8919x + 31.497 (R^2=0.9965); miR-181a: y = -2.032x + 38.82 (R^2=0.9958).

Statistical analysis. The significance of serum miR-181a levels was determined by the Mann-Whitney U test, t-test or Kruskal-Wallis H test as appropriate. Data were expressed as mean values ± standard deviations (SD), and the relative expression of miR-181a and the fold-change of expression was determined by the $2^{-\Delta\Delta CT}$ method: RQ = $2^{-\Delta\Delta CT}$, ΔΔCT = (CT miR-181a - CT miR-16) tumor - (CT miR-181a - CT miR-16) mean normal (17,18). Receiver operating characteristic (ROC) curves were established for discriminating between patients with or without BC. All P-values are two-sided, and values <0.05 were considered statistically significant. All statistical calculations were performed using SPSS software (version 13.0).

Results

Expression of miR-181a in the serum of BC and healthy subjects. We first analyzed the profiles of serum miR-181a in

Table III. Comparison of miR-181a and the conventional tumor markers CA153 and CEA for identifying breast cancer patients.

TNM	miR-181a positive (<0.7486), %	CA153 positive (>25.0), %	CEA positive (>5.0), %
DCIS	50.00 (2/4)	0.00 (0/4)	0.00 (0/4)
I	57.50 (23/40)	2.50 (1/40)	2.50 (1/40)
II	54.43 (43/79)	11.39 (9/79)	10.13 (8/79)
III	69.23 (9/13)	7.69 (1/13)	0.00 (0/13)
IV	75.00 (12/16)	31.25 (5/16)	31.25 (5/16)
Early stage	55.28 (68/123)	8.13 (10/123)	7.32 (9/123)
Later stage	72.41 (21/29)	20.69 (6/29)	17.24 (5/29)
Sum	58.55 (89/152)	10.53 (16/152)	9.21 (14/152)

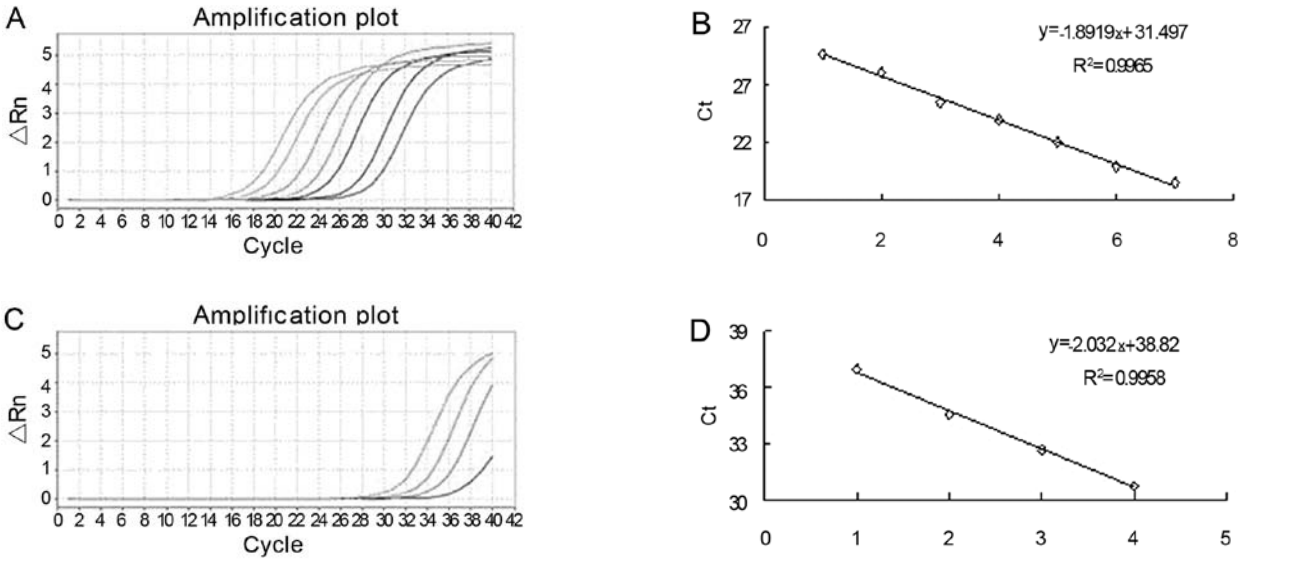

Figure 2. Amplification plots and standard curves for (A and B) miR-16 and (C and D) miR-181a using SYBR-Green. There was excellent linearity between the log of the miRNA concentration and the cycle threshold (Ct) value. Serial 1:4 dilutions of cDNA were used to generate the standard curves.

a small sample of 10 healthy controls and 10 BC patients. The median level of miR-181a was significantly lower in patients with BC than in normal controls (NC): 0.502592±0.44508 vs. 1.412649±0.465748, respectively (P=0.001). The median fold-change of miR-181a for BC/NC was 0.35578. A similar trend was obtained in a larger sample of 75 healthy controls and 152 BC patients: 1.0908±0.50821 vs. 0.8385±0.69453 (P=0.001), with the levels of serum miR-181 significantly downregulated in patients vs. controls (Fig. 3A). A ROC curve analysis showed AUC =0.671±0.031. At the cut-off value of 0.7486 for miR-181a, the sensitivity and specificity for this marker were 70.7 and 59.9%, respectively (Fig. 4).

Comparison of miR-181a with the conventional BC markers CEA and CA153. To investigate whether miR-181a was a potential biomarker for the diagnosis of BC, we compared the sensitivity of miR-181a, CA153 and CEA in each BC stage (*in situ*, I, II, III and IV). As shown in Table I, miR-181a sensitivity for the diagnosis of BC was 58.55%, while the sensitivity of CA153 and CEA were only 10.53 and 9.21%, respectively. Serum miR-181a also showed higher sensitivity (55.28%) for the diagnosis of early stage BC (*in situ*, I, II) compared with CA153 (8.13%) and CEA (7.32%) (Table III).

Association of miR-181a expression with clinical and pathological features. We compared the expression of miR-181a in patients with different clinical TNM classification stages (Fig. 3B), histological grades and hormone receptor status. Statistical analysis revealed no significant correlations between miR-181a and ER or PR status in BC (P>0.05). Study subject characteristics are summarized in Table I.

Discussion

CEA and/or CA153 are currently used as serum markers for BC. Unfortunately, these markers have low sensitivity and specificity for BC screening. miRNAs are small non-coding RNA molecules 20-25 nucleotides in length that help regulate a variety of biological signaling pathways. Some miRNAs are closely related to the development, invasion, metastasis and other characteristics of tumors. Calin *et al* (19) showed that half of the known miRNAs are in cancer-associated genomic

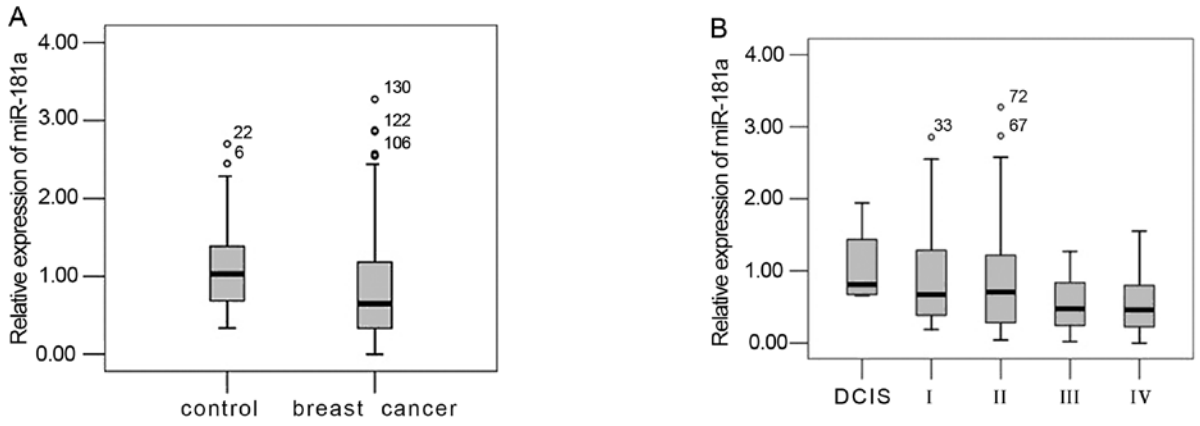

Figure 3. miR-181a expression in healthy controls and in breast cancer patients. (A) Comparison of miR-181a relative expression levels in serum from healthy controls (n=75) and breast cancer patients (n=152) as assessed by qRT-PCR after normalization using the serum miR-16 level. miR-181a expression was significantly lower in breast cancer patients than in healthy controls (P<0.001, Mann-Whitney). (B) Comparison of miR-181a expression levels in serum from breast cancer patients with different TNM stages; there were no significant differences (P=0.285, Kruskal-Wallis H test).

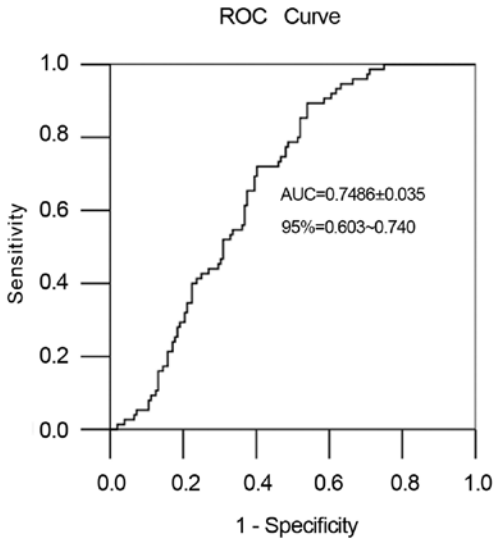

Figure 4. Receiver operating characteristic (ROC) curve for miR-181a. The best cut-off value was 0.7486, and the sensitivity and specificity were 70.7 and 59.9%, respectively. The area under the receiver characteristic curve (ROC-AUC) was 67.1% (95% confidence interval, 60.3-74.0%).

regions/fragile sites, suggesting they may be involved in the initiation and progression of human malignancy. miRNAs are thus potential markers for diagnosing and monitoring patients with BC and may be utilized in novel therapeutic strategies.

Studies have emerged showing that miRNAs derived from epithelial tumors are rapidly released into the blood stream (26,27). Previous reports have shown that miR-181a deregulation is one of the most commonly reported miRNA-related events associated with different cancers. To our knowledge, there are no reports concerning the relationship between serum miR-181a and BC diagnosis.

Since qRT-PCR is the gold standard for gene expression quantification and miR-16 is a commonly used internal reference, we measured serum levels of miR-181a by qRT-PCR and normalized the levels to those of miR-16. In initial experiments in a small set of serum samples (n=20), we found that miR-181a expression was reduced in early BC subjects (0.502592±0.44508) compared with healthy subjects (1.412649±0.465748) (P=0.001). The median fold-change of miR-181a for BC/NC was 0.35578, showing that miR-181a was significantly decreased more than 2-fold. We validated this finding in a larger sample of 75 healthy controls (1.0908±0.50821) and 152 BC patients (0.8385±0.69453), showing that serum miR-181a was significantly downregulated in BC patients compared to controls (P=0.001). ROC curve analysis showed AUC=0.671±0.031. At the cut-off value of 0.7486 for miR-181a, the sensitivity and specificity for this marker was 70.7 and 59.9%, respectively (Fig. 4). Thus, serum levels of miR-181a are potential markers for discriminating BC patients from healthy controls, which is consistent with previous studies (21-25). Gao *et al* (21) also reported that miR-181a (P=0.000) was downregulated in lung carcinoma tissues and showed potential as a novel diagnostic or prognostic biomarker for non small cell lung cancer. Our study indicated that miR-181a was a potential marker for BC. However, previous studies suggest that miR-181a may be a universal serum marker for several cancers and not a specific marker for BC. One dissenting report by Zhao *et al* (28) found that miR-181a levels were upregulated in BC patients compared with healthy controls. The discrepancy might be due to ethnic group, cancer subtype or individual differences. In our study, all of the subjects were Han Chinese, and 80.26% of the BCs were stage I and II, 90.13% were invasive ductal cancer, 69.79% were ER positive and 61.745% were PR positive. It is possible that differences in clinical characteristics, together with the small sample size in the present study, might contribute to differences in the findings. To investigate whether miR-181a could be used as a biomarker in the diagnosis of BC, we compared the sensitivity of miR-181a, CA153and CEA in each BC stage (*in situ*, I, II, III and IV). As shown in Table III, the sensitivity of miR-181a for the diagnosis of BC was 58.55%, while the sensitivity of CA153 and CEA were only 10.53% and 9.21%. We also found that serum miR-181a had higher sensitivity (55.28%) for the diagnosis of early stage (*in situ*, I, II) BC compared with CA153 (8.13%) and CEA (7.32%).

These findings imply that serum miR-181a may be useful in detecting certain carcinomas at early stages, particularly

when the individuals are clinically asymptomatic for extended periods. We speculate that miR-181a may even play a role in the early events of multistep breast carcinogenesis.

We analyzed miRNA expression in groups of tumors classified according to TNM staging and other specific biopathological features, such as hormone receptor expression, grade and disease stage. Our experimental data did not show a significant relationship between miR-181a expression, TNM staging and clinicopathological parameters. These results are consistent with previous studies which reported that circulating miRNAs did not show a correlation to different stages, histological subtypes, or grades of cancer (29-32). These studies also suggested circulating miRNAs may be potential biomarkers for cancer detection.

Although extensive research has been conducted on miRNAs and miRNA-based therapy, there is a limited understanding of miRNA target genes, of the mechanisms of miRNA-mediated gene regulation and of exactly how regulation of target genes relates to tumor progression. The precise targets of miR-181a in BC remain elusive, and it is unclear how miR-181a may be involved in tumor formation or how the levels relate to prognosis. We screened for target genes of miR-181a using the Target Scan program (http://www.targetscan.org) and PicTar (http://pictar). Hundreds of genes were predicted as targets, many of which were involved in cancers, including K-ras, OPN, Hoxb5, E2f5, CARD11 and others). Some of the genes had been validated in previous experiments. In particular, one study demonstrated that miR-181a had a tumor suppressive effect by downregulating K-ras in oral squamous cell carcinoma cells and that miR-181a decreased K-ras protein levels and the luciferase activity of reporter vectors of the K-ras gene (22). It seems clear that KRAS activation occurs in the early stage of lung cancer carcinogenesis (33). In our study, the miR-181a expression level was lower in early stage BC, leading our speculation that the link between K-ras and miR-181a may play a role in the etiology of this malignancy. Bhattacharya *et al* (34) reported that miR-181a regulates OPN-dependent metastatic function in hepatocellular cancer cell lines. Another study (35) showed that miR-181a functioned as a tumor suppressor by inducing apoptosis, triggering growth inhibition and inhibiting invasion in glioma cells. If this is also the case in BC, it raises the possibility that miR-181a could be exploited as a therapeutic intervention for BC. Some studies evaluate the usefulness of miRNAs as both targets and tools in anticancer therapy (36,37). In addition to being used as targeted therapies and chemotherapy, miRNAs could also alter cancer cell sensitivity to radiotherapy, as reported by Weidhaas *et al* (38).

Although our results are promising, the present study has several limitations. First, the sample size is small. Larger samples are needed to validate the feasibility of using serum miR-181a as a non-invasive diagnostic test for BC. Second, miR-181a expression has not been observed in benign tumors or in many different subtypes of BC, so we cannot generalize the findings to all types of BC. Third, the precise mechanisms by which miR-181a and its target miRNAs regulate BC progression remain unclear. Further *in vitro* and *in vivo* studies are needed to determine how miR-181a contributes to breast tumorigenesis.

In conclusion, our data indicate that serum miR-181a is more sensitive for BC detection than either CEA or CA153 and thus shows potential for use as a novel diagnostic biomarker for BC. This study examined the relationship between serum miR-181a levels and target genes *in vivo*, which may be exploited as a minimally invasive treatment for BC. These findings merit further verification in more extensive studies.

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