Int J Clin Exp Pathol 2016;9(6):5912-5920
www.ijcep.com /ISSN:1936-2625/IJCEP0021820

Original Article

Lower expression of Mir-382 is associated with the development, progression and metastasis of breast cancer in the Chinese population

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Received December 14, 2015; Accepted February 25, 2016; Epub June 1, 2016; Published June 15, 2016

Abstract: Objective: MicroRNAs (miRNAs) are a class of small, non-coding RNA molecules that can regulate gene expression, thereby affecting crucial processes in cancer development. miRNAs offer great potential as biomarkers for cancer detection because of their remarkable stability in blood and their characteristic expression in different diseases. In the current study, we focused on Mir-382, one potential tumor-associated miRNA, investigated whether Mir-382 on serum could discriminate between breast cancer patients and healthy controls. Methods: We performed miRNA quantitation assay on serum from breast cancer patients, followed by construction of ROC (Receiver Operating Characteristic) curves to determine the sensitivity and specificity of the assay. A total of 164 breast cancer patients who underwent tumor resection were included in our follow-up study, meanwhile same number healthy subjects were also enrolled as control. Results: In this study, we aimed to investigate whether Mir-382 is associated with the TNM staging and prognosis of breast patients. We found that the Mir-382 expression was significantly lower in tumor tissues compared with that in normal tissue ($P<0.05$). Kaplan-Meier and analysis showed that the Mir-382 expression status is significantly associated with the survival duration (Log-rank test, $P<0.001$), and multivariate Cox regression revealed that patients with low Mir-382 expression were exposed to a 2.352 fold higher death risk (HR=2.352, 95% CI=1.379-4.012, P=0.002) compared with those with high Mir-382 expression. Expression of MiR-382 was also significantly correlated with TNM staging, especially for lymph node metastasis and distant metastasis. By in vitro study, exogenous expression Mir-382 could significantly inhibit the proliferation and migration of breast cancer cell MCF-7; on the other hand, knocking down Mir-382 could significantly reverse these biological characteristics. Conclusion: Our study thus demonstrates that Mir-382 expression in breast tissues is critically associated with the prognosis of patients, suggesting its potential clinical significance.

Keywords: Mir382, breast cancer, survival analysis, migration ability, invasion ability

Introduction

MiRNA (microRNA) is an endogenous, non-encoding RNA molecules found in eukaryotes, the length of miRNA normally ranged from 21 to 25 nucleotides [1, 2]. Been widely found in various animal and plant cells, miRNAs play an important role in the development of the individual, cell proliferation, differentiation, apoptosis, angiogenesis, tumor growth and other processes, miRNAs act mainly through regulating the signaling related molecules such as cells proliferation factors, growth factors, transcription factors, pro apoptotic or anti apoptotic genes [3-5].

MiRNAs has highly conservative in the evolution process. The expression of miRNA has the characters of time dependency and tissue specificity [6, 7]. The mutation of miRNAs could lead to the abnormal function of the cell, including gene mutation, and functional disorder, thus induce a series of diseases (such as cardiovascular disease, Alzheimer's disease, Parkinson's disease, tumor, etc.) [8, 9]. Especially in oncology, the abnormal expression of miRNAs plays an important role in the proliferation, differentiation, apoptosis, migration and invasion of tumor cells. MiRNAs play dual role in the process of tumor suppression and occurring [10, 11]. In addition, recent studies have demon-

strated that miRNAs are corrected with the occurrence and progression of multiple tumors [12, 13].

Finding the early diagnostic markers of tumor conveniently and without obvious trauma has been a key issue in tumor screening and diagnosis. MiRNAs has been widely studied as a potential tumor marker for its close relationship to tumor pathological factors. However, obtaining miRNAs were mainly relied on tumor tissue obtained by surgery or puncture in previous studies, which restricted the applying of miRNAs as tumor markers. Recent research indicated that the mature miRNAs of tumor tissue could be secreted into the peripheral blood and exists stably in the circulating blood [14, 15]. These findings provide the objective possibility for miRNAs being a tumor marker. Compelling studies have revealed that plasma miRNAs is associated with a wide variety of tumors [15, 16].

Breast cancer (BC) is one of the most malignant tumors in women; BC has drawn more and more attention for the increase of death rate. Carbohydrate antigen markers of CEA and CA153 are widely used because of their convenience and relatively low cost, but their also sensitivity are low in BC diagnosis. Mammography is considered as the most effective method for BC examination, but it cannot be popularized in most developing countries due to high cost. Integrated with the advantages of above two methods, plasma miRNAs might be a potential biomarker for BC diagnosis.

Mir-382 has been found to be closely related to the development of many tumors, such as bone sarcoma [17], melanoma [18] and esophageal cancer [19], but the relationship between mir-382 and BC still lack of in-depth and detailed study. This aim of current study is using the medical resources of our hospital, to detect the mir-382 expression in peripheral blood, and to explore the relationship between mir-382 and breast cancer. Meanwhile, miR-382 was transfected to breast cancer cell line MCF-7 to the analyzed effect of miRNA expression on proliferation, invasion and migration in breast cancer cells.

Materials and methods

Study subjects

We enrolled 164 breast cancer patients who underwent tumor resection from January 2008 to December 2012 in Anzhen hospital. Among these patients, mean age was 58.6±7.4 years. The peripheral plasma was obtained from each patient. The tumor specimens were fixed in 10% Formaldehyde and paraffin embedded for pathological diagnosis confirmation. All the patients' diagnoses were confirmed based on the histopathological examination. The histological grade was determined according to the criteria formulated by the World Health Organization in 2007. The numbers of patients in grade I, II, III and IV were 17, 76, 52 and 19, respectively. The patients were followed-up every 3 months, and the follow-up was ended on December 31st 2014. 164 healthy patients were enrolled as controls. We obtained a writing consent from each patient, and this study was approved by the Ethics Committee of An Zhen hospital.

RNA isolation and miR-382 quantification

The RNA from plasma in each patient and healthy subject was isolated using Trizol Reagent (Invitrogen) according to the instructions provided by the manufacturer. To assess the levels of miR-382 in each group, we utilized a TaqMan based quantitative PCR method. The TaqMan PCR assay kits were purchased from Applied Biosystems. The expression of U6 was used as an internal control to adjust miR-382 expression. And the relative expression of miR-382 was determined by the 2-ΔΔCt method.

Cell culture and mir-382 relative expression level test

HBL-100, MCF-7, MDA-MB-231, and MDA-MB--468, these four human breast cancer cell line were purchased from ATCC (Masachussate, USA), when the cell in the flask reach to 90% configuration, Trizol reagent (life technology, USA) was used to isolate the total RNA, and first strand cDNA was synthesized by reverse transcriptase (Promega, USA). Same real-time PCR procedure as above mentioned was used to measure the relative expression of miRNA in these four cell lines.

Inhibition of Mir-382 by miArrest™ miRNA inhibitors

MCF7 was selected for cell assay. The Mir-382 inhibitor was synthesized by GeneCopoeia, Inc (USA). We transfected it into MCF7 cell lines. Real-time PCR was used to test the transfection efficiency and inhibition efficiency. The untransfected MCF7 cells and transfected

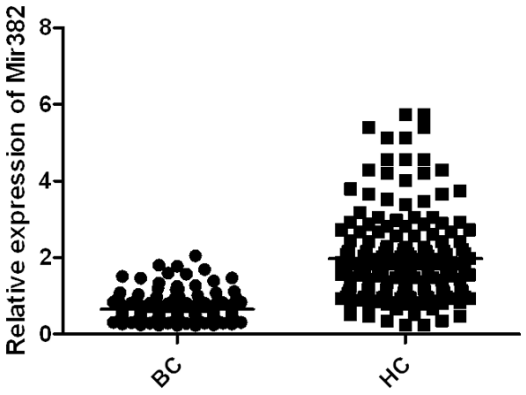

Figure 1. miR-382 expression profile in 164 healthy and breast cancer tissues. BC: breast cancer; HC: health control.

Table 1. Association between miR-382 expression and clinical characteristics

Clinical characteristics	N	miR-382 expression	
		Low	High
Age (years)			
<60	94	58	36
≥60	70	53	17
Tumor size			
<3 cm	89	58	31
≥3 cm	75	54	21
*WHO grade**			
I	17	7	10
II	76	47	29
III	52	39	13
IV	19	13	6
Resection range			
Total resection	71	49	22
Local resection	93	61	32
Adjuvant therapy			
Radiotherapy	13	9	4
Chemotherapy	35	23	12
Radiotherapy and chemotherapy	46	31	15
*Recurrence time**			
<3 months	96	77	19
≥3 months	68	33	35
*Survival duration**			
<15 months	96	72	24
≥15 months	68	35	33

*P<0.05, performed by χ^2 test.

MCF7 cells were used to perform invasion assay and migration assay to demonstrate whether mir382 inhibition influence the bio-characteristics.

Invasion assay

Invasion assay was performed using Boyden chamber system (Neuro Probe) with a fibronectin-precoated (0.5 mg/ml) polycarbonate membrane (8 μm pore size). The lighter side of the polycarbonate membrane was precoated with 250 μg/ml matrigel (BD). The bottom chambers were filled with 30-μl RPMI1640 medium containing 2% BSA while the top chambers were filled with 50-μl RPMI1640 serum-free medium containing 0.2% BSA. 5×10^4 cells per well were added to the top chamber, followed by a 15 h incubation in 37°C, 5% CO_2 incubator. Three independent experiments were performed with triplicate treatment. The cells were fixed in methanol and stained with haematoxylin. The top surface of the membrane was gently scrubbed with a cotton bud, the cells migrated to the lower side of the membrane was counted under the microscope and the numbers of migrated cells were calculated as average plus SD.

Adhesion assay

The same amounts of mir382 inhibitor transfected and untransfected MCF7 cells (3×10^4) were plated onto the matrigel-precoated (50 μg/ml) 96-well plate in triplicate. The cells were washed at 30, 60, and 120 min to remove non-adherent cells. 0.2% BSA precoated wells served as the negative control. After washing, the adhered cells were measured with MTT assay. The relative optical density (OD) was determined at 570 nm using WELLSCAN MK3 ELISA (Labsystems, Dragon, Finland) and a 450 nm reference filter. The OD values reflected the proportion of cells in the matrigel coated 96-well plate.

Statistical analysis

The data in this study were analyzed by SPSS 19.0 software package, Wilcoxon-Mann-Whitney test was used to compare the difference of miR-382 expression between breast cancer and healthy patients. The patient population was dichotomized according to miR-382 expression level. Patients who had a 0.5-fold or above miR-382 expression than the mean expression of control group were categorized as high miR-382 expression, otherwise categorized as low miR-382 expression. Chi square test was performed to examine the association

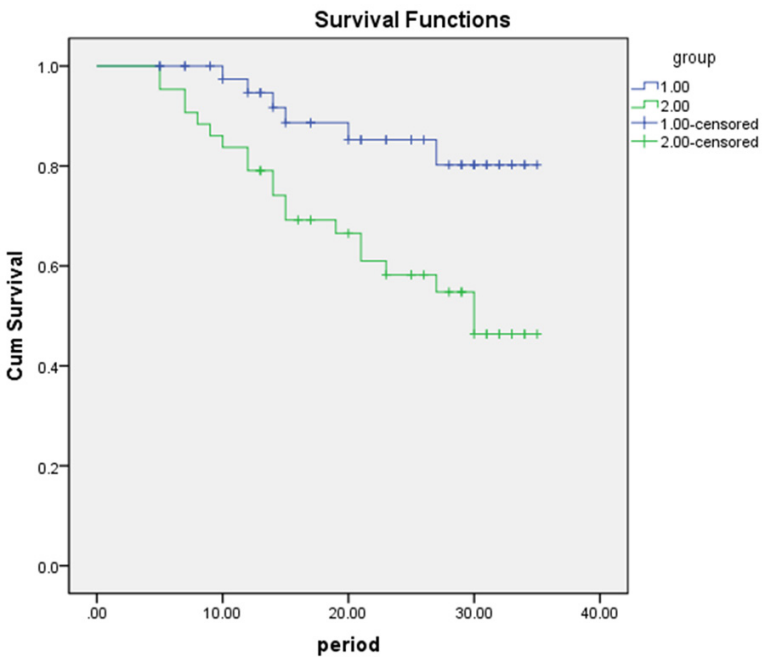

Figure 2. Kaplan-Meier survival analysis of miR-382 expression in breast cancer patients. Group one with blue line referred to patients with higher expression of Mir382, group 2 with green line referred to patients with lower expression of Mir382.

Table 2. Multivariate Cox regression analysis of risk factors associated with overall survival of breast cancer patients

Clinical characteristics	HR	95% CI	*p*
Age	1.005	0.978-1.032	0.719
Tumor size	0.678	0.422-1.088	0.108
WHO grade	0.709	0.435-1.158	0.170
Resection range	0.980	0.620-1.547	0.930
Adjuvant therapy	0.934	0.576-1.516	0.784
miR-382 expression	2.352	1.379-4.012	0.002

between miR-382 expression status and clinical-pathological characteristics. Survival analysis was performed by Kaplan-Meier estimator, comparison of the overall survival between two groups was performed by log rank test. Multivariate Cox regression analysis was also used to determine the hazard ratio of each covariate. A two tailed probability less than 0.05 was considered statistically significant.

Results

miR-382 is down-regulated in breast cancer tissue

We first compared the miR-382 expression profile in 164 healthy and breast cancer tissues. As shown in **Figure 1**, miR-382 level was found to be significantly higher in the blood of healthy subjects than that in breast cancer patients ($P<0.05$).

Association between miR-382 expression and clinical characteristics

To examine the possible association between miR-382 expression and clinical characteristics, we first divided the patients into high-expression group and low-expression group based one the criteria described above. 115 patients were identified as low expression, and 49 patients were identified as high expression. We studied the association between miR-382 expression and general clinical characteristics in the study cohort. As presented in **Table 1**, miR-382 expression was not associated with age, tumor location, tumor size, and resection range and adjuvant therapy. By contrast, association between miR-382 expression and WHO grade, recurrence time or survival duration was observed.

miR-382 correlates with the survival of breast cancer patients

To investigate whether miR-382 expression is correlated with the survival of breast cancer patients, we performed Kaplan-Meier survival analysis. All the cases were followed-up after surgery, and 7 patients lost follow-up due to the changes of their telephone number. As presented in **Figure 2**, patients with high expression of miR-382 exhibited greater overall survival than those with low miR-382 expression. Moreover, multivariate Cox regression analysis showed that the hazard ratio of miR-382 low expression was 2.352 (**Table 2**). Taken together, these data suggested that low expression of miR-382 is associated with a more aggressive type of breast cancer.

Expression of mir-382 in breast cancer cell lines

To observe whether the MIR-382 expression is related to breast cancer in vitro, we examined

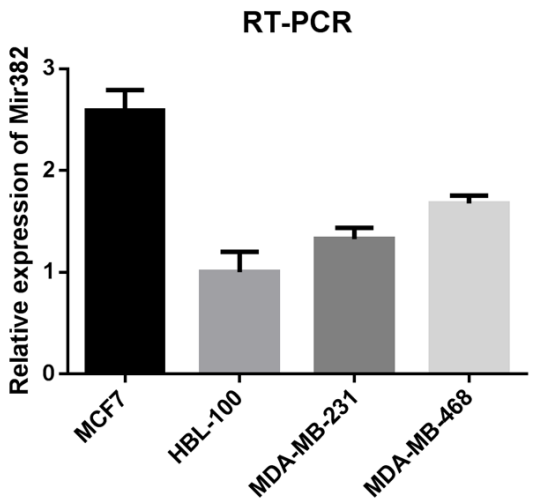

Figure 3. Comparison of MIR-382 in breast cancer cell lines HBL-100, MCF-7, MDA-MB-231, and MDA-MB-468.

the MIR-382 mRNA and protein levels in different breast cancer cell lines (HBL-100,MCF-7,MDA-MB-231, and MDA-MB-468). Real time PCR (**Figure 3**) suggest that HBL-100 has lowest expression and MCF7 has highest expression among these four breast cancer lines, so the MCF7 was selected for further migration and invasion assay.

Effects of MIR-382 on invasive, motive and adhesive abilities and proliferation of MCF7 cells

To study the role of MIR-382 on the cell invasion, motility, and adhesion, which are the major characteristics of the metastasis, we used specific mir382 inhibitor (which was designed and synthesized by GeneCopoeia, Inc) to deplete the endogenous expression of MIR-382 in the MCF7 cells. All the assays were performed after 48 hour transfection. In an invasion assay, we calculated the number of cells that migrated to the bottom side of the membrane on a chamber where the cells were seeded (**Figure 4A**). The data showed that the wild type MCF7 cells had less numbers of the migrated cells with or without transfection of a control Mir382 inhibitor. However, when MIR-382 inhibitor was transfected into the cells, numbers of the migrated cells increased dramatically (**Figure 4B**). These results suggested that the depletion of MIR-382 significantly enhance the migration ability of SK-MES-1 cells.

To examine whether the depletion of MIR-382 has any effect on the motive ability of the cells, we performed a wound-healing experiment using MCF7 cells transfected with mir382 inhibitor. The data showed that compared with wild type MCF7 cells, significant faster in the relative wound closure was observed for the cells transfected with MIR-382 inhibitor (**Figure 5**) both in 12 and 24 h.

The lower expressed MIR-382 correlated to the lung cancer metastasis in the tumor patients reminded us to examine whether MIR-382 has an effect on cell adhesion to ECM (extracellular matrix). For this purpose, we performed a cell adhesive assay using MIR-382 inhibitor transfected cell and wild MCF7 cells. The results showed that the cells, when transfected with mir382 inhibitor, obtained high adhesion ability, compared with the wild type cells (**Figure 6**). The results were in consistence during different times as we observed in 30, 60, and 120 min, respectively. These results indicated that MIR-382 may help the tumor cells adhere to the ECM. Taking together, our data suggest that MIR-382 exerts its effects on metastasis by inhibiting invasion, migration, and adhesion in MCF7 cells.

Discussion

In the current follow-up study, we evaluated the prognostic value of mir-382 in breast cancer patients. Our data showed that the differential expression of mir-382 in breast cancer tissues and normal tissues, Kaplan-Meier survival analysis and Cox regression analysis together showed that patients with low expression of mir-382 have a worse clinical outcome, which confirmed our hypothesis that mir-382 might play a favorable role in the survival of breast cancer patients. Therefore, our study demonstrates that mir-382 expression might be an independent indicator of prognosis of patients with breast cancer.

microRNAs have been recognized as indispensable regulators of normal cell function [20, 21]. Aberrant microRNA expression has been found in several diseases including breast cancer. Several microRNAs have been implicated in the multifacet regulatory network of the pathogenesis of this cancer type. For example, a recent study shows that miR-218 functions as a tumor

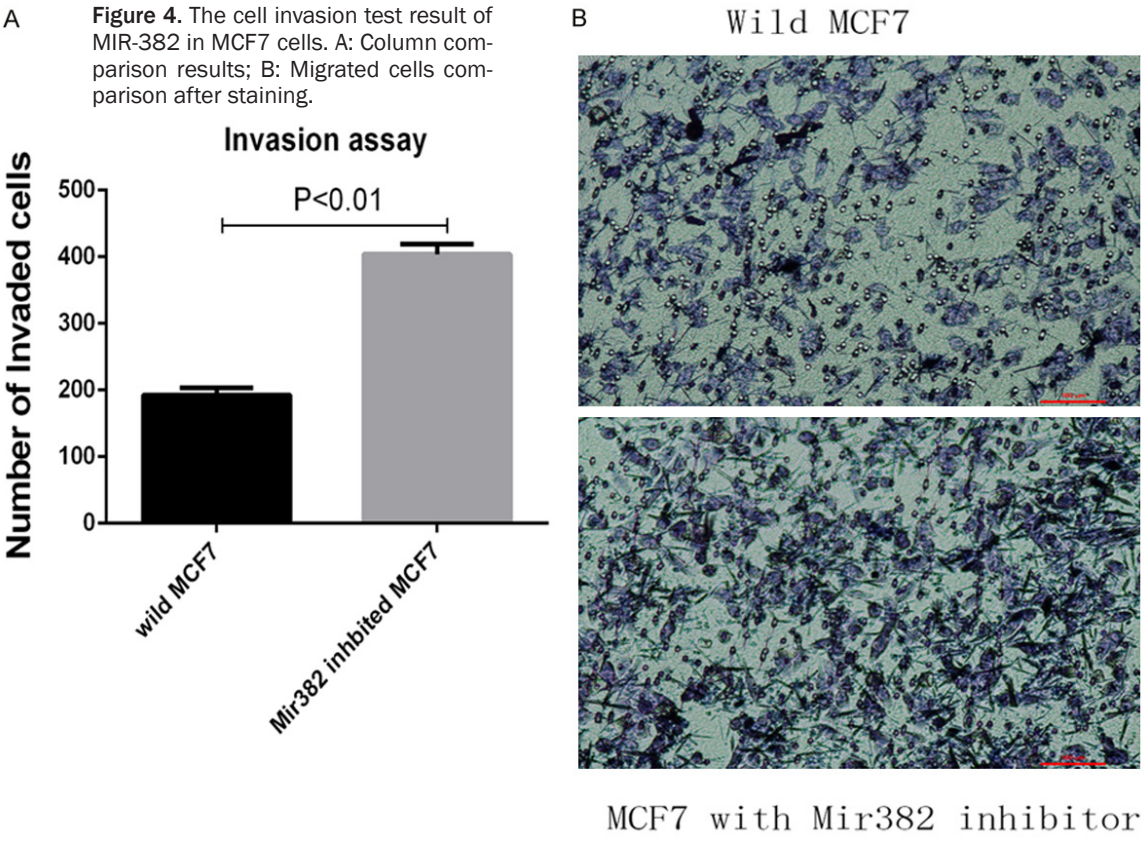

Figure 4. The cell invasion test result of MIR-382 in MCF7 cells. A: Column comparison results; B: Migrated cells comparison after staining.

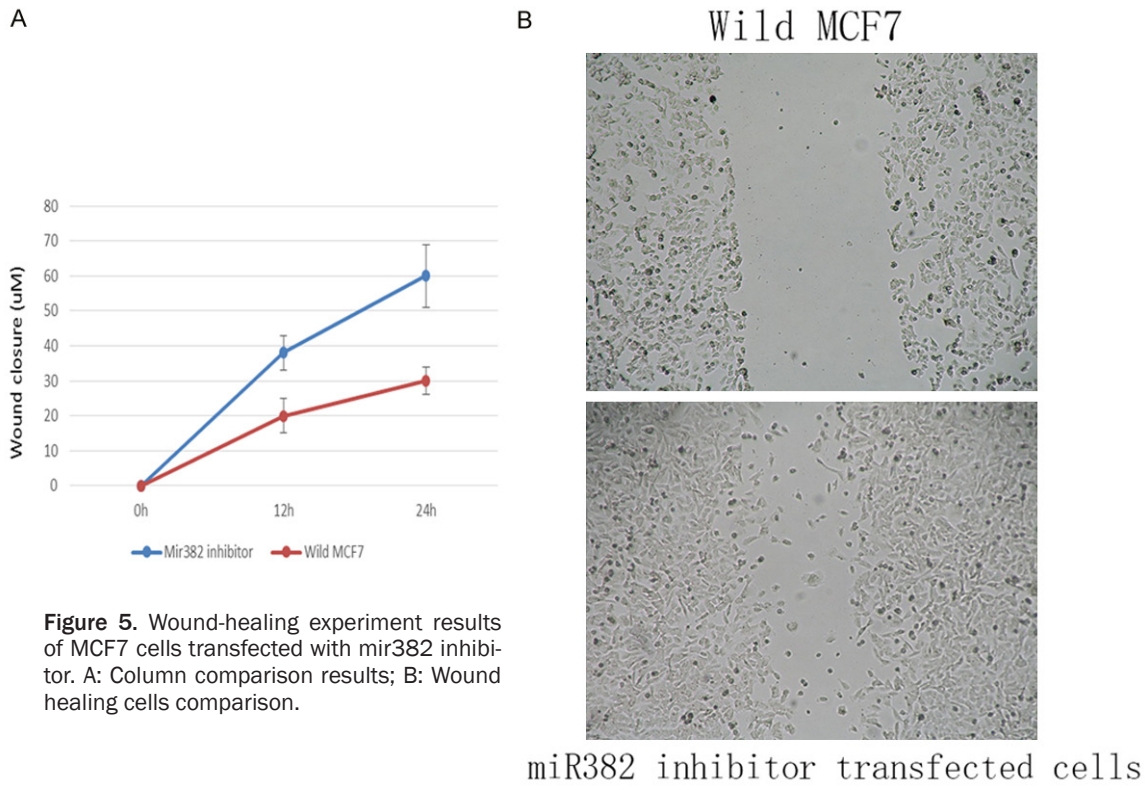

Figure 5. Wound-healing experiment results of MCF7 cells transfected with mir382 inhibitor. A: Column comparison results; B: Wound healing cells comparison.

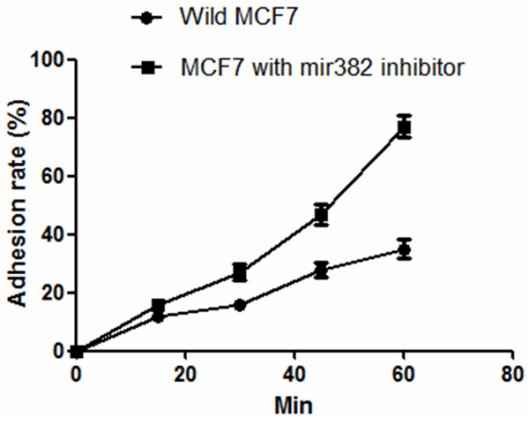

Figure 6. The adhesion test after transfected with mir382 inhibitor.

suppressor by affecting a series of critical biological processes of breast cancer including cell invasion, migration, proliferation and the maintenance of cancer cell stemness [22, 23]. Particularly, growing numbers of studies have revealed their potential clinical values, circulating miR-128 has been identified as a diagnostic marker, and miR-218 has also been reported to show prognostic significance [24-27]. Despite that these studies revealed the pivotal role of microRNA in breast cancer biology, whether other microRNAs are also implicated in this issue is still to be explored. Moreover, given the broad effects of microRNAs are exhibiting, it is reasonable to speculate that a large number of previously uncharacterized microRNAs may show macro effects in breast cancer patients.

The current prognostic model is largely dependent upon the WHO histological grading and immunohistochemistry analysis. However, histological examinations are often subjective and calls for rich clinical diagnostic experience of pathologists, and the false positive of immunohistochemistry also limits its diagnostic power. The application of microRNA in the clinical practice would be expected to meet the needs of clinical practitioner [28-30]. microRNA detection in the paraffin section by the RNA *in situ* method might represents a more reliable diagnostic approach. Moreover, as several circulating microRNAs are emerging as the biomarkers for cancer diagnosis, investigating whether circulating mir-382 level correlates with its expression in tumor site should represent a more convenient alternative to assess the prognosis of patients. Identifying mir-382 as a prognostic marker enables the quantitative evaluation of the death risk at early diagnosis, which might be helpful to tailor individualized treatment. To go further, concerning that we have identified low mir-382 expression is associated with poorer prognosis; it might be an effective therapeutic approach to complement mir-382 in low expression patients.

In summary, despite that our study population is relatively small, we demonstrate the aberrant mir-382 expression in breast cancer patients for the first time; more importantly, the expression status is significantly associated with the survival duration of patients. In the future, large scale studies are still needed to confirm the prognostic significance of mir-382 in breast cancer.

Disclosure of conflict of interest

None.

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