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ORIGINAL ARTICLE

MicroRNA-372-3p promotes the epithelial-mesenchymal transition in breast carcinoma by activating the Wnt pathway

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Summary

Purpose: *To explore the role of microRNA (miR)-372-3p in the epithelial-mesenchymal transition (EMT) of breast carcinoma and its potential mechanism.*

Methods: *The expression of miR-372-3p was detected by real-time PCR (RT-PCR) in 48 samples of breast carcinoma tissues, 30 samples of normal breast tissues, normal breast cells (MCF-10A) and breast carcinoma cells (MCF-7, MDA-MB-231 and HCC38). Transfection efficacy of miR-372-3p mimic and miR-372-3p inhibitor was determined by RT-PCR. Cell viability and invasion were determined by CCK-8 assay and wound healing assay, respectively. The interaction between miR-372-3p and DDK1 was verified by the luciferase reporter assay. Expression levels of DDK1, Wnt3a, E-cadherin and N-cadherin in breast carcinoma cells transfected with miR-372-3p mimic, miR-372-3p inhibitor and DKK1 were detected by Western blot.*

Results: *The expression of miR-372-3p in breast carcinoma tissues was higher than that of normal breast tissues. Correlation analysis revealed that the miR-372-3p expression was negatively correlated with the postoperative survival, but positively correlated with the tumor size and stage of patients with breast carcinoma. No significant correlation was observed between the miR-372-3p expression and age, gender or lymph node metastasis. The receiver operating characteristics (ROC) curve indicated a high diagnostic sensitivity and specificity for miR-372-3p. CCK-8 assay and wound healing assay illustrated that miR-372-3p increased the viability and invasion of MDA-MB-231 and HCC38 cells, respectively. Luciferase activity assay suggested that miR-372-3p was specifically bound to the 3'UTR of DKK1. Overexpressed miR-372-3p markedly increased the expressions of Wnt3a and N-cadherin, but decreased the expressions of DKK1 and E-cadherin, which were reversed by miR-372-3p knockdown. The protein expressions of E-cadherin and N-cadherin were remarkably increased in cells co-transfected with miR-372-3p mimic and DKK1 in comparison with those transfected with miR-372-3p mimic only.*

Conclusions: *MiR-372-3p is upregulated in breast carcinoma tissues, which promotes the viability and invasion of breast carcinoma cells through the Wnt pathway.*

Key words: *breast carcinoma, DKK1, EMT, microRNA-372-3p, Wnt pathway*

Introduction

Breast carcinoma is one of the malignant tumors that seriously endangers women's life and health [1]. Due to the dramatic deterioration of ecological environment and profound changes of lifestyles, the incidence of breast carcinoma has risen significantly in economically developed areas of China. In addition, more younger people tended to be diagnosed of breast carcinoma in West China [2,3]. So far, the effective treatment of breast carcinoma has been greatly improved. However,

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there are still many conditions that would affect the prognosis of patients with breast carcinoma, such as molecular classification and metastasis. It has been reported that the 5-year overall survival rate of patients with distant metastases of breast carcinoma was only 23.3%, and the median overall survival was only 1-2 years [4]. Hence, it is urgent to enlighten the regulatory mechanisms contributing to the occurrence and development of breast carcinoma, especially the mechanisms of distant metastasis, so as to provide a theoretical basis for the development of new treatment approaches.

MiRs are small non-coding RNAs with a length of 22 nucleotides. MiRs can regulate various functional genes by directly targeting the 3'UTR of mRNAs, thus inducing degradation of target genes or inhibition of the translation. Current researches have pointed out that many miRs may participate in the regulation of physiological and pathological processes, such as physical development, organogenesis, viral defense, apoptosis, cell cycle, cell proliferation and tumor formation. In addition, differentially expressed miRs may participate in multiple signaling pathways involved in tumorigenesis, thus affecting the development and progression of tumors. Abnormally expressed miRs, including miR-122, miR-155 and miR-10b, have been observed in breast carcinoma [5-7].

MiR-372-3p was demonstrated to be capable of regulating invasion and EMT of tumor cells in various cancer types, including cervical cancer, liver cancer, renal cell carcinoma, endometrial cancer and prostate cancer [8-12]. EMT is a complex biological process involving multiple genes and biological activities, which is closely associated with invasion and metastasis of tumor cells [13]. However, there is no report on the relationship between miR-372 and EMT in breast carcinoma so far. This study was focused on the role of miR-372-3p in cell invasion of breast carcinoma and its underlying mechanisms.

Methods

Basic patient characteristics

A total of 48 tumor tissues were collected from patients who were newly diagnosed with breast carcinoma in the Affiliated Huai`an No.1 People's Hospital of Nanjing Medical University from March 2010 to March 2012, and another 30 cases of normal breast tissues were collected as the controls. Enrolled patients were not treated with any preoperative chemotherapy, radiotherapy, targeted therapy or other treatment. All the obtained specimens were frozen and preserved in liquid nitrogen within 15 min. The experiment was approved by the Hospital Ethics Committee and all patients signed informed consent.

Cell culture and transfection

Breast carcinoma cells (MCF-7, MDA-MB-231, HCC38) and normal breast cells (MCF-10A) were purchased from the Chinese Academy of Sciences and Shanghai Institute of Biological Cell Resource Center, respectively. Cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C. Cells in logarithmic growth phase were seeded in 6-well or 96-well plates with a density of 1×10^5/mL 1 day prior to the cell transfection. MiR-372-3p mimic or miR-372-3p inhibitor was transfected according to the instructions of Lipofectamine™2000 when the cell confluence was up to 60-70%.

RNA extraction and RT-PCR

The total RNA of breast tissues and cells were extracted by the TRIzol method in strict accordance with the instructions. 500 ng of total RNA were reverse-transcribed into cDNA according to the manufacturer's instructions, which was further amplified using the SYBR Premix Ex Taq II kit (TaKaRa, Tokyo, Japan). The test results were calculated via the $2^{-\Delta\Delta Ct}$ method.

Cell counting kit-8 (CCK-8) assay

After transfection for 48 hrs, MDA-MB-231 cells and HCC38 cells were digested to prepare the cell suspension at a density of 2×10^4/mL. Cells were then seeded in 96-well plates with 6 replicate wells in each group. 10 μL of CCK-8 solution were added to each well for another hr incubation. The absorbance at the wavelength of 450 nm was measured by a microplate reader.

Table 1. Correlation between the microRNA-372-3p expression and pathological features in patients with breast carcinoma (n= 48)

Clinicopathological features	*Number of cases*	*miR-372-3P expression*	
		Low (n=24)	*High (n=24)*
Age (years)			
≤50	27	14	13
>50	21	10	11
Gender			
Male	12	7	5
Female	36	17	19
Tumor size (cm)			
≤2	22	17	5
>2	26	7	19
TNM stage			
I-II	30	19	11
III-IV	34	13	21
Lymph node metastasis			
Absent	19	12	7
Present	29	12	17

Wound healing assay

Transfected MDA-MB-231 cells and HCC38 cells were seeded into 6-well plates at a density of 5×10^5/mL. When the cell confluence was up to 80%, a sterile 10 μL micropipette tip was used to vertically scratch the cell plate. After removing the exfoliated cells with PBS, serum-free medium was placed for 48-h incubation. The cell migration was observed under an inverted microscope, and the width of the scratch was measured and photographed.

Luciferase activity assay

MDA-MB-231 cells and HCC38 cells were co-transfected with DKK1 luciferase reporter vectors containing predicted binding sites as well as miR-372-3p mimic, respectively. PRL-TK was transfected as standard internal control. The luciferase activities in MDA-MB-231 cells and HCC38 cells were determined according to the instructions of luciferase activity assay kit provided by Promega (Madison, WI, USA). Relative luciferase activity was calculated.

Western blotting

The total protein was extracted from cells and tissues, and then quantified by the BCA method. Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis. After transferred to the polyvinylidene fluoride (PVDF) membrane, the protein samples were blocked with 5.0% skimmed milk for 1 hr, and then incubated with the primary antibody (rabbit anti-human DKK1), WNT3A, e-Cadherin, n-Cadherin (Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. After washing with tris buffered saline-tween (TBST) for three times, the membranes were incubated with specific horseradish peroxidase-

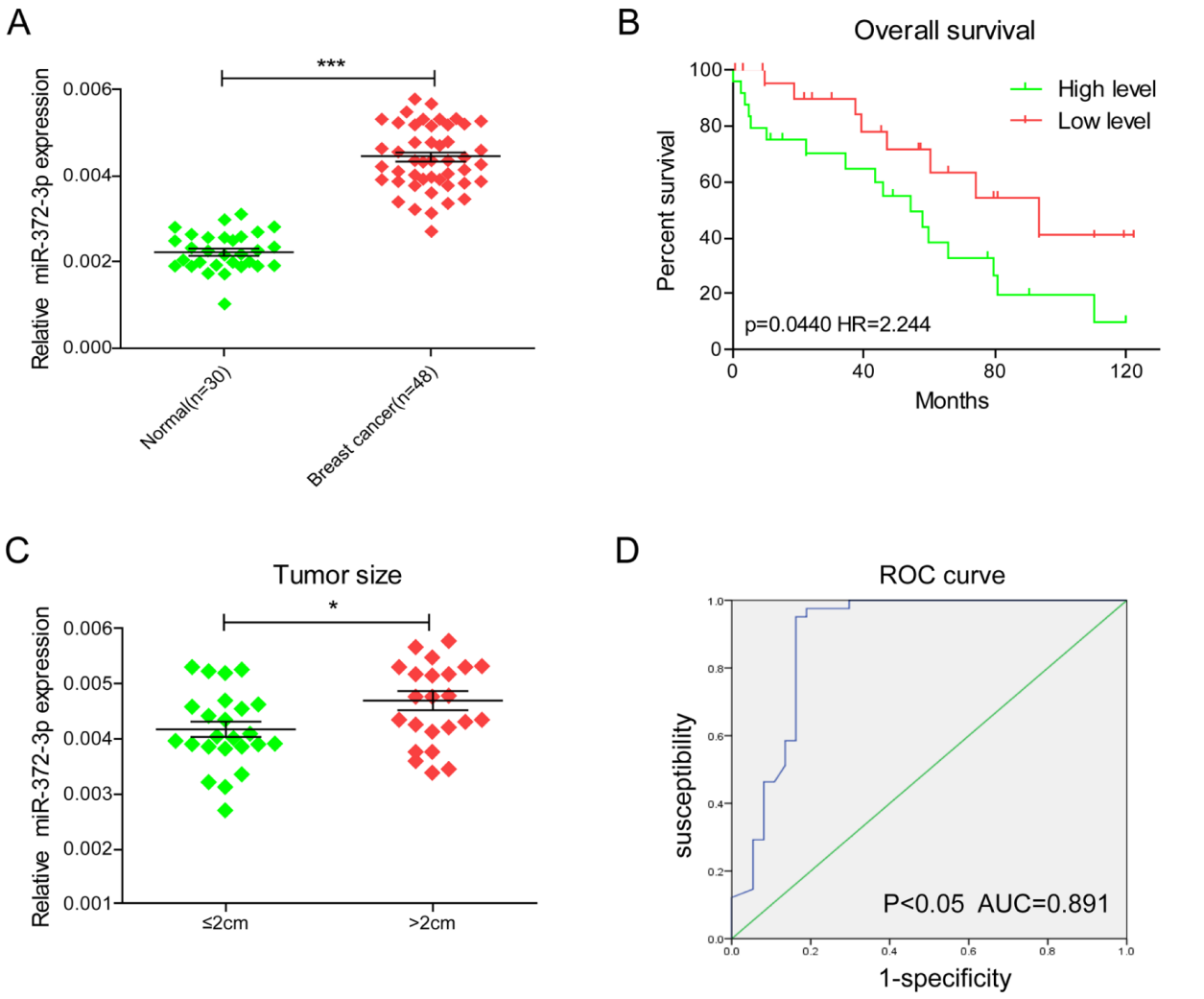

Figure 1. MicroRNA-372-3p was overexpressed in breast carcinoma tissues. **(A):** MicroRNA-372-3p was up-regulated in 48 cases of breast carcinoma tissues and was compared to that of 30 normal tissues and showed that microRNA-372-3p might be involved in the development of breast carcinoma (***p<0.001). **(B):** The survival of microRNA-372-3p high expression group was significantly lower than that of the low expression group. **(C):** The microRNA-372-3p expression in tumor size < 2cm of breast carcinoma was significantly higher compared to ≥ 2cm (*p<0.05). **(D):** ROC curve showed a good sensitivity and specificity of microRNA-372-3p for diagnosing breast carcinoma.

conjugated secondary antibody at room temperature for 1 hr. Immunoreactive bands were exposed by enhanced chemiluminescence method.

Statistics

SPSS17.0, Chicago, IL, USA statistical software was used to analyze the data. The measurement data were expressed as mean ± standard deviation. Comparisons between two groups were conducted by paired *t*-test or independent sample *t*-test. The classification data were analyzed by chi-square test. Survival analysis was assessed by Kaplan-Meier method and log-rank test was performed to evaluate difference between groups. ROC curve was used to evaluate the diagnostic sensitivity and specificity. P<0.05 was considered as statistically significant. Each experiment was independently repeated 3 times.

Results

MiR-372-3p was overexpressed in breast carcinoma tissues

The mRNA expression of miR-372-3p in 48 samples of breast carcinoma tissues and 30 sam-

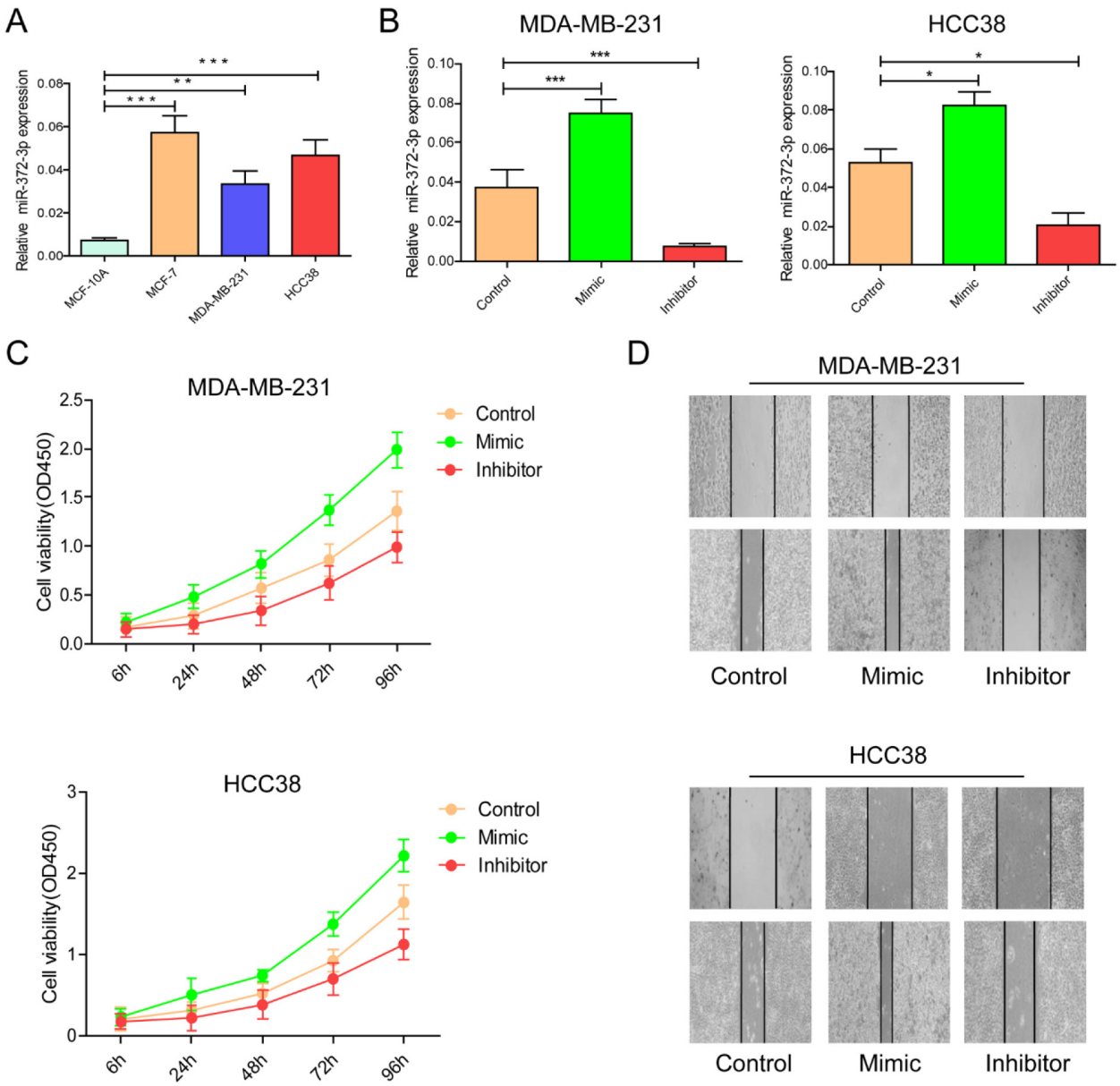

Figure 2. Overexpressed microRNA-372-3p enhanced the proliferation of breast carcinoma cells. **(A):** The expression level of microRNA-372-3p in normal breast cells (MCF-10A) and breast carcinoma cell lines (MCF-7, MDA-MB-231 and HCC38). **(B):** The expression level of microRNA-372-3p in MDA-MB-231 and HCC38 after transfected with microRNA-372-3p mimic or inhibitor. **(C):** CCK-8 assay showed the viability of MDA-MB-231 and HCC38 cells after microRNA-372-3p was overexpressed or knocked out. **(D):** Wound healing assay showed the invasion ability of MDA-MB-231 and HCC38 cells after microRNA-372-3p was overexpressed or knocked out. These results indicated that microRNA-372-3p could promote the proliferative and invasive abilities of MDA-MB-231 and HCC-38 cells (*p<0.05, **p<0.01, ***p<0.001).

ples of normal breast tissues was detected by RT-PCR. Higher mRNA expression of miR-372-3p was found in breast carcinoma tissues compared with normal breast tissues (p<0.05, Figure 1A), suggesting that miR-372-3p might be involved in the development of breast carcinoma. Patients were then assigned into the high expression group and the low expression group based on the miR-372-3p expression level. It was found that patients with higher miR-372-3p expression presented shorter survival compared to the low expression group, suggesting that miR-372-3p may be a prognostic indicator of breast carcinoma (p<0.05, Figure 1B). Additionally, the expression of miR-372-3p was positively correlated with tumor size (p<0.05, Figure 1C) and stage (Table 1, p>0.05) of breast carcinoma. However, no remarkable relationship was observed between the expression level of miR-372-3p and age, gender or lymph node metastasis of patients with breast carcinoma (Table 1, p>0.05). Furthermore, the ROC curve showed a good sensitivity and specificity of miR-372-3p for

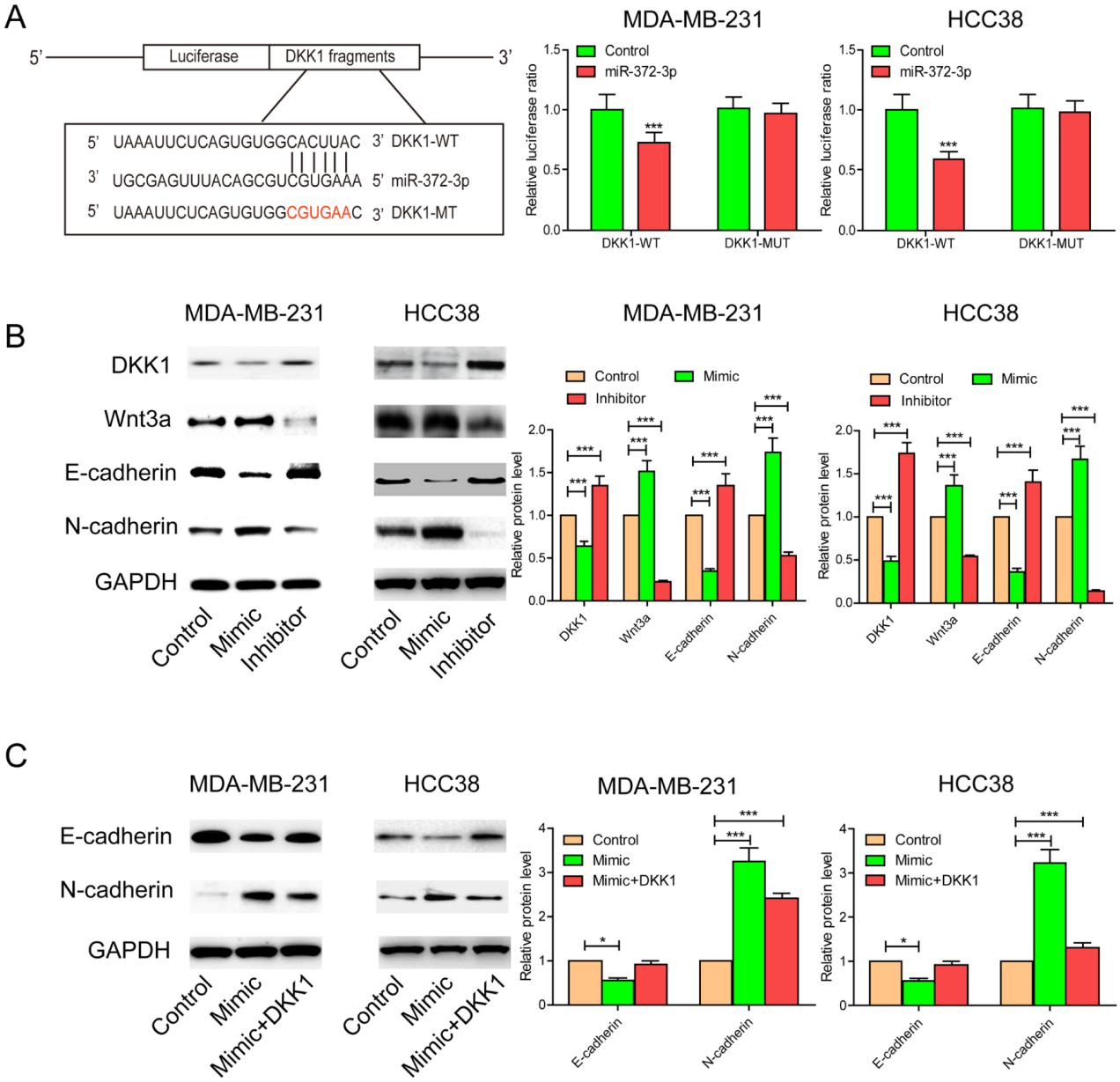

Figure 3. MicroRNA-372-3p promoted the development of EMT of breast carcinoma cells via the Wnt pathway. **(A):** Luciferase activity assay confirmed the direct binding of microRNA-372-3p and DKK1. **(B):** Western blot was used to detect the expressions of the EMT-related proteins after microRNA-372-3p was overexpressed or knocked out. **(C):** The expressions of EMT-related proteins in breast carcinoma cells transfected with microRNA-372-3p mimic, microRNA-372-3p inhibitor and DKK1, respectively. These results demonstrate that microRNA-372-3p promotes EMT of breast carcinoma cells by activating the WNT pathway (*p<0.05, ***p<0.001).

diagnosing breast carcinoma (p<0.05, AUC= 0.891; Figure 1D).

MiR-372-3p overexpression enhanced the proliferation of breast carcinoma cells

MiR-372-3p was highly expressed in breast carcinoma cells compared to that of normal breast cells (p<0.05, Figure 2A). Transfection efficacy of miR-372-3p mimic and miR-372-3p inhibitor is shown in Figure 2B. Cell viability of MDA-MB-231 and HCC38 cells after transfection for 6, 24, 48, 72 and 96 hrs were evaluated. Compared with the control group, overexpressed miR-372-3p significantly enhanced the cell viability in a time-dependent manner, while miR-372-3p knockdown markedly impaired the cell viability (p<0.05, Figure 2C). Furthermore, the wound healing assay also showed that increased miR-372-3p significantly enhanced the invasion ability of MDA-MB-231 and HCC-38 cells, whereas miR-372-3p knockdown showed the opposite result (p<0.05, Figure 2D). The above data indicated that miR-372-3p could promote the proliferative and invasive abilities of MDA-MB-231 and HCC-38 cells.

MiR-372-3p promoted the development of EMT of breast carcinoma cells via the Wnt pathway

Luciferase activity assay suggested that miR-372-3p could directly bind to the 3'UTR of DKK1 (Figure 3A). Moreover, overexpressed miR-372-3p significantly increased the protein expressions of Wnt3a and N-cadherin in MDA-MB-231 and HCC38 cells; however, overexpression of miR-372-3p reduced the protein expressions of DKK1 and E-cadherin (Figure 3B). Subsequently, miR-372-3p mimic and DKK1 were co-transfected in MDA-MB-231 and HCC38 cells, respectively. In comparison to cells transfected with miR-372-3p mimic, E-cadherin expression was increased and N-cadherin expression was decreased in cells co-transfected with miR-372-3p mimic and DKK1 (Figure 3C), demonstrating that DKK1 can partially reverse the EMT phenotype induced by miR-372-3p. All these results further demonstrated that miR-372-3p promoted EMT of breast carcinoma cells by activating the Wnt pathway.

Discussion

Breast carcinoma is one of the major causes of female mortality worldwide. The infiltration and metastasis of breast carcinoma cells is the main cause of disease mortality [1]. During EMT, epithelial tumor cells are transformed into dispersed long-spindle cells without polarity of tight junctions, thus aggregating tumor cell migration, invasion and metastasis [14]. Meanwhile, the expression level of epithelial-indicative proteins (E-cadherin and β-catenin) is decreased, whereas the expression level of interstitial markers (Vimentin and N-cadherin) is significantly increased [15,16]. Besides, a large number of transcriptional factors and signaling pathways have also been found to be involved in EMT [17-21].

Wnt pathway participates in the formation of the brain and nervous system during embryogenesis, while its dysregulation is associated with a variety of tumors [22-24]. Wnt pathway, as one of the most important inducing factors of EMT, is mainly regulated by the phosphorylation/degradation of cytoplasmic β-catenin. Most cytoplasmic β-catenin remains stable by binding to E-cadherin and actin when the Wnt pathway is silenced. However, a small part of β-catenin is utilized to form a "degradation complex" with APC, GSK3β and axin, thus hindering it from entering into the nucleus. When the Wnt signal is activated, it dissociates the "degradation complex" by Dv1, resulting in the accumulated cytoplasmic β-catenin. The β-catenin then translocates into the nucleus and interacts with TCF and LEF, thus regulating the expressions of target genes [25]. It is reported that slug reduces the E-cadherin expression and induces EMT by activating the Wnt pathway [26].

In this study, miR-372-3p was found overexpressed in breast carcinoma tissues, which was associated with tumor size, tumor stage and postoperative survival of patients with breast carcinoma. Luciferase reporter assay showed that miR-372-3p was directly bound to DKK1, the inhibitor of the Wnt pathway. ROC curve also suggested that miR-372-3p could be a potential prognostic indicator of breast carcinoma. Overexpressed miR-372-3p significantly increased the expressions of Wnt3a and N-cadherin in breast carcinoma cells but decreased the expressions of DKK1 and E-cadherin. In contrast, miR-372-3p knockdown showed the opposite results. Moreover, upregulated E-cadherin and downregulated N-cadherin were observed in cells co-transfected with miR-372-3p mimic and DKK1 compared with those transfected only with miR-372-3p mimic, suggesting that DKK1 partially reversed the role of miR-372-3p in promoting EMT.

Conclusions

We confirmed that miR-372-3p activated the Wnt pathway to promote EMT in the development of breast carcinoma. miR-372-3p expression was positively correlated with tumor size and tumor stage and negatively correlated with survival of

patients with breast carcinoma, making it a potential indicator for clinical diagnosis and prognosis of breast carcinoma.

Conflict of interests

The authors declare no conflict of interests.

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