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Original Article

MiR-138 suppresses EMT through degradation KDM6B in breast carcinoma

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Abstract: Breast carcinoma has become the fifth mortality rate in world and the most common cancer in women. MicroRNA is a group of non-coding small RNA which approximal 20-24 nt length, it binding target gene 3'-UTR, thereby degradation its target gene mRNA. Now, more and more microRNA was found participate tumor development. Here, we demonstrate that miR-138 was down-regulated in breast carcinoma samples as well as breast cancer cell lines, and the decreased miR-138 was correlated with Lymph Node Metastasis and TNM stage poor prognosis. Furthermore, we found over-expression of miR-138 could inhibit breast cancer cell proliferation and metastasis. Most important was we uncovered the mechanism of MiR-138 in suppresson of Epithelial-to-mesenchymal transition (EMT) and breast cancer cells invasion. Through degradation of KDM6B, therefore down-regulating KDM6B, and inhibited H3K27me3 demethylation, miR-138 could thereby inhibit EMT. In conclusion, we provide a novel epigenetic mechanism regulating tumor cell invasion and EMT, and provide a new biomarker for breast diagnosis and prognosis.

Keywords: miR-138, EMT, KDM6B, breast cancer

Introduction

MicroRNAs, for short miRNA, are small, high evolutionally conserved, endogenous and non-coding single-stranded RNA molecules of 20-24 nucleotides. In nucleus, primary miRNA undergo a serious of processing, generation approximately 70- to 90-nt precursor miRNA. Pre-miRNAs subsequently catalyze by Dicer to form mature miRNA [1-4]. Recently, more and more study argue that miRNAs as tumor suppressors in many carcinomas [5, 6]. MicroRNA can degradation or translational suppression its target mRNA through binding to partially complementary recognition sequences of the 3'-UTR of its target gene's mRNA [7-9]. And growing number of tumor suppressor and oncogenes genes that have been demonstrated to be control by miRNAs, the importance function of miRNAs in genome stability, cell proliferation, cell cycle progression and malignant transformation is becoming increasingly recognized [10]. A large number of miRNAs were reported to be involved in cancer progression showing an aberrant and variable level of expression [11]. A microRNA expression signature of human solid tumors defines cancer gene targets [12]. Furthermore, increasing studies indicated that the expression level of miRNAs can prediction the medicinal cure effect of the disease [13, 14]. MiR-138 was observed aberrant expression in various malignancies, including non-small lung cancer [15], breast cancer [16] and skin fibroblasts [17]. Functionally, miR-138 has been reported to regulate FOXP4 to inhibit NSCLC and as a putative tumor suppressor [18], multiple results demonstrate mir-138 participate several pathway, such as MAPK pathway [17]. However, the detail mechanism of miR-138 in breast cancer still poorly understood.

Epithelial-to-mesenchymal transition (EMT) is a complex process that regulated by a various molecules and signaling pathways. EMT play a critical role in embryonic development and also promote tumor invasion and metastasis [19-21]. One of the most important features of EMT is that epithelial cell markers were remarkable decrease, including E-cadherin and vimentin, whereas mesenchymal markers were significantly increasing, such as N-cadherin and fibro-

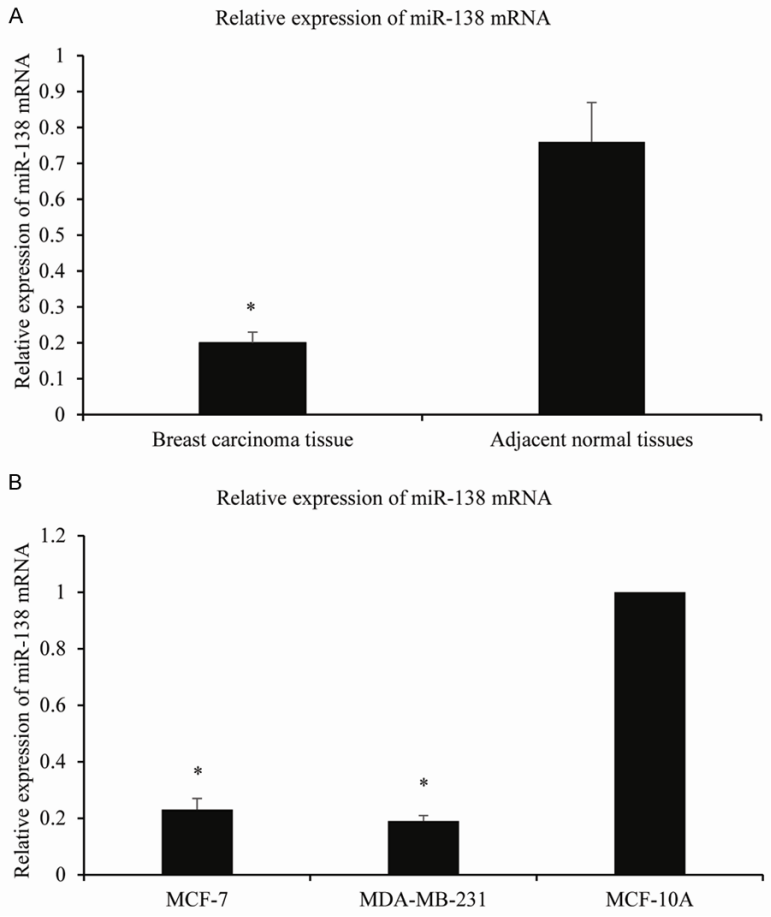

Figure 1. miR-138 is significantly decreased in Breast tissues sample and associated with lymph node metastasis and TNM stage. A. Relative expression of miR-138 mRNA was detected by qRT-PCR in breast carcinoma tissue and adjacent normal tissue from breast carcinoma patients. *P<0.05. B. MiR-138 mRNA level between breast cancer cell lines MCF-7 or MDA-MB-231 and human normal breast cell lines MCF-10A. *P<0.05.

nectin [22]. Recent reports have highlighted the importance of miRNA as a powerful regulator of EMT in cancer cells. In this study, we explored the function of miR-138 in tumor development, and the relationship between miR-138 and the pathogenesis of breast carcinoma, and demonstrated that miR-138 as a tumor suppressor, while overexpression of miR-138 suppress the proliferation, migration and invasion in breast cancer, which are in line with EMT. Furthermore, we found a novel mechanism that miR-138 regulate EMT through degradation KDM6B.

Materials and methods

Antibodies and reagents

KDM6B was from Abcam, E-cadherin, N-cadherin and α-catenin were from BD Biosciences; fibronectin, H4, H3K4me3, H3K9me3, H3K27me3, H4-K36me3, and H4K20me1 were from Abcam. MiR-138 were purchased from RIBOBIO, Inc. MiR-138 mimics (#miR10004607-1-5). MiR-138 inhibitors (#miR20004607-1-5). Mimic negative control (#miR01201-1-5). Inhibitor negative control (#miR02201-1-5).

Cell culture and cell transfection

The breast carcinoma cell lines MCF-7 and MDA-MB-231 cells were maintained in DMEM (Hyclone) supplemented with 2 U/mL penicillin, and 2 mg/mL streptomycin, 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. The normal breast tissue cells MCF-10A was used as control. Lipofectamine 2000 Transfection reagent was used to transfection according to manufacturer's instructions (Invitrogen).

Transwell invasion assay

MDA-MB-231 cells were transfected with miR-138 mimics control, miR-138 mimics, miR-138 inhibitors control or miR-138 inhibitors. After 48 hr, 2×10^5 cells were placed to upper chamber with serum free media and incubate for 24 h at 37°C. The membranes were then stained with 0.5% crystal violet solution and counted.

RNA isolation and real-time quantitative PCR

Total RNA was extracted with TRIzol (Invitrogen). cDNA was prepared by total RNA (2 μg) using the reverse transcription system (TransGen) with dNTPs and random primers. Quantitative real-time PCR (qRT-PCR) under the following conditions: 5 min at 95°C followed by 40 cycles at 95°C for 30 s, 55°C for 40 s, and 72°C for 1 min using an ABI Prism 7300 sequence detection system. Data were normalized by the GAPDH or U6 for each experiment. Data were calculations of $2^{-\Delta\Delta CT}$.

Table 1. Clinic pathologic variables in 156 Breast patients

Variables	No. (n=156)	MiR-138 expression		*P* value
		Low (n=95)	High (n=61)	
Age				
<50	96	60	36	0.604
≥50	60	35	25	
Tumor diameter				
Small (≤3 cm)	64	28	36	<0.001
Large (≥3 cm)	92	67	25	
Pathological grade				
I-II	77	37	40	0.001
III-IV	79	58	21	
pT status				
pT1	86	46	40	0.036
pT2-4	70	49	21	
pN status				
pN0	82	36	46	<0.001
pN1-2	74	59	15	
Lymph node metastasis				
Yes	88	62	26	0.005
No	68	33	35	
Drinking status				
Never drinking	76	45	31	0.674
Drinking	80	50	30	
Differentiation				
Well/moderate	71	38	33	0.084
Poor	85	57	28	

The primer pairs used were as follows: *GAPDH* (forward primer), 5'-CATTTCCTGGTATGACAACGA-3' and *GAPDH* (reverse primer), 5'-TACATGGCAACTGTGAGGAG-3'; *E-cadherin* (forward primer), 5'-AAGAGGACCAGGACTTTGAC-3' and *E-cadherin* (reverse primer), 5'-CACGAGCAGAGAATCATAAGG-3'; *α-catenin* (forward primer), 5'-AAGGTGATTTGATGAAGGCTG-3' and *α-catenin* (reverse primer), 5'-ATCTTCCACAACTTTCAGCT-3'; *γ-catenin* (forward primer), 5'-CTAATCAGTGCCCAGATCCA-3' and γ-*catenin* (reverse primer), 5'-GGATCTCAGCTTTAACTTGACTG-3'; *N-cadherin* (forward primer), 5'-TCATTAATGAGGGCCTTAAAGC-3' and *N-cadherin* (reverse primer), 5'-CCTGAAGTTCAGTCATCACC-3'; *Fibronectin* (forward primer), 5'-GTCTATCAGATATCTGAGGATGGG-3' and *Fibronectin* (reverse primer), 5'-ACTATTCTGTACCCTGTGATGG-3'; *Vimentin* (forward primer), 5'-GCCCTTAAAGGAACCAATGAG-3' and *Vimentin* (reverse primer), 5'-CAGAGAAATCCTGCTCTCCTC-3'; *snail* (forward primer), 5'-TCTAATCCAGAGTTTACCTTCCAG-3' and *snail* (reverse primer), 5'-TGAAGTAGAGGAGAAGGACGA-3'; *KDM6B* (forward primer), 5'-CAAGATGATCAAGTTCTGCC-3' and *KDM6B* (reverse primer), 5'-ACGAACAGGATGTTAAACACC-3; The specific primers of miRNA-138 (#miRQ-0000-4607-1-2) and U6 (#MQP-0201) were purchased from RiboBio (Guangzhou, China). U6 was used to normalized miR-138.

Cell proliferation assay

In order to assess of cell proliferation rates, 5×10^4 MCF-7 and MDA-MB-231 cells were added in 96-well plates and incubated 24 hr. After infected with pre-miR-138 and pre-NC or miR-138-in and NC-inh, added 20 μl MTT (St. Louis, Mo, USA) to each well, then incubated for 4 hr at 37°C. Final, 150 μl DMSO was used to stop reaction and count cell density at OD 490 nm on a microplate reader. Each experiment was repeated at least three times.

Western blot analysis

The total protein was lysed in RIPA lysis buffer and then resolved on SDS-PAGE gel, and then blotted onto the PVDF membrane. The 5% dried skimmed milk was used to blocked PVDF membrane at 37°C for 1 hr, subsequently incubation with appropriate primary antibody at 4°C overnight or 1 hr at room temperature, follow by incubated with a secondary antibody (Santa Cruz) for 1 hr at 37°C. The blots were visualized using Western blotting Luminal reagent on X-ray films (Amersham or Kodak) according to the manufacturer's recommendation.

Statistical analysis

All data were expressed as mean ± standard deviation (SD) from at least four separate experiments. The relationship between miR-138 expression and clinic pathological characteristics was analyzed by χ^2 test. $P<0.05$ (*) or $P<0.01$ (**) was considered statistically significant. The SPSS 17.0 and Microsoft Excel 2013 were used to calculated data.

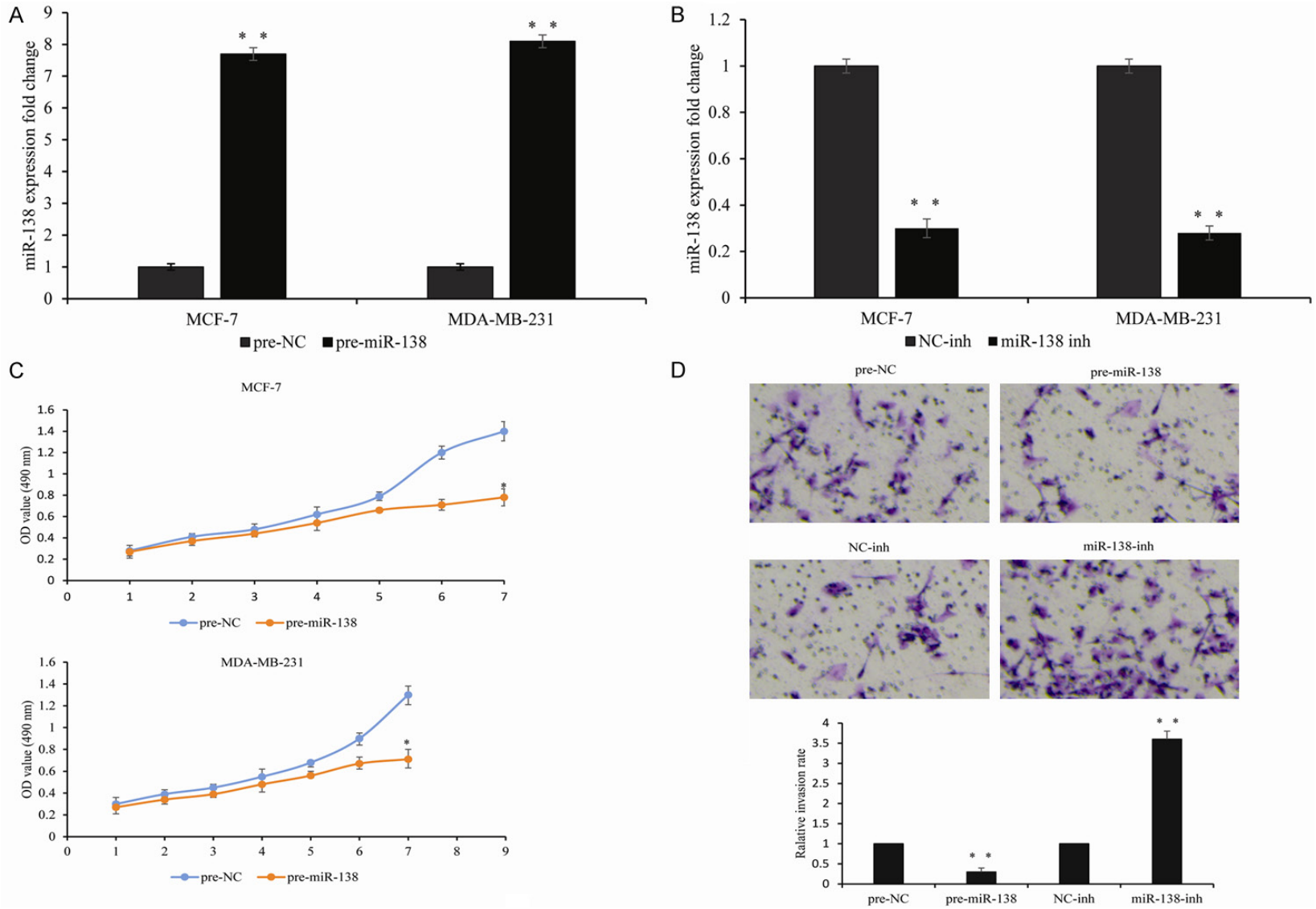

Figure 2. miR-138 inhibits the proliferation of Breast Carcinoma cells and regulates invasion in breast carcinoma. A. qRT-PCR was performed to verify the effect of pre-NC or pre-miR-138 in MCF-7 cells and MDA-MB-231 cells. **P<0.01. B. qRT-PCR was performed to verify the effect of NC-inh or miR-138-inh in MCF-7 cells and MDA-MB-231 cells. **P<0.01. C. The MTT assay was used to assess the proliferation ability of MCF-7 and MDA-MB-231 cells, cells were transfected with pre-miR-138 or pre-NC. *P<0.05. D. MDA-MB-231 cells were transfected with pre-NC, pre-miR-138, NC-inh, miR-138-inh and performed transwell assay in 72 hr later. The number of invaded cells was shown in the graph. Each experiment was carried out three times. *P<0.05. **P<0.01.

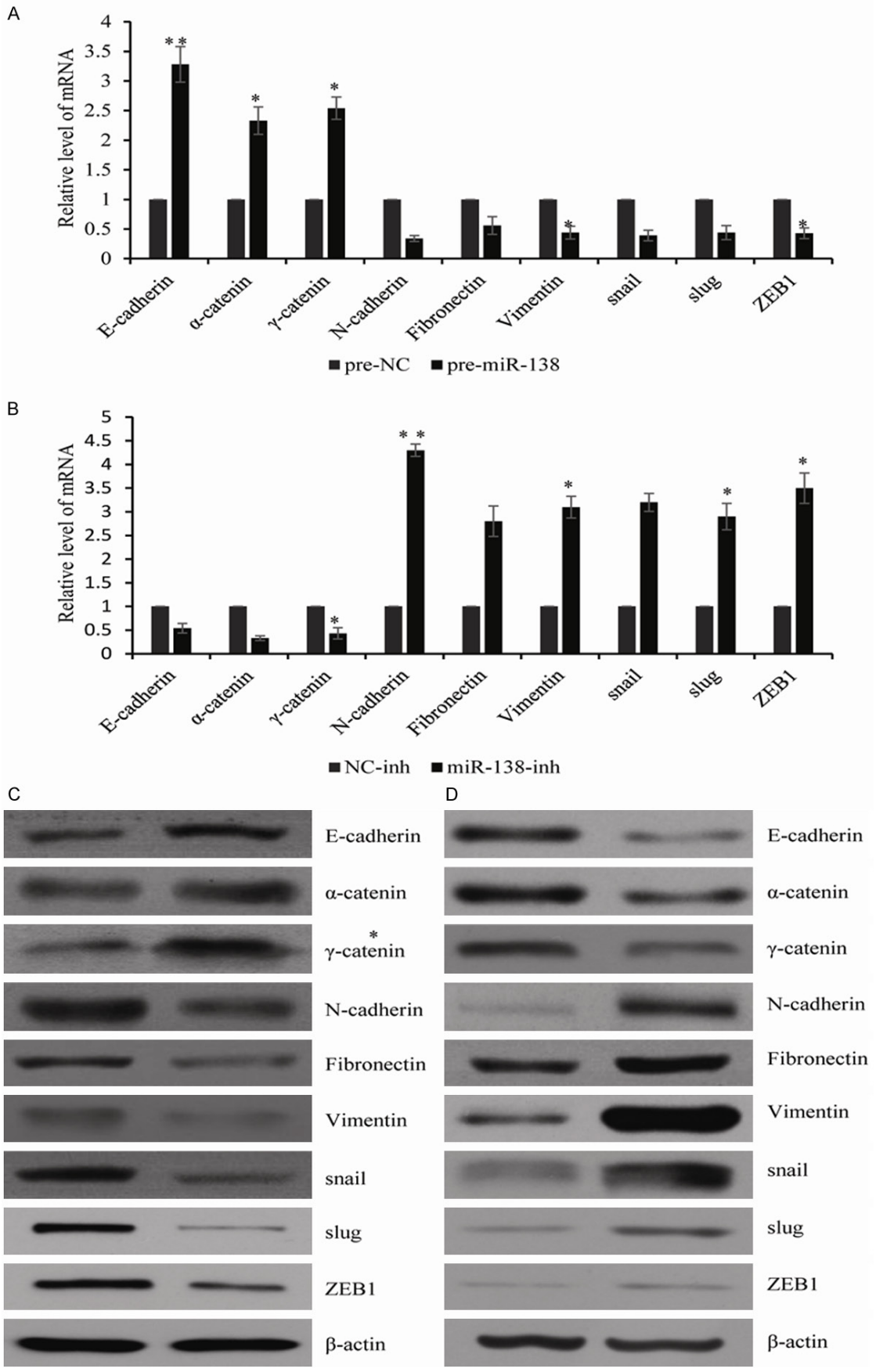
A
Relative level of mRNA
E-cadherin
α-catenin
γ-catenin
N-cadherin
Fibronectin
Vimentin
snail
slug
ZEB1
pre-NC
pre-miR-138
B
Relative level of mRNA
NC-inh
miR-138-inh
C
D
β-actin

Figure 3. miR-138 suppresses breast cancer cells EMT. A. Pre-NC and pre-miR-138 transfected MCF-7 cells, the mRNA levels of epithelial and mesenchymal markers were detected by qRT-PCR. *P<0.05. **P<0.01. B. NC-inh and miR-138-inh transfected MCF-7 cells, the mRNA levels of epithelial and mesenchymal markers were detected by qRT-PCR. *P<0.05. **P<0.01. C. Western blot analysis revealed the change epithelial and mesenchymal markers in MCF-7 cells which transfected pre-NC and pre-miR-138. D. Western blot analysis revealed the change epithelial and mesenchymal markers in MCF-7 cells which transfected NC-inh and miR-138-inh.

Result

MiR-138 is significantly decreased in breast tissues sample and associated with lymph node metastasis and TNM stage

To investigate the function of miR-138 in breast cancer, we collected 156 breast carcinoma samples, and used qRT-PCR to test the expression of miR-138 in breast tissues. As shown in **Figure 1A**, miR-138 was remarkably down-regulate in breast carcinoma tissue samples compared with adjacent normal tissues samples. Subsequently, we found miR-138 was also significantly decreased in two breast cell lines, MCF-7 and MDA-MB-231, compared with that of human normal breast cell line, MCF-10A (**Figure 1B**). Then, we also found there some relationship between miR-138 expression level and clinic pathologic information of breast carcinoma. It was summarized in **Table 1**. Intriguing, a statistically correlation between miR-138 expression levels and with advanced tumor node metastasis (TNM) stage (P<0.001), lymph node metastasis (P=0.005), and tumor size (P<0.001) was observed in this study. However, there are no significant correlation were found between miR-138 expression and other characteristics such as age, drinking status, differentiation.

MiR-138 inhibits the proliferation of breast carcinoma cells and regulates invasion in breast carcinoma

We first used miR-138 mimic or inhibitor (pre-miR-138, pre-NC, miR-138-inh, or NC-inh) to transfect cell, as shown in **Figure 2A** and **2B**, the miR-138 over-expression or depletion in MCF-7 and MDA-MB-231 were confirmed by qRT-PCR (*P*<0.001) (**Figure 2A** and **2B**). Because MiR-138 expression was correlation with tumor size and lymph node metastasis, we assumed that MiR-138 may be play role in cell growth and metastasis. So we subsequently to explore the effect of miR-138 on cell growth, MCF-7 and MDA-MB-231 were infected with miR-138 mimic to examine their growth curve. As shown in **Figure 2C**, MTT assay showed that over-expression of miR-138 inhibit MCF-7 and MDA-MB-231 cell growth compared with their corresponding controls. Then we used miR-138 inhibitor to infected MCF-7 and MDA-MB-231 cells, the opposed result can be seen, cells growth of MCF-7 and MDA-MB-231 were promoted by miR-138 inhibitor treatment (data not shown).

Next to investigate the mechanism that miR-138 participate cancer cells metastasis. MDA-MB-231 cells were transfected by miR-138 or control mimics (pre-miR-138, pre-NC, miR-138-inh, or NC-inh), then transwell assays were performed in vitro. The results show that the cells transient transfection of pre-miR-138 obviously decreased its abilities of invasion compared with negative controls (*P*<0.01), while the inhibition of miR-138 expression significantly increase cell invasion (**Figure 2D**). Above results indicate that miR-138 suppress MDA-MB-231 cells invasion.

MiR-138 suppresses breast cancer cells EMT

EMT is a complex processing, often associated with epithelial markers reduce, such as E-cadherin, and mesenchymal markers increase, such as N-cadherin and vimentin. We detected the epithelial markers and mesenchymal markers in MCF-7 and MDA-MB-231 that transfect miR-138 mimic. It was found that mRNA level of epithelial markers, such as E-cadherin was significantly increase, and mesenchymal markers, such as N-cadherin and vimentin were decrease in MCF-7 cell that over-expression miR-138 compare with control (**Figure 3A**). The oppose result can be seen in MCF-7 cell that transfect with miR-138 inhibitor and control (**Figure 3B**). Transcript factors also play important role in EMT, so we detected some transcript factors associate with EMT. Intriguing, over-expression miR-138 also decrease mRNA level of EMT associated transcript factor expression, such as Slug and ZEB1 (**Figure 3A**). Western blot analysis was further confirmed these differences at the protein levels (**Figure 3C** and **3D**).

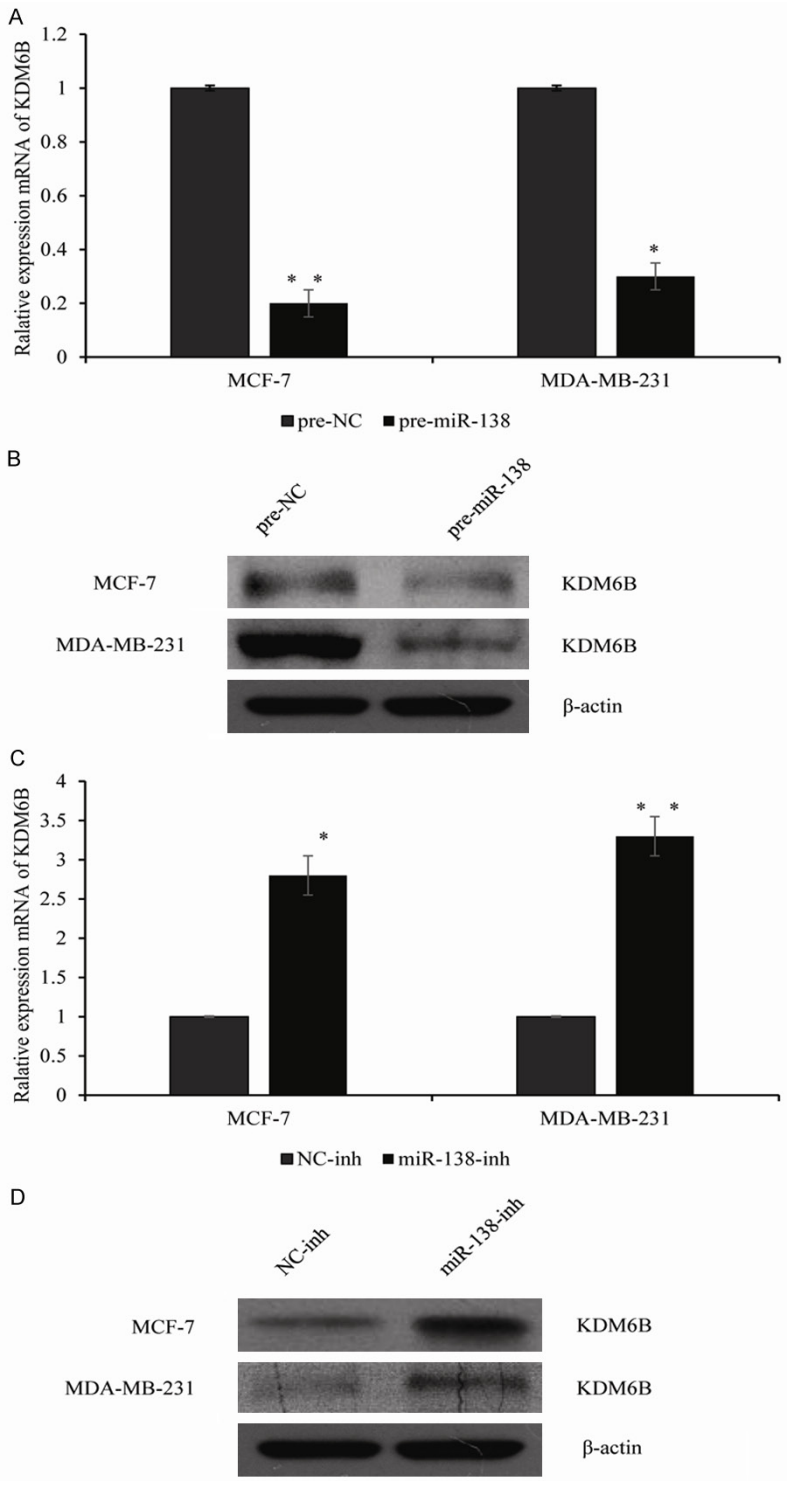

Figure 4. miR-138 suppresses EMT and invasion of breast cancer cells by down-regulating KDM6B. A. MCF-7 and MDA-MB-231 cells were transfected with pre-NC and pre-miR-138 for 48 hr. The relative level of KDM6B mRNA was normalized to GAPDH. *P<0.05. **P<0.01. B. The protein level of KDM6B in MCF-7 and MDA-MB-231 cells which transfected pre-NC and pre-miR-138 was detected by western blotting. C. The KDM6B mRNA change in MCF-7 and MDA-MB-231 cells which transfected NC-inh and miR-138-inh. *P<0.05. **P<0.01. D. NC-inh and miR-138-inh transfect MCF-7 and MDA-MB-231 cells and then detected the protein level of KDM6B by Western Blotting.

MiR-138 suppresses EMT and invasion of breast cancer cells by down-regulating KDM6B

To further explore the mechanism of miR-138 in EMT progress, we sought to identify additional targets for miR-138. We analyzed the potential targets of miR-138 based on public algorithm, Target Scan. It is indicated that KDM6B, as known as JMJD3, maybe regulated by miR-138. There have some studies show that KDM6B induce EMT through activate slug and snail. So we focused our subsequent study on KDM6B.

Based on previous study that microRNA could bind to target mRNA's 3'-UTRs sequence, leading to either target mRNA degradation or translation inhibition. We next explored the effect of miR-138 on the expression of KDM6B mRNA and protein. For this purpose, we used control and miR-138 mimic to transfected MDA-MB-231 and MCF-7 cells, followed by the measurement of KDM6B mRNA and protein expression by qPCR and Western blotting, respectively. The results indicated that miR-138 overexpression led to 80% and 70% decrease in KDM6B mRNA in MCF-7 and MDA-MB-231 cells, respectively (**Figure 4A**), and the protein level of KDM6B were also remarkable in cells transfected with pre-miR-138 (**Figure 4B**). Consistently, treatment of the cells with miR-138 inhibitors resulted in a 2.5-fold increase in KDM6B mRNA (**Figure 4C**) and a pronounced

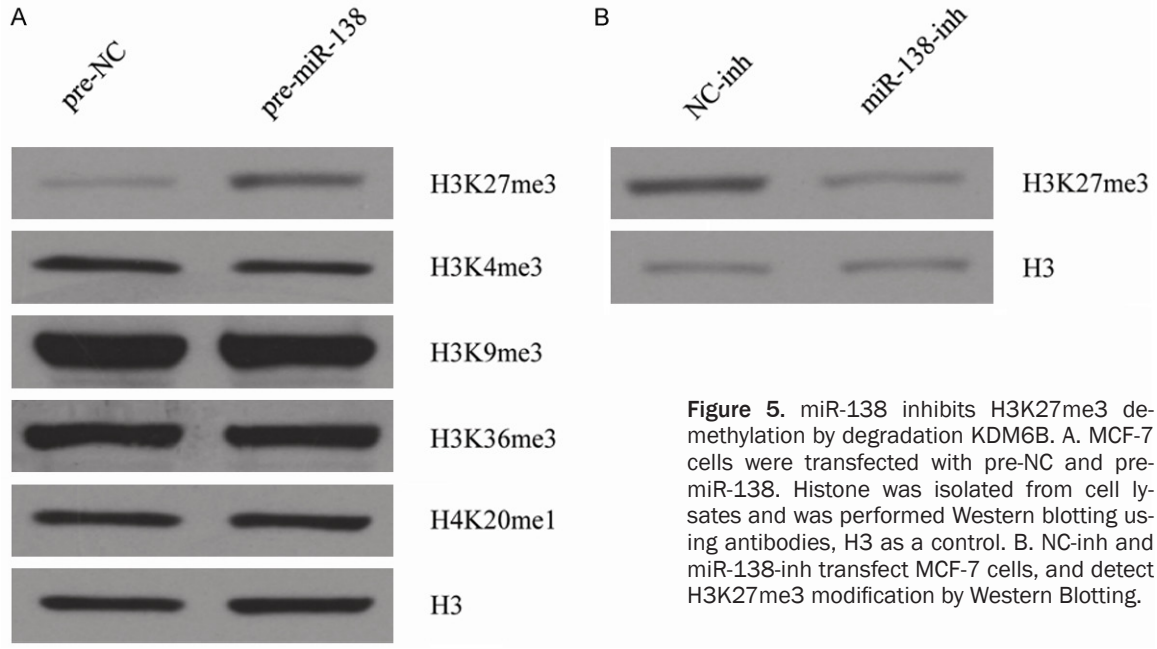

Figure 5. miR-138 inhibits H3K27me3 demethylation by degradation KDM6B. A. MCF-7 cells were transfected with pre-NC and pre-miR-138. Histone was isolated from cell lysates and was performed Western blotting using antibodies, H3 as a control. B. NC-inh and miR-138-inh transfect MCF-7 cells, and detect H3K27me3 modification by Western Blotting.

elevation in KDM6B protein expression (**Figure 4D**). Moreover, there are some study indicated that KDM6B promote EMT by its demethylase activity. So we hypothesis that miR-138 suppress EMT through degradation KDM6B. In order to confirm our hypothesis, MCF-7 cells transfect miR-138 and then co-transfected with the KDM6B expression construct containing *KDM6B* coding sequence but lacking the *KDM6B* 3'-UTR, which is resistant to miR-138, succeeded to rescue epithelial/mesenchymal marker changes induced by miR-138 (data not shown). Together, above data support a novelty mechanism that miR-138 suppresses EMT of breast cancer cells through degradation KDM6B.

MiR-138 inhibits H3K27me3 demethylation by degradation KDM6B

As stated above, KDM6B is a member of JmjC domain-containing demethyltransferase family, the function of which is histone H3 Lys 27 (H3K27me3) demethylation. The observation that miR-138 reduce the mRNA level of KDM6B prompted us to explore whether miR-138 could influence on trimethylation of H3K27. Consistent with decreased expression of KDM6B protein, increase of H3K27me3 was observed in miR-138 mimic transfected MCF-7 cells, whereas no significant difference in H3K4me3, H3K9me3, H3K36me3, H4K20me1 were detected (**Figure 5A**). In contrast, H3K27me3 was decreased in cells that were treated with miR-138 inhibitors (**Figure 5B**). Together, these results indicate that miR-138 promote H3K27 trimethylation by promoting KDM6B mRNA degradation.

Discussion

Breast cancer is the fifth mortality rate in general and the most common cancer in women. Further-more, the incidence of breast carcinoma is still increasing. It is necessary to identify a new clinical biomarker to predict the prognostic and developing a novel therapeutic target. Now growing evidence has indicated that several miRNAs take part in cancer apoptosis, proliferation and metastasis Liu [23]. Thus far, almost 1500 miRNAs regulate approximal more than 30% of the total genomic mRNAs [24]. MiR-138 has been reported interaction with cyclin D3 to regulate on-small cell lung cancer cells [15]. But the role of miR-138 in breast carcinoma is still unknown. In this study, we identify the expression level of miR-138 in breast cancer tissue sample and breast cell line. The results show that miR-138 was remarkable decrease in both tissue sample and cell lines compare with adjacent non-tumor tissues or normal breast cell. We also found the expression level of miR-138 has significant correlation with advanced tumor node metastasis (TNM)

stage (P<0.05), lymph node metastasis (P< 0.001), and tumor size. Subsequently, a serious of MTT assay and transwell assay demonstrate that miR-138 participate in cell proliferation and metastatic.

EMT is determined as a complex process that promote metastatic ability of breast cancer cell, it regulated by multiple mechanism, such as signal pathway, transcript factors and so on. One of the most characters is the change of epithelial as well as mesenchymal markers expression levels. For instance, E-cadherin, an epithelial marker, is significantly decreased, whereas N-cadherin, a mesenchymal marker, is increase. To our surprise, miR-138 suppresses EMT in MCF-7 and MDA-MB-231 cell line. But miR-138 how to inhibit EMT is poor understand. We next found the target gene of miR-138, based on public database public, Target Scan, we found KDM6B was potential target of miR-138.

KDM6B, also known as JMJD3, is member of JmjC family. KDM6B has histone H3K27me3 demethylase. Several cancers are in touch with H3K27me3 alternation, including kidney cancers, colon, gastric, ovarian and breast [25, 26]. Recent studies argue that KDM6B induce EMT in several cancer through its H3K27me3 demethylase, because low H3K27 trimethylation could increasing EMT associated transcript factors expression [27]. We hypothesis that miR-138 regulated EMT maybe through influence KDM6B. Our data confirmed that both mRNA and protein level of KDM6B was effect by miR-138, that means miR-138 degradation KDM6B.

Collectively, our data indicated that miR-138 was remarkable decrease in breast carcinoma cell lines and tissue samples, and overexpression miR-138 could suppress breast cancer cell proliferation and metastatic. Intriguing, EMT also was regulated by miR-138, and the detail mechanism was miR-138 degradation KDM6B, thereby influence EMT. In this study, we demonstrate that miR-138 act as tumor suppressor in breast carcinoma, in addition, our founding provide a new therapeutic target and a prognostic marker in breast carcinoma.

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Disclosure of conflict of interest

None.

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