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The oncogenic impact of RNF2 on cell proliferation, invasion and migration through EMT on mammary carcinoma

Jin Wu[a,1], Hao Wang[a,1], Qing Li[c,1], Qian-Ying Guo[a], Si-Qi Tao[a], Yu-Xian Shen[b,**], Zheng-Sheng Wu[a,*]

[a] Department of Pathology, Anhui Medical University, Hefei, Anhui, China
[b] Institute of Biopharmaceuticals, School of Basic Medical Science, Anhui Medical University, Hefei, Anhui, China
[c] Department of Pathology, Fuyang Hospital of Anhui Medical University, Fuyang, Anhui, China

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ABSTRACT

Mammary carcinoma (MC) is one of most common malignancy in women, and ring finger protein 2 (RNF2) possesses various roles in vast human tumors. In MC tissues as well as in cell lines RNF2 exhibited high expression, had significant association with tumor size, lymph node status, TNM stage, patients' poor survival, and promoted cell proliferation, colony formation, cell migration and invasion of MC cell lines which was mediated by downregulation of E-cadherin protein. These data reveal that RNF2 protein plays a vital role in the development of MC and may be a potential therapy target of MC.

1. Introduction

Mammary carcinoma (MC) is the most common malignancy in women worldwide, accounting for approximately 32% of new cancer cases in women in African Americans [1]. While in China, MC alone is expected to account for 15% of all new cancers in women from 72 cancer registries [2]. Although massive advances in diagnostic and therapeutic strategies have been made to date, MC still contributes to most mortality rate in women [1,2]. Over the past decades, numerous tumor suppressor genes and oncogenes have been demonstrated in MC and further studies of these gene alterations and functions will result in deeper understanding of molecular mechanisms of MC development and progression.

RNF2, also named Ring1B, a core member of Polycomb group proteins (PcGs) which is known as transcription repressors of several thousand genes implicated in differentiation pathways, plays an important role in different types of human cancer dependent on its E3 ligase activity [3–5]. In mammalians, PcGs contain two main distinct Polycomb core complexes known as Polycomb repressive complex 2 and 1 (PRC2 and PRC1 respectively). As one of the PRC1 core members, RNF2 catalyzes lysine 119 mono-ubiquitylation (K119) of histone H2A (H2AUb1) which might lead to gene silencing through the induction of chromatin compaction and inhibition of transcriptional elongation [3,6–8]. Apart from its monoubiquitination activity, the PRC1 complex also has poly-ubiquitination activity. For example, Geminin, a DNA replication inhibitor, could be poly-ubiquitinated by PRC1 to sustain adult hematopoietic stem cell activity [9]. Previous studies demonstrated that several E3 ligases regulate p53 activity through direct degradation or altering its transcriptional activity [10,11]. Furthermore, a previous study revealed that RNF2 negatively regulates p53 expression and promotes tumor development in selective cancer cell types [12]. Knockdown of RNF2 in HeLa cells could lead to morphologic changes and/or a dramatic inhibition of cell proliferation [13]. A recent study reported that RNF2 was overexpressed in ovarian carcinoma and urothelial carcinoma, further being an independent prognostic marker [14,15].

These observations reply that RNF2 may play an important role in the progression of several types of human cancer. Thus, in this study, we firstly detected the expression of RNF2 in mammary carcinoma tissues and cell lines, and then analyzed the correlation between RNF2

Abbreviations: MC, Mammary carcinoma; RNF2, ring finger protein 2; miRNAs, microRNAs; IHC, immunohistochemistry; siRNA, small interfering RNA; RT-qPCR, quantitative real-time polymerase chain reaction; RFS, relapse-free survival; OS, overall survival

* Corresponding author at: Department of Pathology, Anhui Medical University, Hefei, Anhui, 230601, China.
** Corresponding author at: School of Basic Medical Science, Anhui Medical University, Hefei, Anhui, 230601, China.
E-mail addresses: shenyx@ahmu.edu.cn (Y.-X. Shen), woozson@yahoo.com (Z.-S. Wu).
[1] Authors contributed equally to this project.

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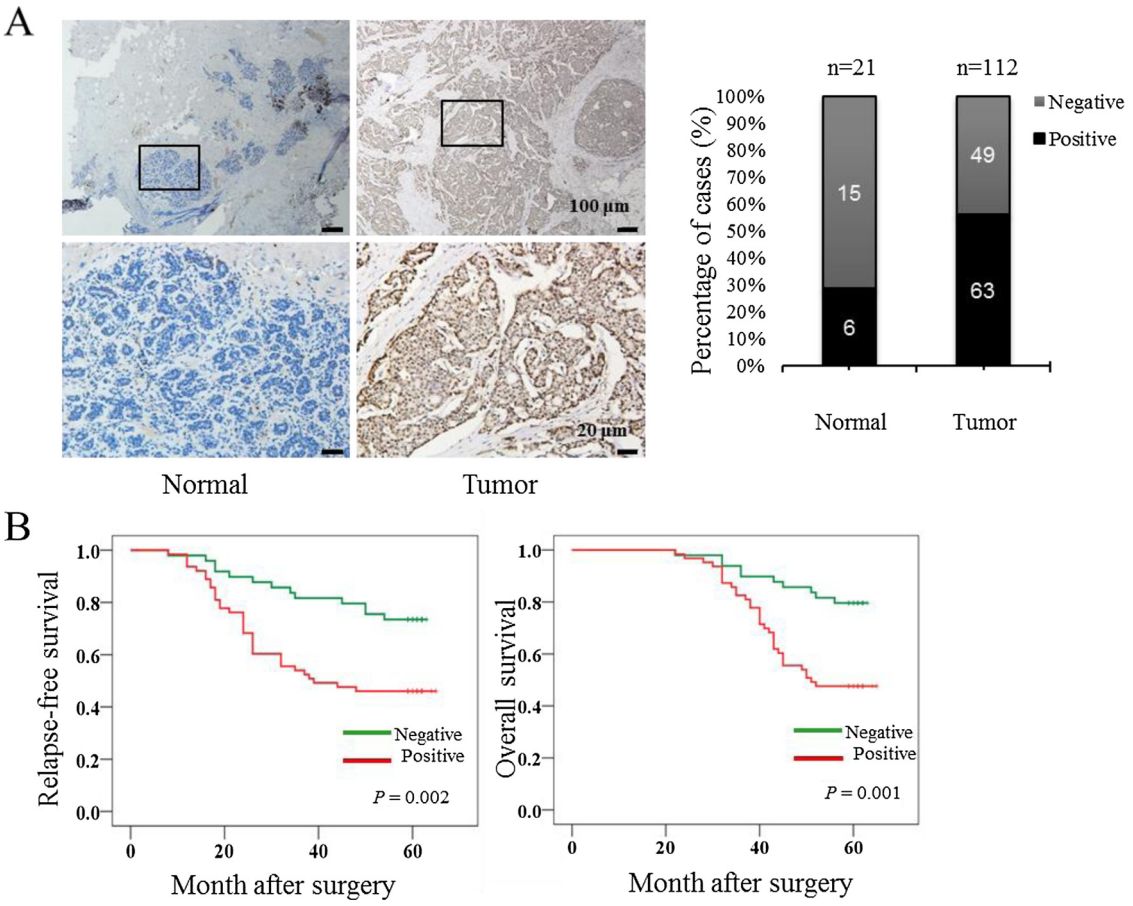

Fig. 1. Expression of RNF2 protein and mRNA in mammary carcinoma (MC) and benign mammary disease.
(A) Expression of RNF2 protein in MC (left) and adjacent non-cancerous tissue samples (right) was detected by immunohistochemistry. Scale bars represent 100 μm and 20 μm. ***(B)*** Kaplan-Meier analyses of the relapse-free survival or overall survival according to RNF2 protein expression.

Table 1
RNF2 protein expression in adjacent non-cancerous and MC tissues.

	n	RNF2 Positive, n(%)	
adjacent non-cancerous	21	6(28.6)	
MC	112	63(56.3)	0.02

protein level and clinicopathological characteristics of patients as well as survival rate. Secondly, we explored the role of RNF2 expression in mammary carcinoma cell proliferation, migration and invasion. Thirdly, the potential molecular mechanisms underlying the intrinsic process were revealed.

2. Material and methods

2.1. Mammary tissue specimens

In this study, 112 MC and 21 adjacent non-cancerous samples with available clinical information and paraffin-embedded blocks were enrolled. All the patients received tumor resection and tissues were obtained from patients during the surgeries. The All MC patients were female and received radical mastectomy or modified radical mastectomy. These 112 MC patients were followed-up for a median 60 months. A protocol to use patient samples was approved by the Biomedical Ethics Committee of Anhui Medical University and a written informed consent was obtained from each patient.

2.2. Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue specimens were cut into 4-μm-thick sections. The specific immunohistochemistry procedure was operated as we previously described [16]. And the antibody used here was a rabbit anti-RNF2 polyclonal antibody obtained from Origene technologies (Origene, Beijing, China).

Expression of RNF2 protein in mammary tissue specimens was reviewed and scored by two pathologists using a light microscope (Olympus) who were blind to the clinical and histopathological features of each slide. The extent of RNF2 specific nuclei immunostaining was divided into two groups: one for negative staining as lower than 10% positive staining, while the other as positive staining as equal or higher than 10% staining. The experiment has designed negative and positive controls.

2.3. Cell culture

All human breast cancer cell lines (BT549, BT474, SKBR3, MCF-7, MDA-MB-231, MDA-MB-453 and T47D)and human breast epithelial cell line (MCF-10A) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). MCF-7, BT474, SKBR3, T47D and BT549 cells were maintained in the RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Hyclone). MDA-MB-231 were cultured in DMEM medium supplemented with 10% FBS. MDA-MB-453 cells were cultured in L15 medium supplemented with 10% FBS. MCF-10A were cultured in DMEM/F12 medium supplemented with 5% super horse serum (SHS, Sangon biotech). In addition, MDA-MB-453 were cultured in an incubator under CO2-free at 37°C. All cell lines except

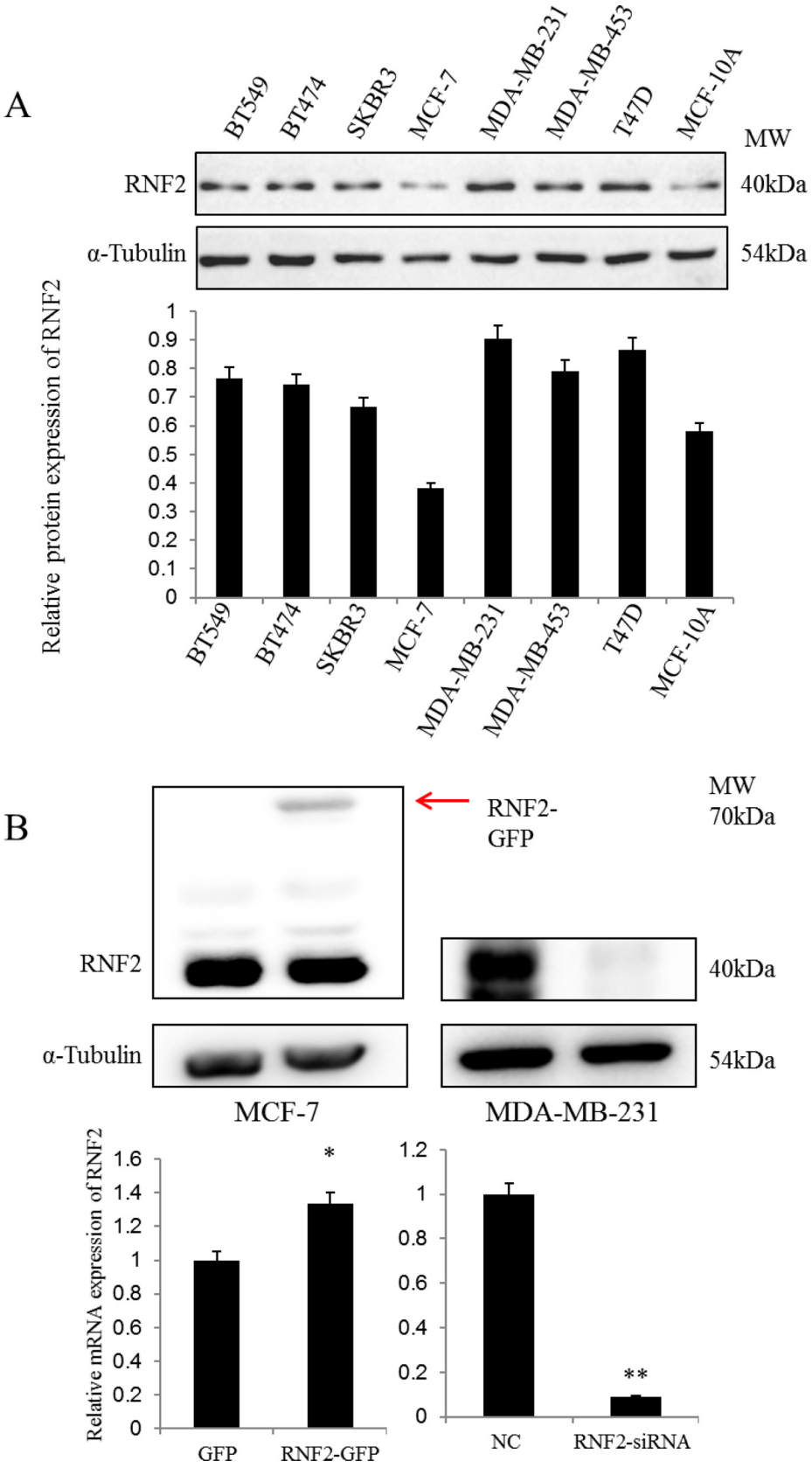

Fig. 2. The Efficient of Transfection RNF-GFP and siRNA in MCF-7 and MDA-MB-231.
(A) Expression of RNF2 protein in MC cell lines and non-tumorigenic mammary epithelial cells was analyzed by western blot. ***(B)*** Relative plasmids and siRNAs were transfected into MCF-7 and MDA-MB-231 cell lines for 48 h, which were confirmed by western blot and qRT-PCR. *, $P < 0.05$; **, $P < 0.01$.

Table 2
Associations between RNF2 expression in mammary carcinoma tissues and the clinicopathological characteristics of the tumor cohort.

Characteristics	n	RNF2		*P* value
		Positive, n(%)		
Age (years)				
≤35	10	8 (80.0)		0.197
35–55	61	35 (57.4)		
> 55	41	20 (48.8)		
Tumor size (cm)				
≤2	6	3 (50.0) ab		0.011
2–5	82	40 (48.8) a		
> 5	24	20 (83.3) b		
Lymph node metastasis				
0	35		7 (20.0) a	0.001
1–3	45		28 (62.2) b	
> 3	32		28 (87.5) c	
Stage				
I	7		4 (57.1)	0.261
II	71		36 (50.7)	
III	34		23 (67.6)	
Grade				
I + II	58		16 (27.6)	0.001
III + IV	54		47 (87.0)	
ER				
-	70		44 (62.9)	0.069
+	42		19 (45.2)	
PR				
-	69		40 (58.0)	0.642
+	43		23 (53.5)	
HER-2				
0–1 +	76		39 (51.3)	0.126
2 + −3 +	36		24 (66.7)	

Note: The different alphabet of "abc" indicate that the difference between the two groups is statistically significant ($P < 0.05$), and the same alphabet indicates that there is no statistically significant difference between the two pairs ($P > 0.05$).

MDA-MB-453 were cultured in the atmosphere of 5% CO2 at 37℃.

2.4. RNA isolation and quantitative polymerase chain reaction

Total cellular RNA was extracted with TRIZOL reagent (Life technologies) according to manufacturer's instructions. Then qRT-PCR was performed to detect expression of RNF2 and GAPDH as described previously [17,18]. The primer sequences were following: RNF2 5′-GCGA GGCAAGAAACAACAG-3′ (forward), 5′-CAACAGTGGCGTTACCA GAA-3′ (reverse), GAPDH 5′ − CCACTCCTCCACCTTTG-3′ (forward) and 5′-CACCACCCTGTTGCTGT-3′ (reverse).

2.5. Transfection of siRNA and plasmid

To knockdown or overexpress RNF2 protein, we choose MDA-MB-231 and MCF-7 cells as a pair of model for gene transfection. In brief, cells were seeded into 6-well plates and transiently transfected with RNF2 small interfering RNA (siRNA) *vs*. its negative control (GenePharma, Shanghai, China) or RNF2 overexpression plasmid with GFP tagged *vs*. its vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The sequences of RNF2 siRNA and negative control were listed following: 5′-UUAAUUCACUGUGUAGACUUCUAGG-3′ and 5′-UUCUCCGAACGU GUCACGUTT-3′. All EP tubes and tips used for siRNA transfection were dipped in DEPC treated water before.

2.6. Protein extraction and immunoblotting

Total cellular proteins were extracted and immunoblotting was performed according to previous studies [19]. The antibodies used for immunoblotting were displayed as follows: a rabbit anti-RNF2 polyclonal antibody (Origene, Beijing, China), a mouse anti-E-cadherin monoclonal antibody (BD, New York, USA) and a mouse anti-α-Tubulin monoclonal antibody (Sigma, St Louis, MO, USA).

2.7. Cell proliferation and colony formation assays

After 48 h of transfection, cells were counted and subcultured in 96-well plates for up to 4 days. Then at each certain time, MTT work reagent was added into each well, and the cells were incubated at 37 °C for 4 h. Absorbance was detected at 490 nm using a microplate reader (BioTek, Vermont, USA). At the same time, cells were counted and planted into 6-well plate, subcultured for almost 10 days, washed with PBS solution, dyed with crystal violet and then colony numbers were counted. The experiment was repeated thrice. Data were expressed as mean ± standard deviation (SD).

2.8. Transwell migration and invasion assays

Cell migration and invasion assays using a Transwell insert (8 μm; Corning, New York, USA) were performed as follows: cells were harvested with trypsin, washed with PBS and resuspended with a serum-free medium. The lower chamber was filled with a medium supplemented with 10% fetal bovine serum as a chemoattractant. And certain numbers of cells were seeded in the upper chamber and incubated in 37 °C and 5% CO_2 for 24–48 h. Thereafter cells remaining in the upper chamber were removed using a cotton swab, and filters were fixed using ethanol, and stained with 0.1% crystal violet. The number of migration cells was counted under light microscope in five random low-magnification areas. For invasion assays, the inserts were precoated with extracellular Matrigel (BD Biosciences, Bedford, USA). Both assays were performed in triplicate.

2.9. Immunofluorescence

Cells were seeded uniformly in a 24-well plate, transfected for 48 h, fixed with 4% formalin, permeabilized by 0.1% Triton X-100, blocked in 5% BSA, and incubated with indicated primary antibodies followed by fluorescent secondary antibodies. Nuclei were counterstained with DAPI (Sigma). Images were obtained using Olympus Microscope BX53/IX71.

2.10. Statistical analysis

All statistical analyses were performed using SPSS Software for Windows version 17.0 (SPSS Inc., Chicago, USA). The chi-squared (x^2) test was used to analyze the difference in RNF2 expression between MC and adjacent non-cancerous and the patient clinicopathological parameters. Bonferroni correction was performed for multiple comparisons. All the statistical have been tested for normality and equivariance. $P < 0.05$ was considered statistically significant.

3. Results

3.1. RNF2 was overexpressed in MC tissues and MC cell lines

We detected the expression of RNF2 protein in 112 MC tissues and 21 adjacent non-cancerous samples using immunohistochemistry and the data showed that expression of RNF2 protein was significantly higher in MC tissues than in adjacent non-cancerous tissues (56.3% *vs*. 28.6%, $P < 0.05$, Fig. 1A and Table 1). Similarly, in MC cell lines, RNF2 protein levels were higher than in non-tumorigenic mammary

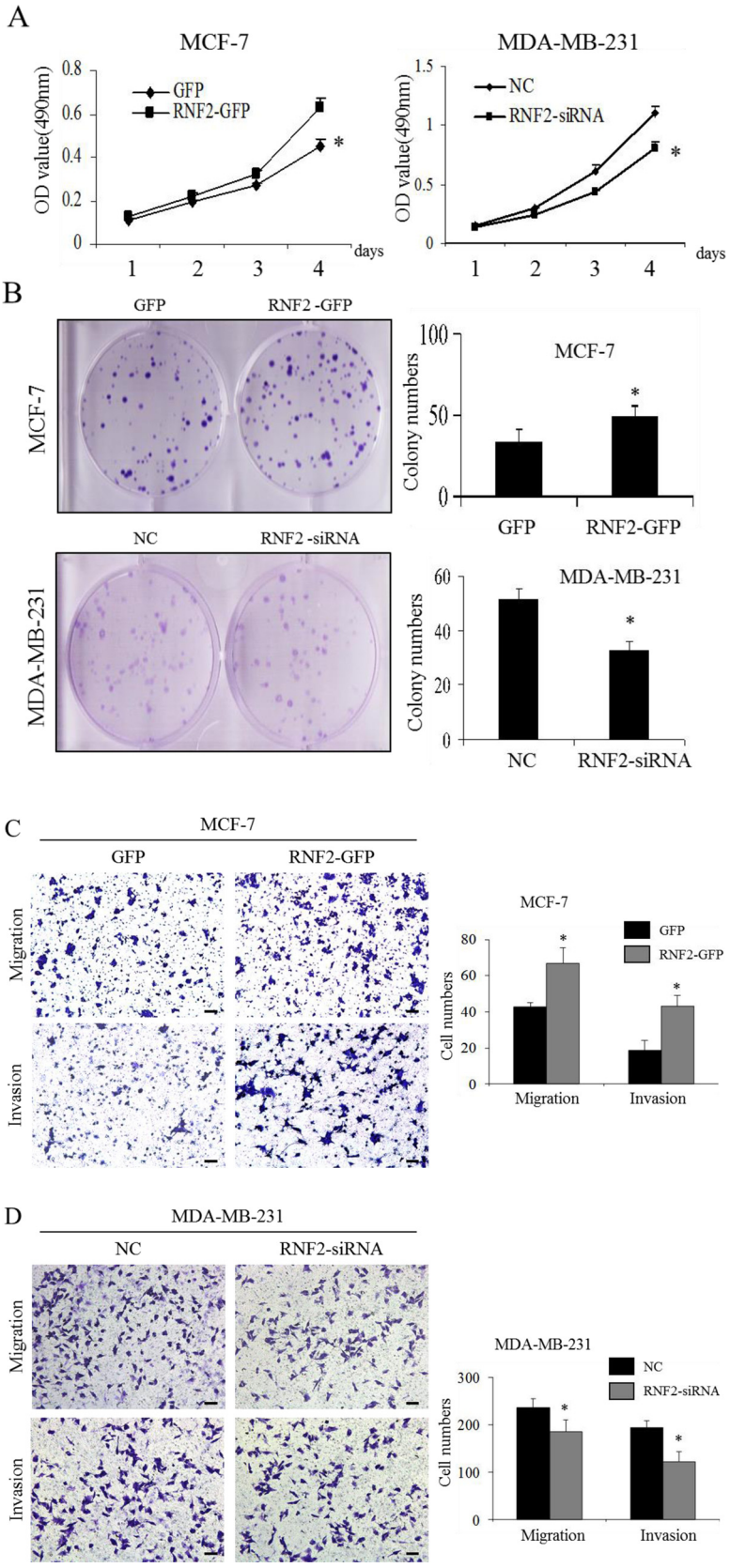

Fig. 3. Expression of RNF2 promotes mammary carcinoma cell proliferation, colony formation, migration and invasion *in vitro*.
(A) After transfected for 48 h, MTT assay was performed to detect cell proliferation in MCF-7 and MDA-MB-231 cells. ***(B)*** Colony formation was observed after 48 h transfection in MCF-7 ***(B upper)*** and MDA-MB-231 ***(B below)*** cells. *, $P < 0.05$. ***(C)*** Transwell migration and invasion assay. MCF-7 cells were grown and transiently transfected with RNF2-GFP or GFP for 48 h. *, $P < 0.05$. ***(D)*** Transwell migration and invasion assay. MDA-MB-231 cells were grown and transiently transfected with RNF2 siRNA or negative control (NC) for 48 h. *, $P < 0.05$. Scale bars represent 50μm.

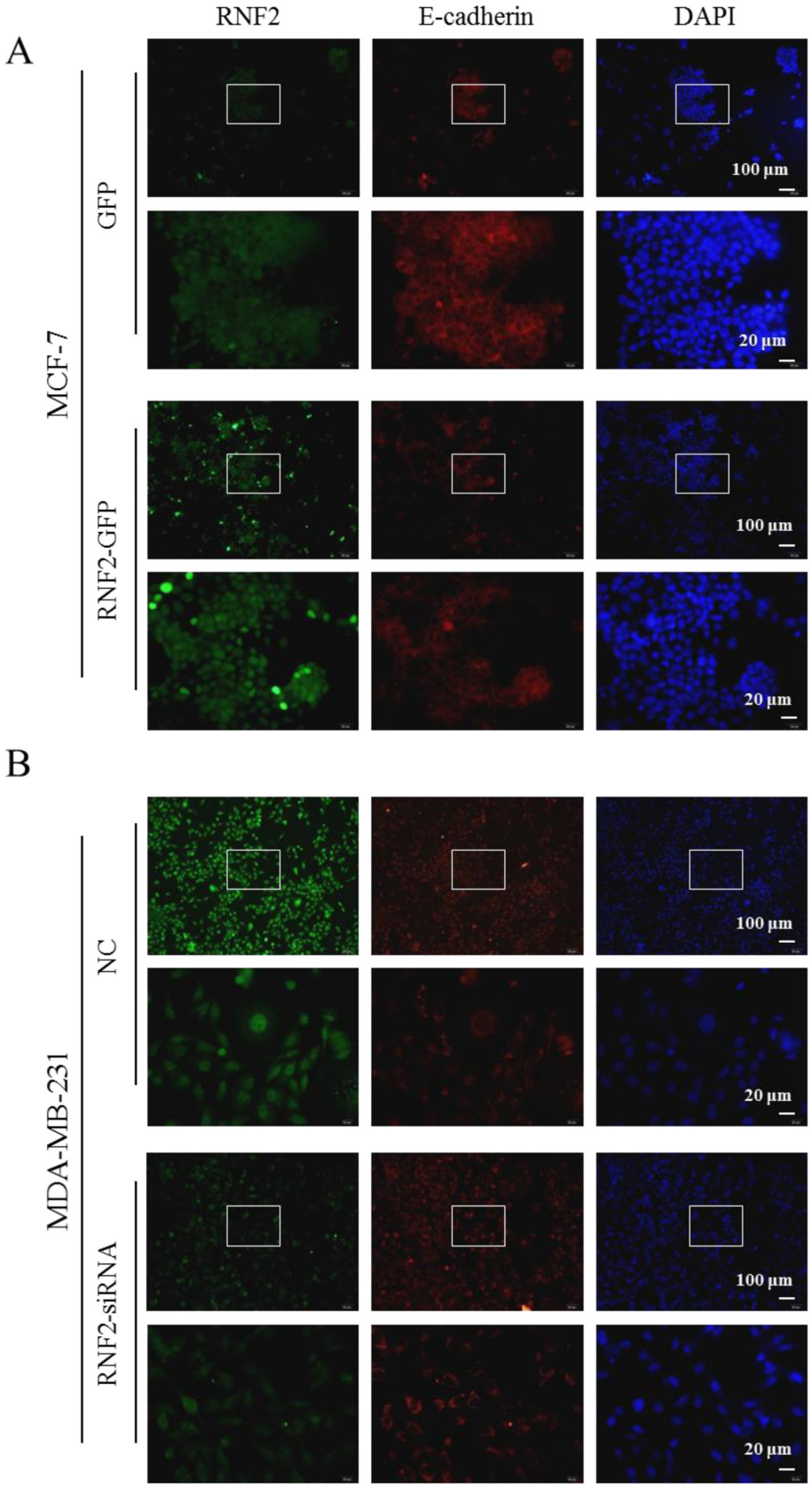

Fig. 4. Effects of RNF2 expression on E-cadherin expression.
(A) Immunofluorescence. MCF-7 cells were transiently transfected with GFP or RNF2-GFP for 48 h and stained with anti-RNF2 (green) and anti-E-cadherin (red) antibodies, respectively. ***(B)*** Immunofluorescence. MDA-MB-231 cells were transiently transfected with RNF2 siRNA or NC for 48 h and stained with above antibodies respectively. Scale bars represent 100 μm and 20 μm.

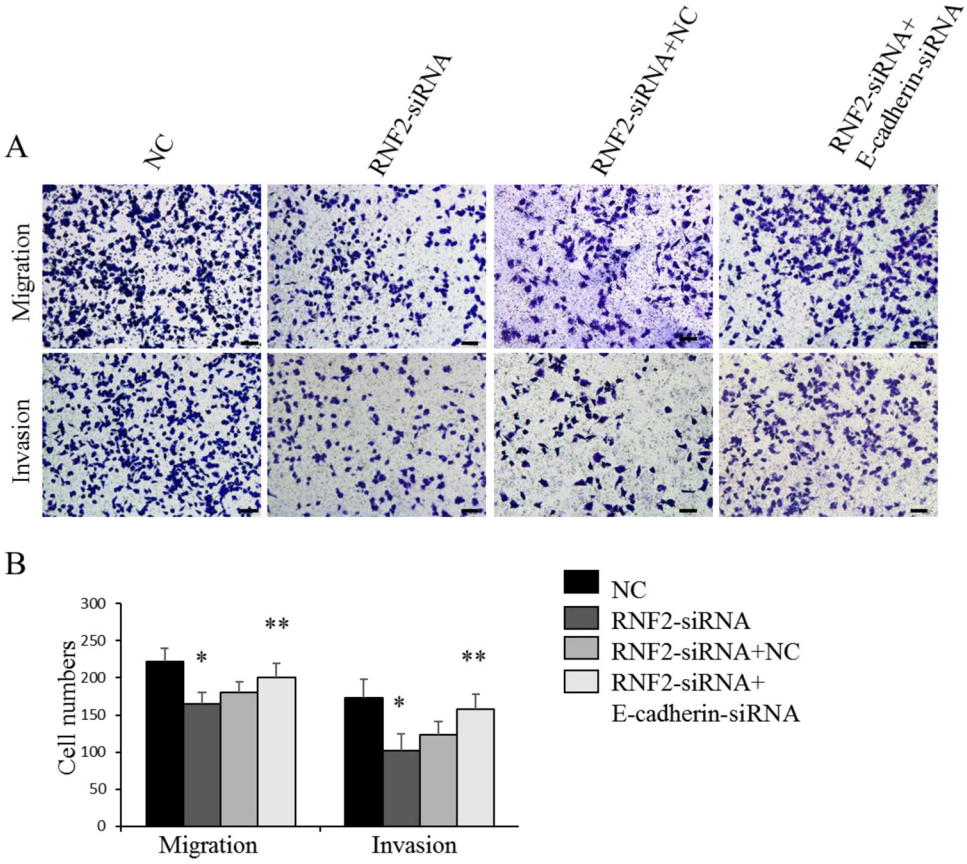

Fig. 5. Effects of RNF2 on cell migration and invasion were dependent on E-cadherin. ***(A)*** NC, RNF2-siRNA single or combined with E-cadherin-siRNA were transfected into MDA-MB-231 cells for 48 h and transwell assays were performed subsequently. ***(B)*** Cells were counted under light microscope in five random low-magnification areas. *, $P < 0.05$; **, $P < 0.01$. Scale bars represent 50μm.

epithelial cells by western blot (Fig. 2A).

3.2. Association between RNF2 protein expression and clinicopathological characteristics in MC patients

We found that RNF2 protein was significantly associated to tumor size, lymph node status and TNM stage ($P < 0.05$), but not apparently related to patients' age, histological stage, the expression of ER, PR and HER-2 ($P > 0.05$, Table 2). Kaplan-Meier analyses revealed that patients with RNF2 protein-positive primary tumors exhibited lower five-year relapse-free and overall survivals than patients with RNF2 protein-negative tumors ($P = 0.002$ and 0.001, respectively; Fig. 1B).

3.3. Expression of RNF2 promoted MC cell proliferation in vitro

Next we explored the impact of RNF2 on the biological behavior of MC cells. Based on the expression levels of RNF2 protein in MC cell lines (Fig. 2A), we therefore selected MCF-7 and MDA-MB-231 cells for the gain-of-function and loss-of-function assays. RNF2 siRNA and plasmid was transiently transfected into MDA-MB-231 cells respectively which was confirmed by immunoblotting and qRT-PCR ($P < 0.05$, Fig. 2B). We noticed that overexpression of RNF2 promoted MCF-7 cell viability ($P < 0.05$, Fig. 3A left), whereas knockdown of RNF2 expression reduced MDA-MB-231 cell viability ($P < 0.05$, Fig. 3A right). Colony formation assays showed that overexpression of RNF2 protein significantly promoted colony formation of MCF-7 cells compared with the vector control ($P < 0.05$, Fig. 3B upper). While we silenced the expression of RNF2 protein in MDA-MB-231 cells, cell colony formation was significantly decreased compared with the negative control ($P < 0.05$, Fig. 3B below).

3.4. Expression of RNF2 promoted cell migration and invasion in vitro

We also detected the impact of RNF2 expression on cell migration and invasion through traditional Transwell assay. Forced expression of RNF2 significantly promoted migration and invasion of MCF-7 cells ($P < 0.05$, Fig. 3C). In contrast, silencing the expression of RNF2 decreased the capacity of MDA-MB-231 cells migration and invasion compared to the control cells ($P < 0.05$, Fig. 3D).

3.5. Expression of RNF2 affects E-cadherin expression in vitro

As a previous study revealed that E-cadherin was upregulated in shRing1A/B cells in pancreatic cancer [23], we were drived to detect what changes of E-cadherin expression would be in MC as RNF2 expression changes. Thus, we artificially changed the expression of RNF2 in relevant two MC cell lines, and observed the change of E-cadherin expression. Immunofluorescence staining showed that E-cadherin protein was down-regulated in RNF2-GFP MCF-7 cells (Fig. 4A) cells and upregulated in si-RNF2 MDA-MB-231 cells (Fig. 4B).

3.6. Effects of RNF2 on cell migration and invasion were dependent on E-cadherin in MC cell lines

In order to observe whether the effects of RNF2 on cell migration and invasion were dependent on E-cadherin or not in MC cell lines, we chose MDA-MB-231 cell lines to perform Transwell assays. The result showed that after downregulation of RNF2 protein cells transferred the chamber decreased largely, while additional downregulation of E-cadherin protein reversed this phenomenon ($P < 0.05$, Fig. 5A and B).

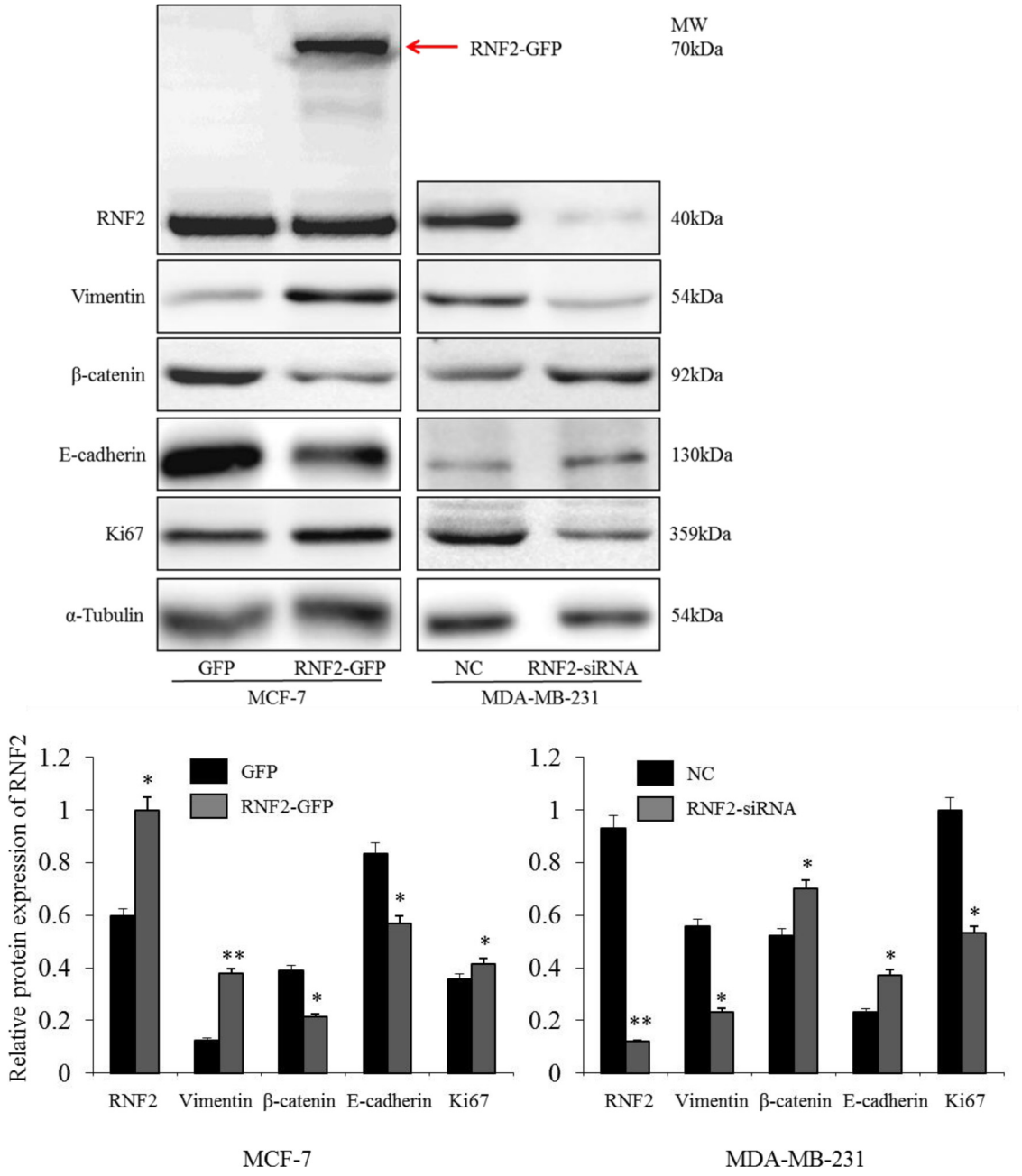

Fig. 6. Expression of RNF2 affects the Epithelial mesenchymal transition (EMT) and ki-67 of MC *in vitro*.
MCF-7 and MDA-MB-231 cells were transfected with RNF2-GFP/GFP or RNF2 siRNA/NC for 48 h, and subjected to western blot. *, $P < 0.05$; **, $P < 0.01$.

3.7. Expression of RNF2 affects the Epithelial mesenchymal transition (EMT) and ki-67 of MC in vitro

As E-cadherin is an important marker of EMT, western blot was performed to detected the level of more EMT markers. we artificially changed the expression of RNF2 in relevant two MC cell lines, observed the change of EMT markers expression. Western blot showed that level of E-cadherin and β-catenin was down-regulated in RNF2-GFP MCF-7 cells while vimentin and ki-67 was up-regulated. More importantly, we observed the similar phenomenon in si-RNF2 MDA-MB-231 cells ($P < 0.05$, Fig. 6).

4. Discussion

In the current study, we firstly detected the protein expression of RNF2 in MC tumors and benign mammary disease tissues, and analyzed the association between RNF2 expression and patients' clinicopathological characteristics. As higher expression of RNF2 protein was detected in MC tumors and significant correlation between RNF2 protein and clinicopathological characteristics was seen, we next explored the potential roles of RNF2 in cell proliferation, migration and invasion of MC cell lines. Finally, we made tiny efforts to observe how RNF2 protein affects MC cell biological behaviors.

RNF2, belongs to the PcGs which was first discovered to repress several homeotic genes expression in the developing embryo in drosophila, catalyzes H2AUb1 dependent on its *N*-terminal Ring finger domain and plays critical roles in stem cells renewal, differentiation, and tumorigenesis [20,21]. In addition, we observed higher expression of RNF2 protein in a cohort of MC tissues compared with benign mammary disease specimens, revealed that RNF2 might act as an oncogene in MC. Overexpression of RNF2 was observed in numerous types of human neoplasms including ovarian carcinoma [14], urothelial

carcinoma [15], Ewing sarcoma [22], pancreatic cancer [23], and esophageal carcinoma [24]. Thus correlation between RNF2 expression and patients' clinicopathological characteristics was analyzed and significant association between RNF2 expression and several characteristics including tumor size, lymph node status, TNM stage and patients' poor survival was observed, which gave an indication that RNF2 might be involved in MC cell proliferation and tumor migration and play as an oncogene.

Next, we detected the potential effects of RNF2 on MC cell lines *in vitro*. Using gain and loss-of-function experiments, our data displayed forced expression of RNF2 promoted cell viability, colony formation, cell migration and invasion in MC while knockdown of RNF2 protein inhibited these biological behaviors. As Chen et al. reported that RNF1 and RNF2 are required for Snail-mediated cell migration in pancreatic cancer [23], Yang et al. showed that in esophageal carcinoma downregulation of RNF2 expression inhibited tumor growth in nude mice and promoted cell apoptosis in cell lines [24]. Bosch et al. demonstrated that downregulation of RNF2 expression inhibited cell migration and invasion in breast cancer cell lines [25]. In melanoma, it has great controversy over the role of RNF2 whether E3 ligase or not [26,27]. All these data showed the oncogenic roles of RNF2 expression in several kinds of human tumor.

Epithelial mesenchymal transition (EMT) is one of several important steps in the process of tumor metastasis. In this process, cells lose their epithelial characteristics, including cell-cell adhesion proteins such as E-cadherin, ZO-1, and loss of cellular polarity; meanwhile obtain the ability of mesenchymal tissue cells, such as motility activity, for promoting migration of cytoskeletal proteins such as vimentin and protease such as matrix metalloproteinase 9 (MMP9) and other markers [28–30]. Cells with EMT subtypes, even if there is only one cell, can still transfer [31–33], which have an extremely adverse impact on the prognosis of patients. We then speculate that RNF2 promotes the migration and invasion capacity of MC cells mediated by EMT. With immunofluorescence and Western blot experiments, we confirmed this conjecture. Upregulation of RNF2 protein decreased E-cadherin protein expression, E-cadherin protein expression increased when silencing the expression of RNF2 protein, and regulation of E-cadherin protein could reverse the effects of RNF2 protein on cell migration and invasion, which suggested that RNF2 protein can inhibit the expression of E-cadherin protein and promote tumor metastasis. At last, we performed the western blot to check the protein expression of other EMT marker proteins in RNF2 knockdown and overexpressed cells.

In summary, our study revealed that RNF2 protein was higher in MC tissues compared with adjacent non-cancerous specimens. In addition, RNF2 expression was significantly associated with tumor size, lymph node status and TNM stage in MC patients. The oncogenic roles of RNF2 expression were confirmed through loss and gain-of–function approaches *in vitro* in MC cell lines. To the best of our acknowledgement, we demonstrated the relationship between RNF2 expression and clinicopathological characteristics in MC patients for the first time, indicating that RNF2 might be a potential therapeutic target in MC. Thus a conclusion was reached that RNF2 protein plays a vital role in the development of MC, which may be mediated by down regulation of E-cadherin protein.

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