Original Article

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Silence of MACC1 decreases cell migration and invasion in human malignant melanoma through inhibiting the EMT

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Summary **Metastasis-associated colon cancer 1 (MACC1) has been demonstrated to promote metastasis of several cancers via regulating epithelial-mesenchymal transition (EMT). However, its biological behavior in human malignant melanoma remains unclear. In this study, MACC1 downregulation was established in two melanoma cell lines (A375 and G361 cells) using RNA interference, as confirmed by quantitative real time PCR (qRT-PCR) and Western blot analysis. Subsequently, we investigated the effects of MACC1 silencing on cell mobility, migration and invasion using scratch wound and Transwell assays. Our results indicated that knockdown of MACC1 significantly suppressed cell migration and invasion ability of both melanoma cell lines. Moreover, downregulation of MACC1 upregulated E-cadherin, N-cadherin and Vimentin, as confirmed by qRT-PCR, Western blot and immunofluorescent Staining analysis. These findings suggest MACC1 might serve as a new molecular target for the treatment of melanoma by a novel mechanism underlying the metastasis of melanoma cells.**

Keywords: Melanoma, Metastasis-associated colon cancer 1 (MACC1), migration, invasion, epithelial-mesenchymal transition (EMT)

1. Introduction

Human malignant melanoma is characterized as highly aggressive with metastatic potential, which has been considered as one of the deadliest forms of skin cancer (*1*). It is estimated that more than seventy thousand melanoma cases were diagnosed in 2014 in the United States (*2*) and is still rapidly increasing compared to any other solid tumors (*3*). Over the past years, some progress has been made in surgery removal, chemotherapy and radiotherapy (*4,5*). However, the incidence and mortality rate have been increasing for higher recurrence and metastasis. Considering the lack of effective therapies, better understanding of molecular mechanisms underlying malignant melanoma metastasis and development is urgently required to improve the treatment strategies for this devastating disease.

Metastasis-associated colon cancer 1 (MACC1), was first identified in colon cancer by genome-wide data analysis (*6*), and subsequently has been found to be overexpressed in various other types of cancer, including lung cancer (*7*), gastric cancer (*8*) and hepatocellular carcinoma (*9*). Moreover, MACC1 could promote tumor proliferation and invasion *in vitro* in these cancer types. In addition, MACC1 has been demonstrated to be an independent prognostic indicator of recurrence and disease-free survival, as well as be involved in cancer initiation and development (*10*). Recently, various studies have revealed that MACC1-induced tumorigenesis is closely correlated with hepatocyte growth factor (HGF)/c-MET signaling pathway activation (*6,10*) leading to enhanced cell motility, invasion and metastasis (*11*). Furthermore, MACC1 could increase vimentin and suppress E-cadherin in colon cancer cells, but its silencing reversed these changes (*12*). To our knowledge, there is no report on its role regulating cell metastasis, as well as the underlying mechanism in carcinogenesis of malignant melanoma in the literature.

In the present study, RNA interference was first used to knockdown MACC1 expression in melanoma cell

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lines. Then the biological behavior of MACC1 silencing was analyzed on melanoma cells, including cell mobility, migration and invasion, as well as possible mechanisms underlying these functional assays.

2. Materials and Methods

2.1. *Cell lines and culture*

Two human malignant melanoma cell lines, A375 and G361 were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum and 100 U/mL each of penicillin and streptomycin (Gibco, Grand Island, NY, USA). All cells were cultured in a fully humidified atmosphere containing 5% CO^2 at 37°C.

2.2. *Small interfering RNA (siRNA) sequences and transfection*

The targeted MACC1 and one scrambled siRNA sequences (5'-AAAGACAGAAGGAGAAAGGAA-3' and 5'-AAAGACAGAAGGAGAAAGGAA-3') were chemically synthesized and purified. For MACC1 silencing, A375 or G361 cells were placed in 6-well plates, seeded at a quantity of 1×10^5 cells, and transfected with siRNA against MACC1 (siMACC1) or negative scrambled siRNA (siCtrl) using Lipofectamine™ RNAiMAX (Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were harvested for mRNA expression detection and Western blot analysis. Stably transfected cells were selected for migration and invasion assays.

2.3. *Quantitative real time PCR (qRT-PCR)*

Total RNA was extracted from cells using Trizol reagent (Ambion/Thermo-Fisher Scientific, Grand Island, NY, USA) according to the manufacturer's instructions. For cDNA synthesis, 1 μg RNA was transcribed using the SuperScript First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed using Power SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol on a LightCycler® 480 II (Roche, West Sussex, UK). Primers used included the following: MACC1 (forward): 5'-TTCTTTTGATTCCTCCGGTGA-3'; MACC1 (reverse): 5'-ACTCTGATGGGCATGTGCTG-3'; E-cadherin (forward): 5'-TCTGGAAGGAATGGA GGAGTC-3'; E-cadherin (reverse): 5'-AATTGGGCAAATGTGTTCAGC-3'; N-cadherin (forward): 5'-GCTCCCTTAATTCCTCAAGT AGTG-3'; N-cadherin (reverse): 5'-TTCAGTCATCACCT CCACCATAC-3'; Vimentin (forward): 5'-ATTCCACTTTGCGTTCAAGG-3'; Vimentin (reverse): 5'-CTTCAGAGAGAGGAAGCCGA-3'; GAPDH (forward): 5'- TGACTTCAACA GCGACACCCA-3'; GAPDH (reverse): 5'- CACCCTG TTGCTGTAGCCAAA-3'. GAPDH was used as a control to normalize amounts of cDNA among samples. Differences were calculated using the threshold cycle (Ct) and comparative Ct methods for relative quantification. Results were expressed as the relative expression of mRNA levels compared to controls. The experiment was performed in triplicate.

2.4. *Western blot analysis*

Total cellular protein were extracted from A375 and G361 cells using 2 × SDS sample buffer (100 mM Tris-HCl, 10 mM EDTA, 4% SDS, 10% Glycine) supplemented with protease inhibitors (PMSF, Sigma-Aldrich). Approximately 30 μg of protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto Polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). Membranes were than blocked with 5% skim milk for 2 h at room temperature, and then incubated with primary antibodies against MACC1 (Sigma, Louis, MO), E-cadherin (Cell Signaling Technology, Danvers, MA), N-cadherin (Cell Signaling Technology, Danvers, MA), Vimentin (Cell Signaling Technology, Danvers, MA) and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. GAPDH was used as the loading control. The signals were detected by enhanced chemiluminescence (Millipore, Billerica, MA).

2.5. *Scratch wound assay*

Forty-eight hours after transfection with siRNAs, cells were incubated overnight until grown to about 90% confluency. Then a standard 200 μL plastic filter tip was drawn across the well to produce a wound. The floating cells were eliminated and wells were washed with PBS. The lodged cells were incubated in fresh complete medium for another 24 h. The wound areas were observed at 0 and 24 h time points and photographed under a fluorescence microscope. Wound healing effects were determined by measuring the percentage of the wound area compared with the area of total cells. Each sample was analyzed in triplicate. The experiment was performed in triplicate.

2.6. *Cell migration assay*

For cell migration assay, A375 (2.0×10^4 cells/well) and G361 cells (3.0×10^4 cells/well) were seeded in serum-free media in upper Transwell culture chambers (8 μm pore size, Millipore, MA, USA) coated with collagen

for 24 h. Then 1 mL medium was added to the bottom chamber. After 24 h incubation, migrated cells in the membrane were fixed with 4% paraformaldehyde and stained with 0.02% crystal violet. Finally, the number of migrating cells on the lower membrane surface was counted in five random 200× fields under a microscope. The mean number of cells per field was calculated as cell counts. Each sample was analyzed in triplicate. The experiment was performed in triplicate.

2.7. *Cell invasion assay*

For cell invasion assay, A375 (2.0×10^4 cells/well) and G361 cells (3.0×10^4 cells/well) were seeded in serum-free media in upper Transwell culture chambers (8 μm pore size, Millipore, MA, USA) coated with matrigel (BD Biosciences) for 24 h. Complete media containing 10% FBS was added to the lower chamber as chemoattractant. Finally, the number of invasive cells on the lower membrane surface was counted in five random 200× fields under a microscope. Similarly, the mean number of cells per field was calculated as cell counts. Each sample was analyzed in triplicate. The experiment was performed in triplicate.

2.8. *Immunofluorescent staining*

The A375 cells were cultured in a 24-well plate after 24 h transfection. The cells were then washed three times with PBS for 5 min and fixed with 4% paraformaldehyde in PBS, followed by permeabilization with 0.1% Triton X-100 in PBS for 5 min, and then blocked for 1h with PBS containing 5% bovine serum albumin. The cells were incubated with E-cadherin, N-cadherin or Vimentin antibody overnight and washed with PBS three times for 5 min. They were then incubated with the corresponding secondary antibodies for 1 h and washed three times with PBS for 5 min. A drop of 4', 6-diamidino-2-phenylindole (DAPI, Invitrogen) was added into the 24-well plate. The cells were visualized using a fluorescence microscope (VANOX-S, Olympus, Melville, NY, USA).

2.9. *Statistical analysis*

Numerical data were analyzed using SPSS13.0 software and expressed as means ± standard deviation (SD) of at least three independent determinations. Statistically significant differences between groups were assessed using analysis of the Student's *t* test. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. *Establishment of MACC1 silencing A375 and G361 cells*

It has been reported that MACC1 is closely associated with metastatic carcinomas in various tumors. To investigate the potential role of MACC1 in human malignant melnoma, the expression of MACC1 was specifically downregulated in melanoma cell lines A375 and G361 using siRNA transfection, as confirmed by qRT-PCR and Western blot analysis. As shown in Figure 1A, the mRNA ($p < 0.001$) and protein levels of MACC1 were significantly decreased in A375 cells following siMACC1 transfection, indicating a considerable knockdown efficiency. Similar results were also observed in G361 cells (Figure 1B, $p < 0.001$). Taken together, we successfully constructed a MACC1 silencing melanoma cell model.

3.2. *MACC1 silencing inhibits the motility of A375 and G361 cells*

To investigate whether MACC1 silencing inhibits A375 and G361 cell mobility, a wound healing assay was performed. As shown in Figure 2A and C, a continuous rapid movement was observed in the control group compared with cells following siMACC1 transfection. Quantitative analysis further indicated that the wound areas in the siCtrl group was significantly decreased compared to the siMACC1 group in both A375 (Figure 2B, $p < 0.001$) and G361 cells (Figure 2D, $p < 0.001$) after incubation for 24 h.

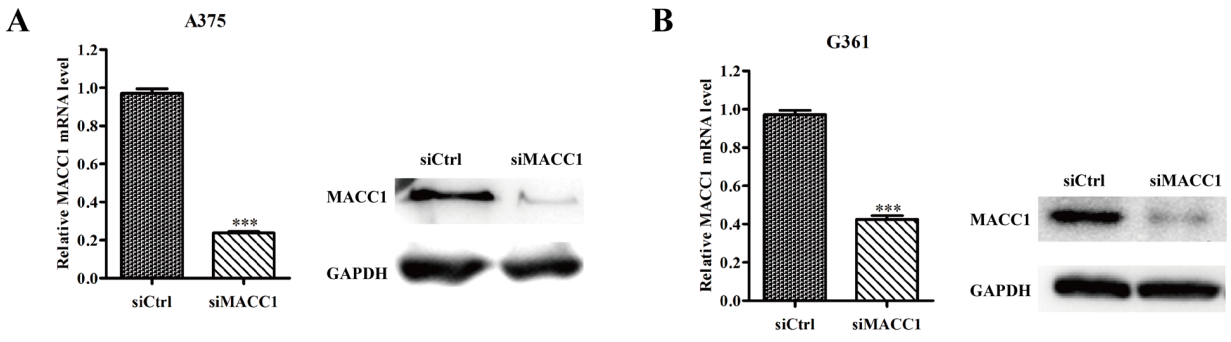

Figure 1. Stable knockdown of MACC1 was successfully constructed in melanoma cells. The mRNA and protein levels of MACC1 were analyzed in A375 **(A)** and G361 **(B)** by qRT-PCR and Western blot analysis. GAPDH was used as a loading control. ***$p < 0.001$, shown siMACC1 *vs*. siCtrl group.

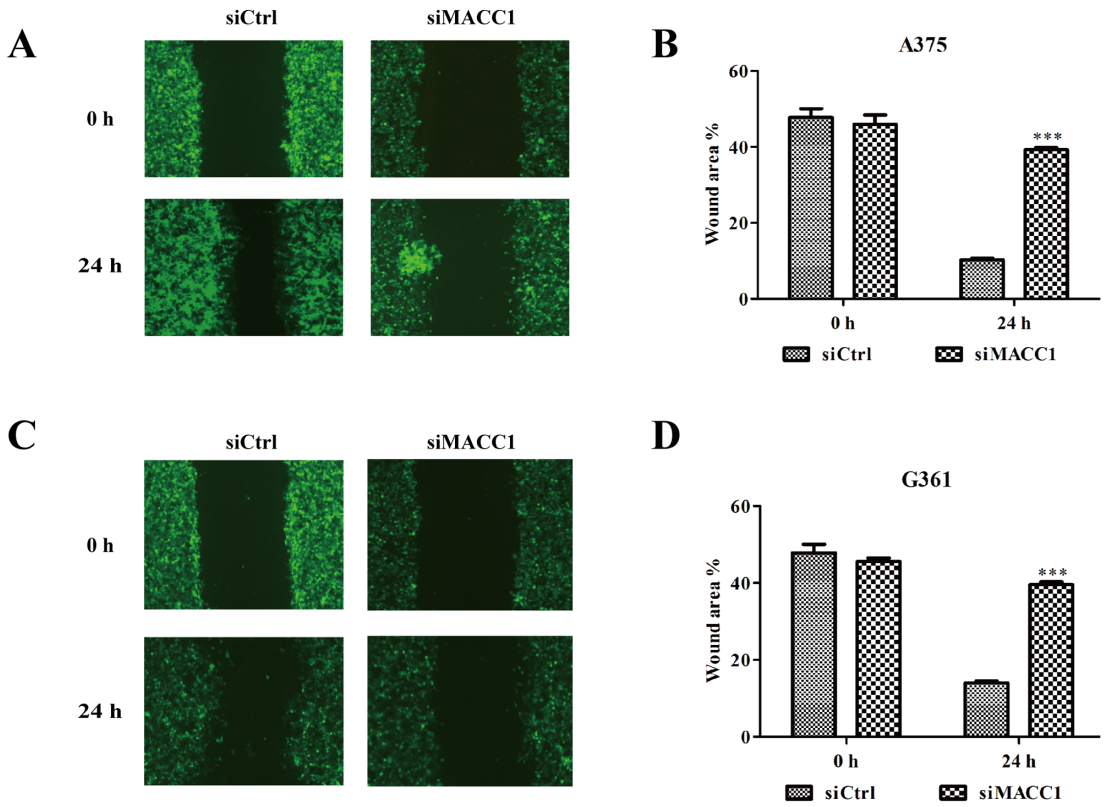

Figure 2. MACC1 silencing inhibits the mobility of melanoma cells. Cell in monolayers were wounded by 200 μl plastic filter tip and the remaining cell monolayers were incubated in the medium for 24 h. At the 0 h and 24 h, the wound areas were photographed in A375 **(A)** and G361 **(C)** under a fluorescence microscope and the percentage of wound area was calculated A375 **(B)** and G361 **(D)** as described in the methods section. ***$p < 0.001$, shown siMACC1 *vs*. siCtrl group.

3.3. *MACC1 silencing suppressed migration and invasion of melanoma cells*

It has been demonstrated that cancer metastasis is inseparable with cell migration and invasion. Therefore, the effects of MACC1 on A375 and G361 cell migration were determined by Transwell cell migration assay. As shown in Figure 3A and B, treatment of A375 and G361 cells with siMACC1 led to a significant decrease in cell vertical migration through the Transwell chamber ($p < 0.001$). Subsequently, cell invasion was measured by Transwell matrigel invasion assay and the results are shown in Figure 3C and D. This indicated that the invasive ability of A375 and G361 cells was remarkably reduced by siMACC1 treatment. These consistent results suggested that MACC1 silencing could effectively reduce the metastatic potentials of the melanoma cells.

3.4. *MACC1 silencing altered EMT expression in melanoma cells*

To further investigate the possible mechanisms underlying MACC1 silencing suppressed cell migration and invasion, the expression of the epithelial markers E-cadherin and N-cadherin, as well as the mesenchymal marker Vimentin were determined in A375 cells after transfection with siMACC1 or siCtrl using qRT-PCR and Western blot analysis. As shown in Figure 4A, the mRNA and protein levels of E-cadherin were obviously upregulated in A375 cells after MACC1 knockdown, whereas those of N-cadherin (Figure 4B) and Vimentin (Figure 4C) were both significantly downregulated in A375 cells induced by MACC1 silencing. Immunofluorescent staining of E-cadherin, N-cadherin and Vimentin showed the same results as those from qRT-PCR and Western blot analysis (Figure 5). Collectively, these results support the idea that knockdown of MACC1 decreased migratory and invasive ability through alteration of EMT expression in melanoma *in vitro*.

4. Discussion

Malignant melanoma is the most deadly type of skin cancer with a high metastatic potential. Due to the complicated mechanism underlying its metastasis, there is currently no effective treatment for malignant melanoma. In this study, we investigated for the first time the biological behavior of MACC1 in melanoma pathogenesis. We found decreased MACC1 resulted in significant inhibition of cell migration and invasion in melanoma through modulating EMT expression. Our

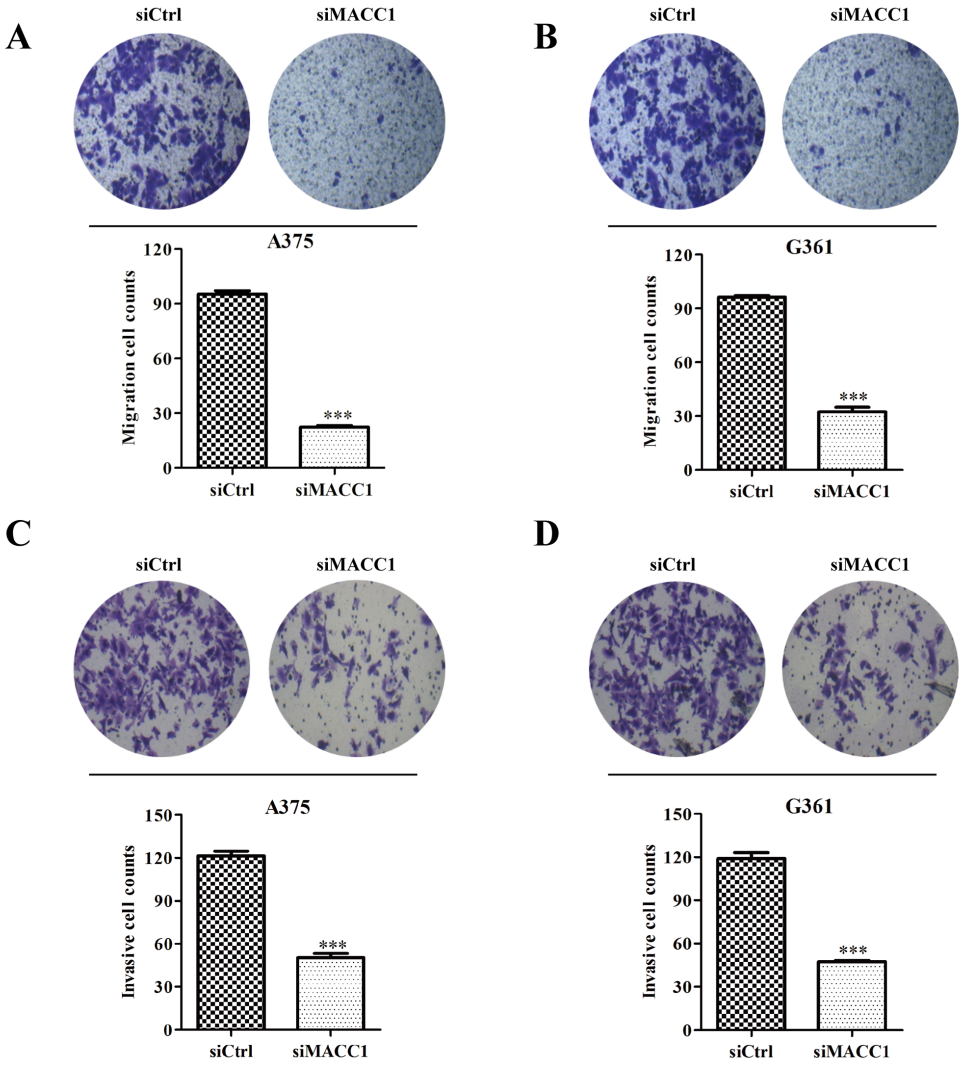

Figure 3. MACC1 silencing inhibited cell migration and invasion ability of melanoma cells. A375 and G361 cells were placed in upper Transwell culture chambers, which were coated with collagen or matrigel. Cell penetration through to the lower surface were stained with crystal violet and photographed under a light microscope, then counted in A375 **(A)** and G361 **(B)** cells. Cells that penetrated through with matrigel to the lower surface were stained with crystal violet and photographed under a light microscope, then counted in A375 **(C)** and G361 **(D)** cells. Quantitative results were obtained from three independent experiments. ***$p < 0.001$, shown siMACC1 *vs*. siCtrl group.

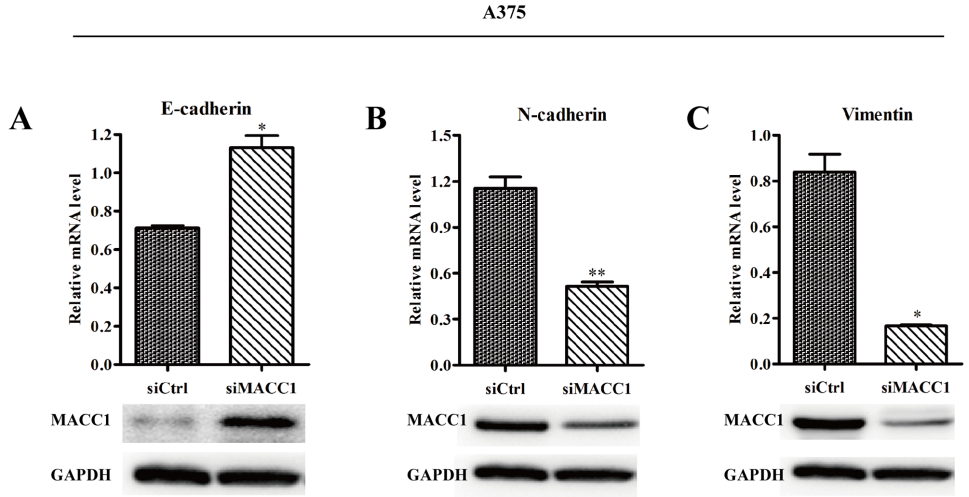

Figure 4. MACC1 silencing altered EMT expression in A375 cells. Knockdown of MACC1 increased E-cadherin expression, but decreased N-cadherin and Vimentin expression as shown by qRT-PCR and Western blot analysis. GAPDH was used as a loading control. **$p < 0.05$, *$p < 0.01$, shown siMACC1 *vs*. siCtrl group.

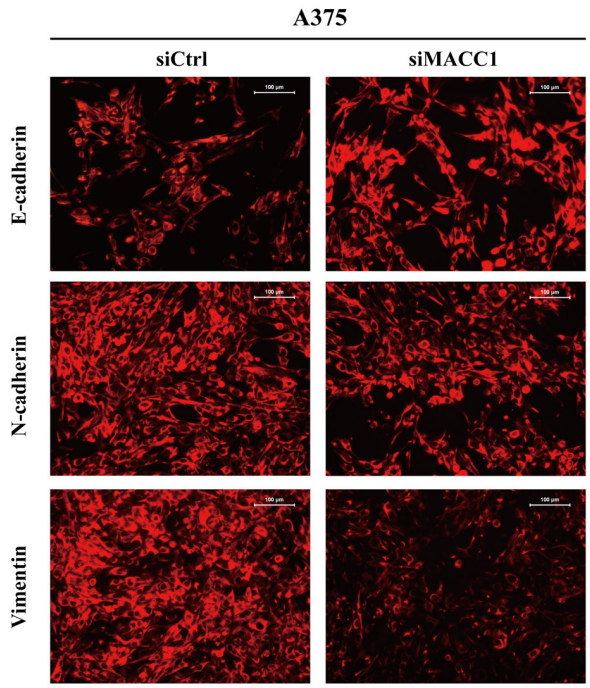

Figure 5. The expression of E-cadherin, N-cadherin and Vimentin in A375 cells was analyzed by immunofluorescent staining. The results showed that MACC1 silencing could increase the expression of E-cadherin, and decrease the expression of N-cadherin and Vimentin.

results may provide a new target for intervention in melanoma treatment.

Accumulating evidence have indicated there is a close relationship between MACC1 and proliferation and metastasis in a variety of tumors. Consistent with our results, downregulation of MACC1 remarkably inhibited cell migration and invasion in gastric cancer (*13*), glioma (*14*) and ovarian cancer (*15*). Previous studies have demonstrated that deregulation of HGF/c-MET signaling, governed by MACC1 induces various malignant behaviors in cancer (*16*). EMT plays an important role in HGF/c-MET signaling regulation, which are mainly involved in the initiating step for the cascade of tumor invasion driven by various genes (*11,17,18*). Therefore, we speculate MACC1 might be associated with the EMT phenotype. To confirm this speculation, we focused on hallmarks of EMT. Our results showed that MACC1 silencing could upregulate expression of E-cadherin, while downregulating expression of N-cadherin and vimentin.

E-cadherin, as the hallmark of EMT (*19*) is an essential adhesive tumor suppressor (*20*) in the establishment of epithelial adhesion junctions and a tight polarized cell layer, whose downregulation is a leading event in the progression of various tumors into the metastatic cascade (*21,22*). Thus, the upregulation of E-cadherin induced by MACC1 silencing in cells implicated that MACC1 plays a crucial role in impairing epithelial characteristics. N-cadherin is a mesenchymal marker and is also correlated with the migratory and invasive phenotype (*23*). Vimentin, an intermediate filament during EMT, is shown to be required for remodeling of cytoskeleton elongation and facilitating mesenchymal cell migration (*24*). After downregulation of MACC1, we found that these two representative mesenchymal markers were consistently decreased. Collectively, MACC1 plays an important role in melanoma metastasis.

In summary, it is not hard to suggest how the oncogene MACC1 affects the malignancy of melanoma, as originally shown in our current cellular experiments. Our findings demonstrate MACC1 might be a potential therapeutic target for inhibiting EMT in melanoma invasiveness. Furthermore, more experiments are still needed to determine whether MACC1 silencing will reduce melanoma tumorigenicity in mouse xenograft models, as well as the specific mechanisms.

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