Capn4 promotes epithelial-mesenchymal transition in human melanoma cells through activation of the Wnt/β-catenin pathway

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Received May 26, 2016; Accepted July 1, 2016

DOI: 10.3892/or.2016.5247

Abstract. Melanoma, as one of the most highly metastatic types of cancer, is resistant to current treatment methods, including popular targeted molecular therapy. Consequently, it is essential to develop a deeper understanding of the mechanisms involved in melanoma progression so that alternative treatments may be identified. To date, accumulating evidence supports the use of calpains, including calpain small subunit 1 (also known as Capn4 or CAPNS1), which affect cancer progression through many pathways, such as epithelial-mesenchymal transition (EMT), the Wnt/β-catenin (β-catenin) and the nuclear factor κB (NF-κB) signaling pathways. The EMT pathway is well known as one of the most important events in tumor metastasis. The present study observed cross-talk among the EMT, β-catenin and NF-κB pathways. To identify the underlying mechanisms of Capn4 activity in melanoma cells, we determined Capn4 expression by gene chip and immunohistochemistral analyses in melanoma tissues and cells *in vitro*. The extent of apoptosis as determined by TUNEL assay, DAPI staining, and cleaved-caspase-3 assay was increased in human melanoma cells in which Capn4 expression had been knocked down when compared with untreated cells. Transwell assays and xenograft tumorigenicity studies were also performed to assess the effects of Capn4 on migration and invasion *in vitro* and tumor growth *in vivo*, respectively. The levels of β-catenin, vimentin, E-cadherin and N-cadherin were altered in human melanoma cells as determined by western blot analysis assay. Our study demonstrated that Capn4 is an underlying target for melanoma treatment.

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Key words: melanoma, Capn4 calpain small subunit 1, epithelial-mesenchymal transition, β-catenin, E-cadherin

Introduction

Melanoma is the most aggressive form of skin cancer (1). Patients with advanced-stage melanoma have a very low survival rate (15%) and an overall survival rate of 8-18 months (2). Both the incidence and mortality rates of melanoma are increasing worldwide (3), with melanoma as one of the fastest growing malignancies in North America (4). Melanoma incidence has been increasing annually by 4% in the United States (3) and by 3-7% in European countries (5). There are ~20,000 newly diagnosed cases of melanoma in China every year (6). In addition, melanoma is known to have a high risk of relapse after resection of the primary tumor. Metastatic melanoma is difficult to treat due to its resistance to high-dose IFN-α2b, chemotherapy, radiotherapy, immunotherapy, and targeted molecular therapy. Several chemotherapeutic agents show single-agent activity in melanoma at a level of 10-15% (7).

Cells undergoing EMT expand out of and change the surrounding microenvironment to subsequently migrate from the primary site (8). When EMT is ongoing, it was observed that E-cadherin is downregulated and N-cadherin and vimentin are upregulated (9). It has been reported that calpains play a role in EMT. Calpains are members of a family of calcium-dependent intracellular cysteine proteases, which include μ-calpain and m-calpain (10,11). Both μ-calpain and m-calpain form heterodimers consisting of a large catalytic subunit (80 kDa) encoded by the genes Capn1 and Capn2, respectively, and a small regulatory subunit (28 kDa) encoded by the gene Capn4 (11). Calpains, including Capn4, are implicated in several physiological processes, including cytoskeletal remodeling, cellular signaling, apoptosis and cell survival (12). The involvement of μ-calpain and m-calpain in tumor invasion has been extensively studied during the last decade. In several types of cancer, tumor cells, such as

those from osteosarcomas (13), rhabdomyosarcomas (14), hepatocellular carcinoma (HCC) (15,16), colorectal (17) or breast cancer (18), present abnormally high activity of μ-calpain and/or m-calpain.

Capn4 is involved in regulating invasion, migration and cell survival (19). Downregulation of Capn4 suppressed the migration and invasion of glioma cells by reducing the level of vimentin and N-cadherin expression in glioblastoma multiforme (GBM) cells (20). Capn4 also promoted the migration and invasion of HCC (16). Capn4 overexpression is implicated in intrahepatic cholangiocarcinoma (ICC) metastasis/invasion (21). Capn4 expression was found to be higher in clear cell renal cell carcinoma (ccRCC) tumor tissues than that noted in adjacent non-tumor tissues (22). Capn4 is overexpressed in nasopharyngeal carcinoma (NPC) and its downregulation in NPC cells is associated with reduced levels of matrix metalloproteinase 2 (MMP2) and vimentin (23). Capn4 promotes non-small cell lung cancer (NSCLC) progression via upregulation of MMP2 (24).

Previous studies have reported that Capn4 is involved in the proliferation and metastasis of solid tumor cells, but few studies on Capn4 have been performed on melanoma. We determined that Capn4 was overexpressed in melanoma as determined by gene chip analyses from the GEO:GSE3189 database (25). The β-catenin signaling pathway is reported to play an important role in embryogenesis, stem cell maintenance and tumorigenesis, including melanoma progression (26). New research suggests that the NF-κB/p65 signaling pathway plays an important role in the EMT pathway (27). The present studies identified cross-talk among the β-catenin, NF-κB and EMT pathways. Therefore, we hypothesized that Capn4 promotes the migration and invasion of human melanoma cells through the EMT or β-catenin signaling pathways.

Materials and methods

Overexpression of Capn4 in melanoma was determined by gene chip analyses from the GEO:GSE3189 database (Fig. 1) (25). There was a statistically significant difference in Capn4 mRNA expression between the melanoma tissues and normal tissues ($P<0.01$).

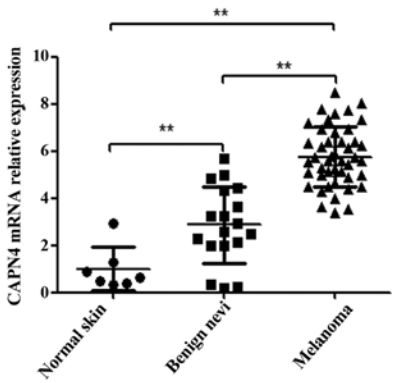

Figure 1. Overexpression of calpain small subunit 1 (Capn4) in melanoma was determined by gene chip analyses from the GEO database (25). There was a statistically significant difference in Capn4 expression between the melanoma tissues and normal tissues; $^{**}P<0.01$.

Immunohistochemistry (IHC) of clinical specimens. All of the clinical specimens used in this study were obtained from the Department of Pathology, Chongqing Medical University (Chongqing, China). The clinical specimens included 120 resected melanoma tumor tissues and 34 normal tissues obtained from patients who underwent surgery for melanoma or other diseases. The most representative tumor area in each biopsy was carefully selected and marked on the hematoxylin and eosin-stained (H&E-stained) slides. IHC was performed on the targets of interest. None of the enrolled patients had received chemotherapy or radiation therapy before surgical resection. An anti-Capn4 antibody (diluted 1:300; Proteintech, Wuhan, China) was used for IHC for Capn4 expression in melanoma tissues and normal tissues. IHC was performed as previously described (21).

Ethics statement. The clinical processes in this study were approved by the Ethics Committees of the Chongqing Medical University, and the patients who participated in the studies provided informed consent. The animal studies described here were approved by the Institutional Animal Care and Use Committee of Chongqing Medical University (Chongqing, China).

Cell culture and reagents. The human keratinocyte cell line HaCaT (HaCaT) and human melanoma cell lines M14, SK-MEL-1 and MV3 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The human melanoma cell line A375, obtained from the Shanghai Institute of Cell Biology in the Chinese Academy of Sciences (Shanghai, China) was cultured in a growth medium of DMEM (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% FBS, 2000 U/ml penicillin G (1,000,000 U=1 g) and 500 μg/ml streptomycin solution. The HaCaT cell line and human melanoma cell lines M14, SK-MEL-1 and MV3 were cultured in RPMI-1640 (Thermo Fisher Scientific, Inc.) containing 10% FBS, 2000 U/ml penicillin G and 500 μg/ml streptomycin. All cells were grown in a humidified incubator at 37˚C and 5% CO_2.

Capn4 targeting short hairpin RNA (shRNA) and cell transfection. An shRNA plasmid directed against Capn4 was purchased from Genomeditech Co., Ltd. (Shanghai, China) and was used to downregulate Capn4 expression in the A375 and M14 cells. The shRNA targeting sequences for Capn4 were as follows shRNA, CCGGCGCCACAGAACTCATGAACATCTCGAG ATGTTCATGAGTTCTGTGGCGTTTTTG and control shRNA, CCGGTTCTCCGAACGTGTCACGTTTCAAGA GAACGTGACACGTTCGGAGAATTTTTG.

We generated human melanoma A375 and M14 cells that were stably transfected with Capn4 shRNA (shRNA-infected cells). The shRNA-infected cells (shRNA) were compared with the control cells (con) in which Capn4 expression had not been knocked down. The shRNA-infected A375 and M14 cells were assessed via western blot analysis.

Western blot (WB) analysis. The expression levels of Capn4, cleaved-caspase-3, β-catenin, E-cadherin, N-cadherin, and vimentin in the shRNA-infected A375, M14, SK-MEL-1, MV3 and HaCaT cells were determined by WB. The WB

samples were prepared from whole-cell lysates. Total protein was quantified, and equal amounts of protein were separated on SDS-PAGE gels and electrotransferred onto PVDF membranes. The blots were subsequently probed with primary antibodies as follows: rabbit anti-Capn4 (Proteintech), anti-cleaved-caspase-3 (Abcam, Cambridge, MA, USA), anti-β-catenin, anti-E-cadherin, anti-N-cadherin, anti-vimentin, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, Inc., USA) and anti-cyclin-dependent kinase 4 (CDK4; diluted 1:100; LifeSpan Biosciences, Seattle, WA, USA). The primary antibodies were detected with peroxidase-conjugated goat anti-rabbit immunoglobulin antibodies as secondary antibodies that were diluted 1:2,000 in blocking solution for 2 h at 37˚C. Antibody-bound proteins normalized to GAPDH or CDK4 were used as internal references to quantitatively analyze protein content and were detected and analyzed using the Quantity One WB analysis system.

MTT assay. The metabolic activity in each well, which indicated the effect of Capn4 on the proliferation and viability of the A375 and M14 cells, was determined using a standard MTT assay. In this assay, both shRNA-infected cells and control cells were seeded into 96-well plates at low cell concentrations ($1.5x10^5$ cells/well) and cultured for 72 h. The medium was replaced with 10 μl MTT solution (5 mg/ml). After being cultured for 1-5 days in the dark, 150 μl dimethyl sulfoxide (DMSO) was added to each well, and the plates were incubated for 4 h. The absorbance at 450 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader. The relative number of cells was calculated using comparable standard curves from the obtained optical density values.

Apoptosis assay. shRNA-infected A375 and M14 cells along with their comparable controls were seeded onto glass coverslips in 6-well plates and incubated overnight. Apoptotic A375 or M14 cells were quantified using an *in situ* apoptosis detection kit. The cells were then fixed in 4% formaldehyde solution before being permeabilized with proteinase K for 25 min at room temperature. Endogenous peroxidase was blocked with 2% H_2O_2 for 5 min at room temperature, and the sections were incubated with 100 μl TUNEL solution (Beyotime Institute of Biotechnology, Shanghai, China) for 1 h at 37˚C in the dark. The stained cells were analyzed by fluorescence microscopy. Six microscopic fields were randomly selected from each sample, and 100 cells were randomly selected from every field. The apoptotic rate was calculated as follows: (TUNEL-positive cells) = (number of total apoptotic cells/100) x 100%. The A375 and M14 cells were washed with phosphate-buffered saline (PBS) and 400 μl of DAPI (Beyotime Institute of Biotechnology) staining solution was added to each well. The staining solution consisted of a mixture of methanol and DAPI as follows: 1 ml methanol and 2 μl DAPI (from a stock solution of 1 mg/ml). The cells were incubated for 10 min in the dark with this staining solution and afterwards washed with PBS. The stained cells were analyzed by fluorescence microscopy. The TUNEL assay and DAPI staining were merged *in situ*. The expression of cleaved-caspase-3 was assayed by WB.

Xenograft tumorigenicity. Five-week-old BALB/c nude mice were obtained from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China) and maintained in a sterile animal facility. shRNA-infected and control A375 cells were harvested, centrifuged, and washed thoroughly with PBS and adjusted to appropriate concentrations. A total of $5x10^6$ shRNA-infected A375 cells, suspended in 0.1 ml of PBS, were subcutaneously injected into the left flank regions of 5-week-old BALB/c nude mice (n=3). Control BALB/c nude mice were injected with the untreated A375 control cells (n=3). All animal studies were approved by the Institutional Animal Care and Use Committee of Chongqing Medical University. After 10 days of treatment, tumor sizes were monitored weekly. At the end of treatment, the tumor tissues were harvested and weighed.

Migration and invasion assays. The Cell Invasion Assay kit (Merck Millipore, Darmstadt, Germany) was used to measure cell migration and invasion. For Transwell migration analysis, $3x10^5$ cells (shRNA-infected or control cells) were suspended in medium with growth factors and plated in the top chamber lined with a non-coated membrane. For invasion analysis, the chamber inserts were coated with 200 mg/ml Matrigel and dried overnight under sterile conditions. Then, $3x10^5$ cells (shRNA-infected or control cells) in medium with growth factors were plated in the top chamber. For both assays, medium-containing serum was added in the lower chamber as a chemoattractant. After incubation at 37˚C for 48-96 h, the top chambers were wiped with cotton wool to remove the non-migratory or non-invasive cells. The migrating and invading cells on the underside of the membrane were fixed in 100% methanol for 10 min, stained with crystal violet, and counted using light microscopy.

Statistical analysis. In our study, the values are represented as the means ± SD (standard deviations) for three independent experiments. Statistical analysis was performed using SPSS software version 20.0. The differences between the groups were analyzed using t-tests. P values <0.05 ($P<0.05$) were considered statistically significant.

Results

IHC of the clinical specimens. Cells were considered positive for Capn4 expression when brown granules distributed in the nucleus or cytoplasm could be observed under a microscope. Cell staining intensity was graded on a 0-3 scale as follows: 0 (absence of staining), 1 (weakly stained), 2 (moderately stained) and 3 (strongly stained). The percentage of positive tumor cells ranged from 0-3 as follows: 0 (absence of positive cells), 1 (<33% positive tumor cells), 2 (33-66% positive tumor cells) and 3 (>66% positive tumor cells). The staining score, which ranged from 0-9, was calculated by multiplying the intensity score by the percentage score. The expression of Capn4 in melanoma tissues vs. normal tissues was described as low (IHC, 0-4) or high (IHC, 5-9). The Capn4 expression in the melanoma tissues was higher compared than the expression in the normal tissues (Fig. 2A). The percentage of cases with high Capn4 expression was ~66.67% (80/120) in the melanoma tissues, compared with 20.59% (7/34) in the normal tissues (Fig. 2B) ($P<0.01$).

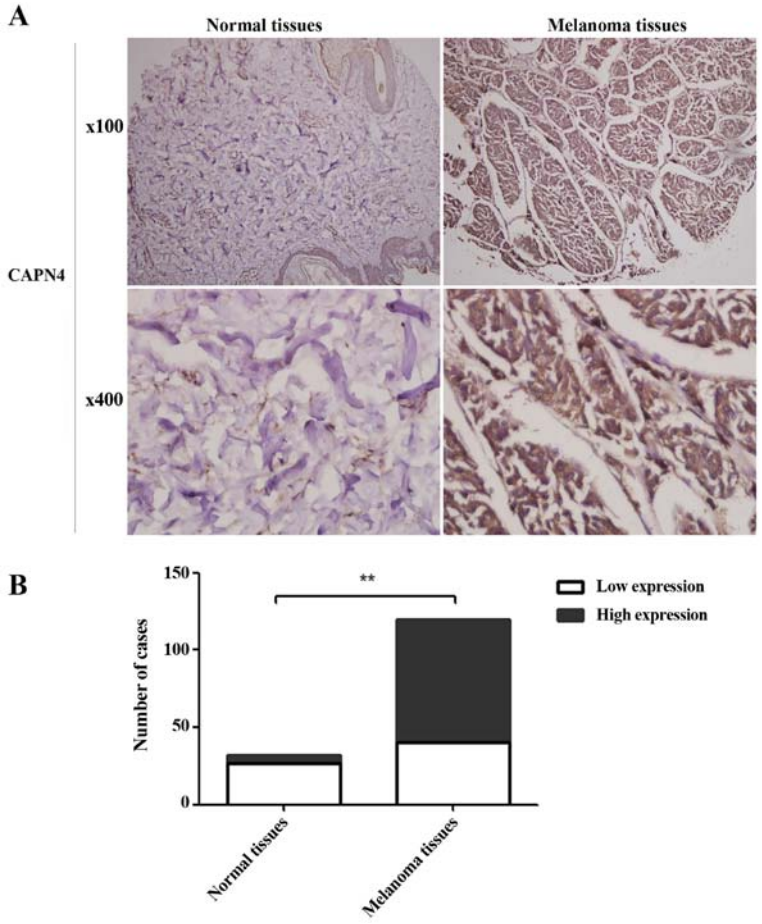

Figure 2. Capn4, is higher in melanoma tissues than that in normal tissues collected for the study. (A) An anti-Capn4 antibody was used in immunohistochemical staining for Capn4 expression in melanoma tissues and normal tissues. Cells were considered positive for Capn4 expression when brown granules distributed in the nucleus or cytoplasm could be observed under a microscope. Objective magnification, x100 and objective magnification, x400. (B) The percentage of cases with high Capn4 expression was ~66.67% (80/120) in the melanoma tissues, compared with 20.59% (7/34) in normal tissues; **P<0.01; Capn4, expression of calpain small subunit 1.

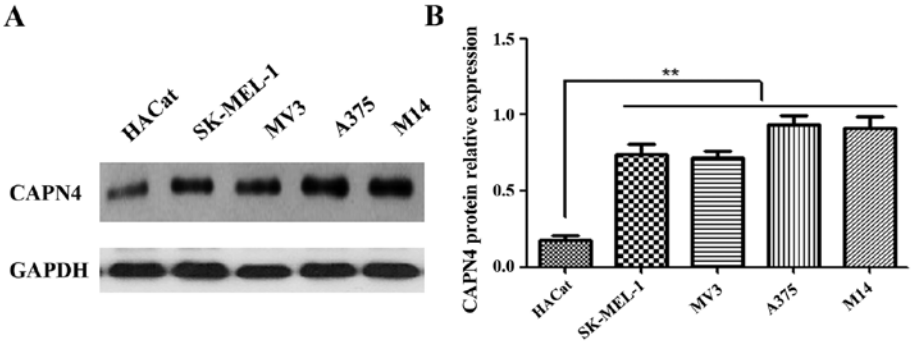

Figure 3. Expression of Capn4 in human keratinocyte HaCaT cells and human melanoma cells (A375, M14, SK-MEL-1 and MV3). (A) Western blot analysis (WB) was performed to detect the protein expression of Capn4, normalized to that of GAPDH in all studied human melanoma cells (A375, M14, SK-MEL-1 and MV3) and HaCaT cells. (B) The protein expression of Capn4 in all studied human melanoma cells (A375, M14, SK-MEL-1 and MV3) was higher than that in HaCaT cells; **P<0.01. Capn4, calpain small subunit 1.

Capn4 protein expression in melanoma and HaCaT cells. WB analysis showed that the protein expression of Capn4, normalized to that of GAPDH in all the studied human melanoma cells (A375, M14, SK-MEL-1 and MV3) was higher than that in the HaCaT cells (Fig. 3A) and there was a statistically significant difference between the melanoma cells when compared with the HaCaT cells (Fig. 3B) (P<0.01.

shRNA targeting Capn4 and cell transfection. A375 and M14 cells were stably transfected with Capn4 (shRNA-infected cells, knockdown of Capn4) and compared with the control cells (con, controls). The protein expression of Capn4 in the shRNA-infected cells was downregulated. WB analysis showed that the protein expression of Capn4 in the shRNA-infected cells (A375, M14), normalized to that of

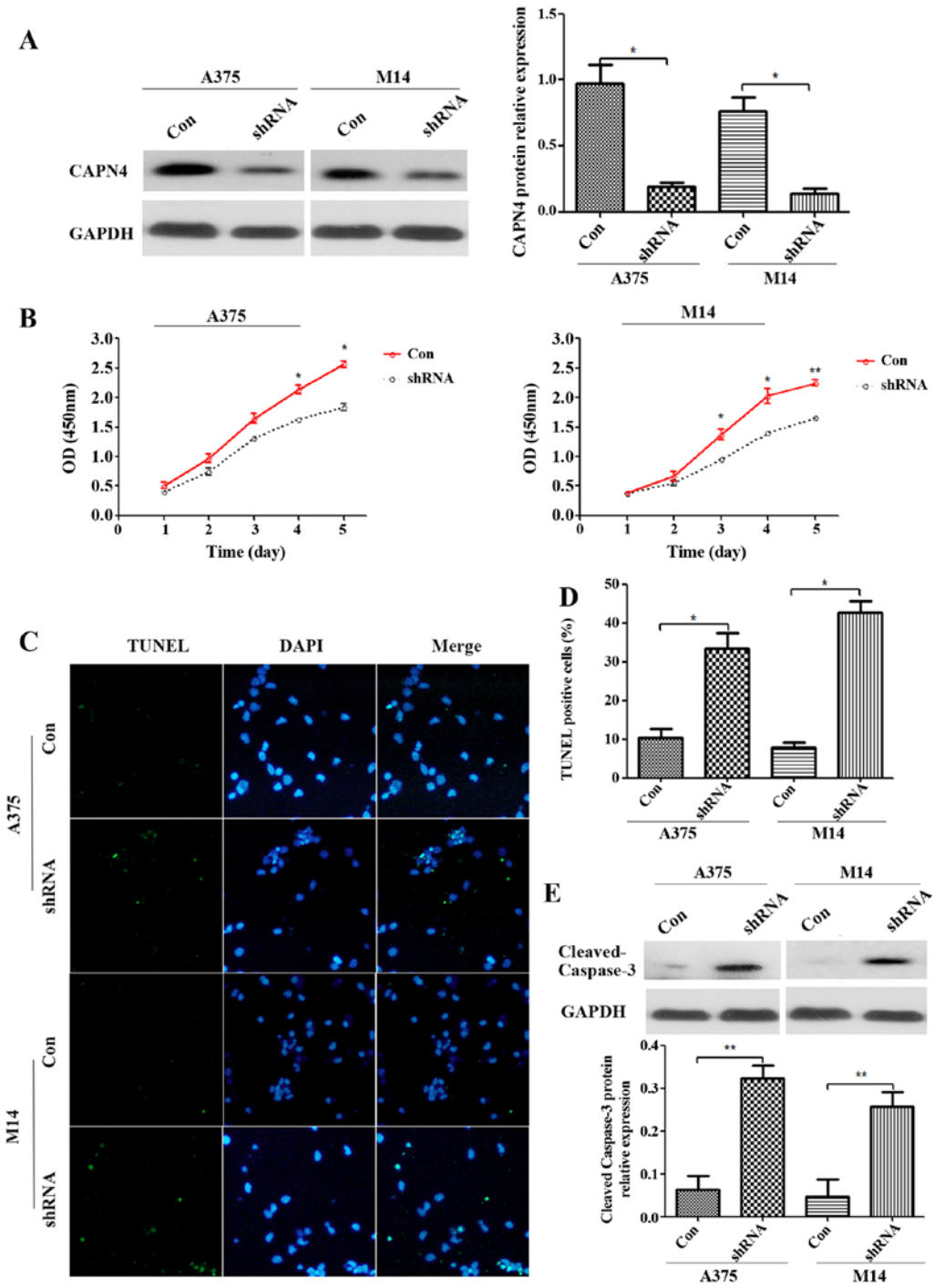

Figure 4. MTT and apoptosis assays. (A) Western blot analysis (WB), normalized to GAPDH, showed that the protein expression of calpain small subunit 1 (Capn4) in the short hairpin RNA (shRNA)-infected cells was lower than that in the controls. (B) The proliferative ability of the shRNA-infected cells was less than that of the controls by MTT assay. (C) By immunofluorescence assay, the DNA fragmentation with TUNEL in shRNA-infected cells was much more that those of the controls. Loss of cell membrane integrity was detected in the shRNA-infected cells when compared with the controls by DAPI staining. The TUNEL assay and DAPI staining were merged *in situ*. (D) The percentage of TUNEL-positive cells in the shRNA-infected cells was higher than that in the controls. (E) The expression of cleaved-caspase-3 in the shRNA-infected cells was higher than that in the controls; *P<0.05, **P<0.01. Capn4, calpain small subunit 1.

GAPDH, was lower than that noted in the controls (Fig. 4A) (P<0.05).

MTT assay. The proliferative ability of the shRNA-infected cells was less than that of the controls. The difference in cell viability was statistically significant between the shRNA-infected and the control cells after 4 or 5 days, both in the A375 and M14 cells (Fig. 4B) (P<0.05).

Apoptosis assay by immunofluorescence and cleaved-caspase-3 expression. DNA fragmentation, as assessed by a TUNEL assay (green: fragmented DNA), was increased in

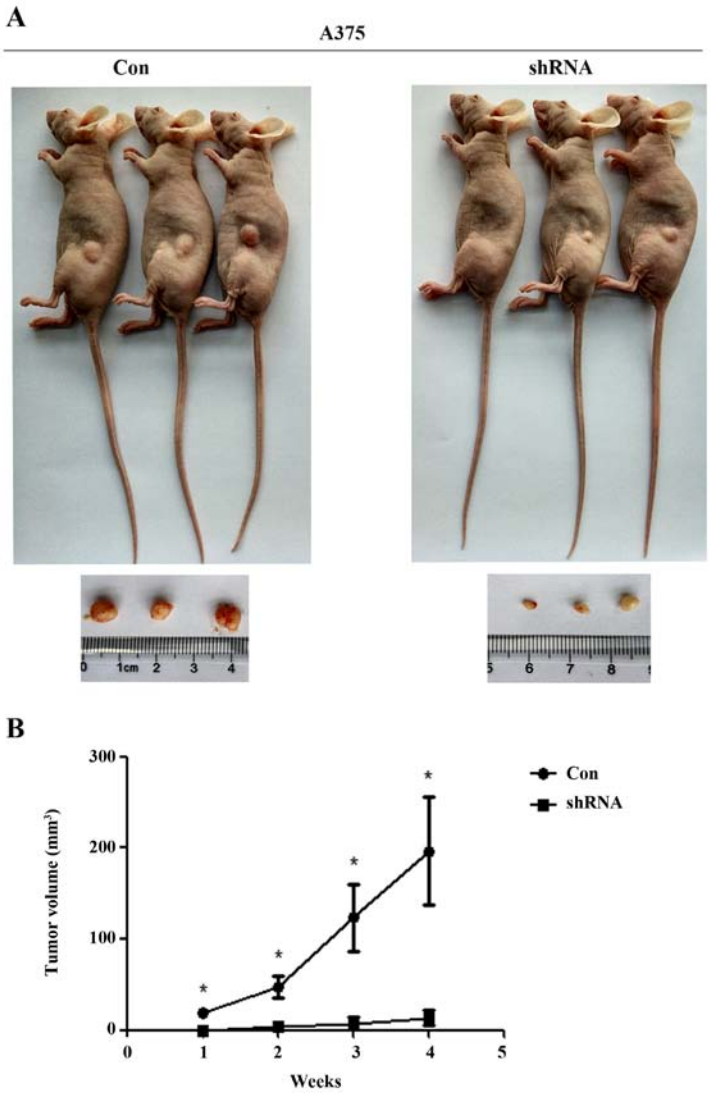

Figure 5. Results of xenograft tumorigenicity. (A) Tumors growth in the nude mice injected with the Capn4 short hairpin RNA (shRNA)-infected A375 cells and control cells (n=3/group). (B) The tumors grew slower in the nude mice injected with shRNA-infected cells than the rate in the controls; *P<0.05.

the shRNA-infected cells than that noted in the control cells for both the A375 and M14 cells (Fig. 4C). The percentage of TUNEL-positive cells in the shRNA-infected cells was higher than that in the controls for both the A375 and M14 cells (Fig. 4D) (P<0.01). In addition, *in situ* DAPI staining was performed in the shRNA-infected cells and controls. Cell nuclei were stained blue allowing them to be observed using fluorescence microscopy. A number of cells exhibited nuclear fragmentation, condensed chromatin filaments or nuclear condensation, which are signs of cell membrane integrity loss. These characteristics were observed more frequently in the shRNA-infected cells than that noted in the controls (Fig. 4C). The expression of cleaved-caspase-3 in the shRNA-infected cells was higher than that in the controls for both the A375 and M14 cells (Fig. 4E) (P<0.01).

Xenograft tumorigenicity. To evaluate the effect of Capn4 on melanoma tumor formation in nude mice, a xenograft tumorigenicity assay was performed. shRNA-infected and control A375 cells were injected into 5-week-old BALB/c nude mice (n=3/group). The tumors grew slowly in the nude mice injected with the shRNA-infected cells compared with this rate in the controls. Nude mice treated with the shRNA-infected A375 cells generated smaller tumors in contrast to the control group (Fig. 5A). The volume of the tumors produced by the shRNA-infected cells was significantly lower than that of the controls (Fig. 5B) (P<0.05).

Knockdown of Capn4 reduces both migration and invasion of A375 and M14 cells in vitro. To determine the functional role of Capn4 in the metastasis of human melanoma cells, stably shRNA-infected cells (Capn4 knockdown) were generated. Capn4 knockdown in the shRNA-infected cells was verified by WB as previously described above. Transwell assays were then performed to assess the migration and invasion of the shRNA-infected and control A375 and M14 cells. Notably, shRNA-infected cells displayed a statistically significant lower rate of migration and invasion than did the controls for both the A375 and M14 cells (Fig. 6) (P<0.01, P<0.05, respectively).

Capn4 decreases E-cadherin and increases the expression of β-catenin-N (β-catenin in the nucleus), N-cadherin and

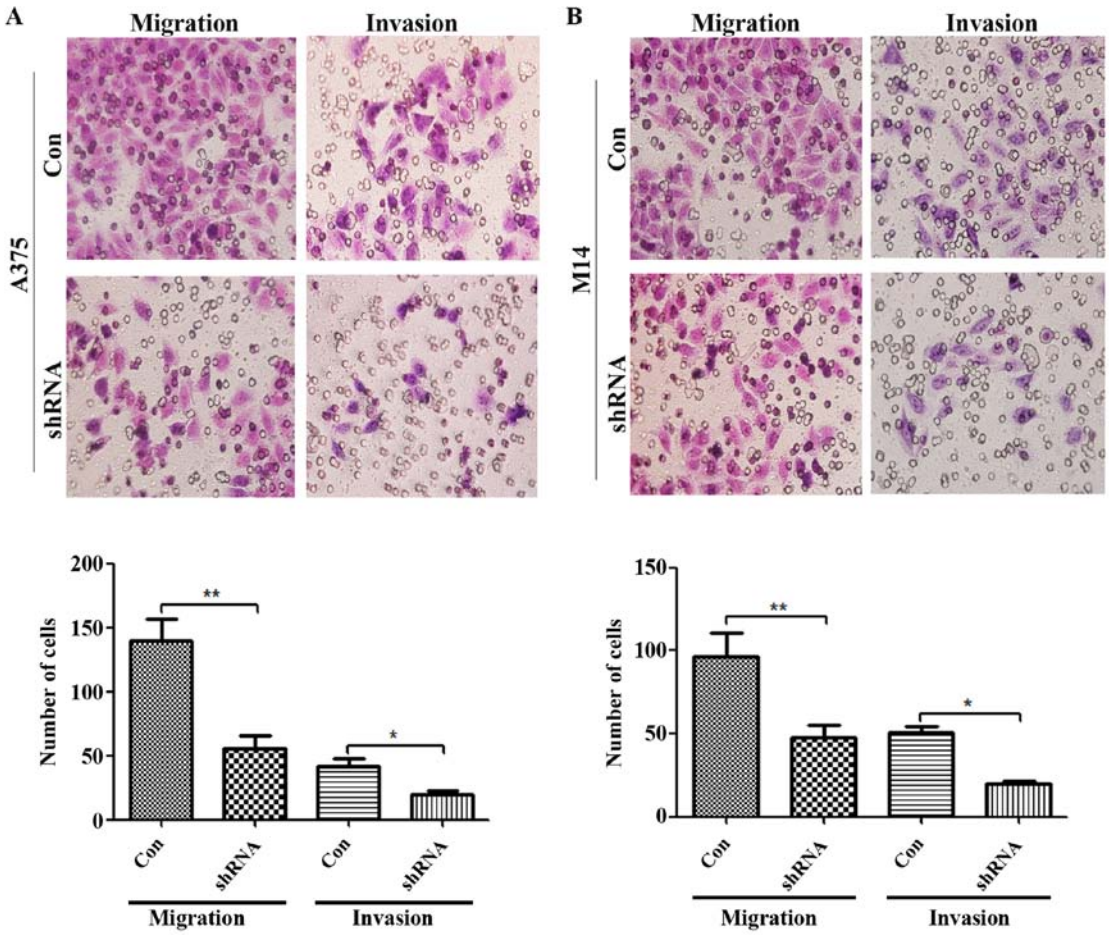

Figure 6. Migration and invasion assay of (A) A375 and (B) M14 cells *in vitro*. The Transwell assays were performed to assess the effects of Capn4 on migration and invasion in A375/M14 cells and control cells. The Capn4 shRNA -infected cells displayed a lower rate of migration and invasion than that of the controls; $^{*}P<0.05$, $^{**}P<0.01$. Capn4, calpain small subunit 1.

vimentin. To determine the effects of Capn4 expression on the EMT pathway, the protein levels of EMT markers were detected by WB in the A375 and M14 cells. The expression of E-cadherin was increased in the shRNA-infected cells compared with that of the controls (Fig. 7A and B) ($P<0.01$). The expression of β-catenin-N, N-cadherin and vimentin was decreased in the shRNA-infected cells compared with the controls (Fig. 7A and B) ($P<0.05$). No changes were observed in the expression of β-catenin-T (total β-catenin, included in the nucleus and in the cytoplasm) in the shRNA-infected cells compared with the controls (Fig. 7A and B). These results suggest that Capn4 plays an important role in the EMT pathway in human melanoma cells.

Discussion

Previous studies have reported that Capn4 is overexpressed in tumor tissues, including ICC (21), ccRCC (22) and NPC (23). Our study confirmed that Capn4 expression was increased in both melanoma tissues and cells *in vitro*. Lower levels of Capn4 expression were detected in the shRNA-infected cells when compared with the controls and the viability of the shRNA-infected cells was lower when compared with the control cells by MTT assay. The TUNEL assay, DAPI staining and cleaved-caspase-3 level assay proved that apoptosis was enhanced in the shRNA-infected cells compared with the controls. In other words, Capn4 enhanced the activity, inhibited apoptosis and promoted proliferation in human melanoma cells. *In vivo*, the tumor cells grew more slowly in nude mice injected with the shRNA-infected cells than in those injected with the control cells. *In vitro*, the Transwell assays demonstrated that the shRNA-infected cells displayed a statistically significant lower rate of migration and invasion than did the controls. Our study demonstrated that Capn4 promotes the migration and invasion of human melanoma cells *in vitro* and tumor growth *in vivo*.

The migration and invasion of tumor cells are regulated by several pathways, such as the Wnt/β-catenin, NF-κB/p65 and EMT pathways. β-catenin influences many cellular processes, including cell adhesion, growth and differentiation (28). High levels of β-catenin have been reported in tumor cells (29). β-catenin is also ubiquitously referred to as an 'oncogenic' pathway that promotes tumor progression (30) and is reported to play an important role in melanoma tumorigenesis (26). In addition, the NF-κB pathway exhibits tumor-promoting functions in different models of carcinogenesis. The NF-κB pathway is constitutively and highly activated in melanoma cells (31). Inhibiting the NF-κB/p65 signaling pathway also promotes melanoma cell death (32). The EMT hyperactivated pathways

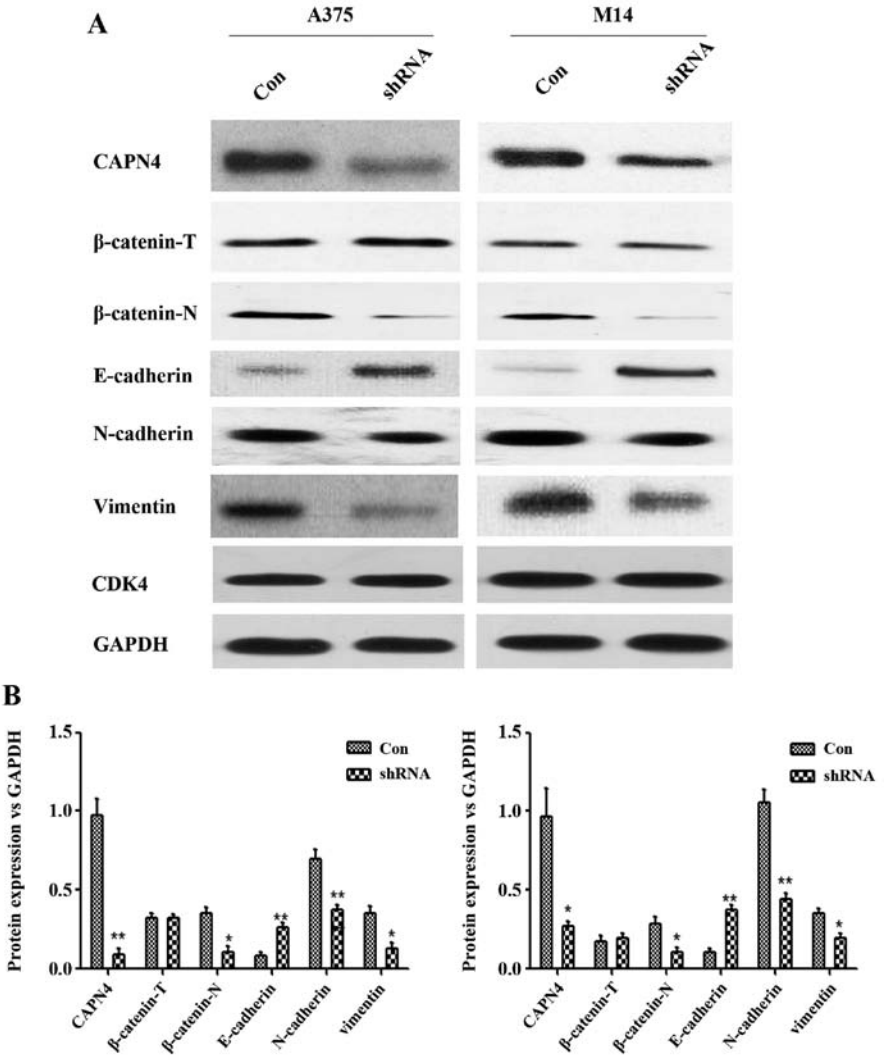

Figure 7. The expression of E-cadherin, β-catenin-N, β-catenin-T, N-cadherin and vimentin in Capn4 shRNA-infected cells. (A) Western blot analysis (WB), normalized to that of GAPDH or anti-cyclin-dependent kinase 4 (CDK4), showed the expression levels of E-cadherin, β-catenin-N, β-catenin-T, N-cadherin and vimentin. (B) E-cadherin was increased in the Capn4 short hairpin RNA (shRNA)-infected cells when compared with the controls. The expression of Capn4, β-catenin-N, N-cadherin and vimentin were decreased in the shRNA-infected cells compared with the controls, and no changes were observed in the level of β-catenin-T; *P<0.05, **P<0.01; Capn4, calpain small subunit 1.

regulate the expression of genes targeting the initiation of the metastatic cascade. Upregulation of E-cadherin and dowregulation of N-cadherin and vimentin expression leads to reduced migration and invasion of bladder carcinoma cells (9). Evidence has accumulated that these pathways cross-talk and regulate each other's activities and functions. β-catenin interacts with NF-κB subunits in colon cancer cells (33) and also regulates the activation of NF-κB (34). NF-κB/p65 promotes lung cancer proliferation through Wnt/β-catenin signaling (35). For instance, the NF-κB pathway directly regulates EMT through the transcription of EMT-associated gene products (3). Inactivity of β-catenin signaling in lung adenocarcinoma cells suppressed tumor cell growth and EMT *in vitro* and *in vivo* (36). Downregulation of Capn4 suppressed the migration and invasion of glioma cells and reduced the level of vimentin and N-cadherin (20). It has also been reported that upregulation of Capn4 promotes migration of hepatoma cells via the NF-κB/p65 pathway (37). In our experiments, the protein levels of EMT markers were detected by WB assay. The levels of β-catenin-N, N-cadherin and vimentin were decreased, and the level of E-cadherin was increased in Capn4-knockdown cells. There was no change in the level of total β-catenin expression in the shRNA-infected cells compared with that of the controls. Evidence has accumulated in support of the theory that Wnt/β-catenin signaling promotes EMT in cancer cells (38-40). Therefore, we concluded that Capn4 contributes to the EMT pathway through the promotion of β-catenin nuclear entry but not through the increase of total β-catenin expression.

In conclusion, our findings revealed that Capn4 was overexpressed in melanoma tumor tissues and melanoma cells. Moreover, Capn4 promoted the migration and invasion of human melanoma cells *in vitro* and tumor growth *in vivo*. Furthermore, we also unveiled a potential mechanism by which Capn4 promoted the migration and invasion of human melanoma cells through the activation of the EMT pathway via the increase of β-catenin-N expression. We presume that Capn4 is an underlying target for melanoma treatment, and we will continue to study Capn4 in regards to the prognosis in melanoma in the future.

Acknowledgements

This study was supported by the General Program of the National Natural Science Foundation of China (no. 81171365) and the Natural Science Foundation of Chongqing in China (cstc2013jcyjA10136).

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