

# Angiomotin promotes breast cancer cell proliferation and invasion

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Received November 25, 2014; Accepted January 21, 2015

DOI: 10.3892/or.2015.3780

**Abstract.** Angiomotin (Amot) is a multifunctional protein involved in endothelial cell migration and tube formation and angiogenesis. However, the biological role and molecular mechanism for the abnormal expression of Amot in breast cancer is poorly understood. The aim of the present study was to examine the function of and relationship between Amot and the Hippo-Yes-associated protein (YAP) pathway. The expression and location of Amot was examined in breast cancer tissues and cell lines using immunohistochemistry, real-time polymerase chain reaction analysis (RT-PCR), western blotting and immunofluorescence. ANOVA, Student's t-test, Wilcoxon and Chi-square tests were utilized to determine the association of Amot expression with clinically relevant parameters. Stable Amot knockdown MCF-7 cells (MCF-7 Amot KD) were generated to investigate the impact of Amot downregulation on the growth and invasion of MCF-7 cells *in vitro*. Western blotting was applied to detect the expression of the Hippo-YAP pathway protein in the MCF-7 cells. It was observed that Amot was highly expressed in breast cancer tissues, but weakly expressed in adjacent non-cancerous tissues. Additionally, the expression level of Amot was correlated with that of Ki-67. In MCF-7 cells, Amot downregulation resulted in a significant decrease of cell proliferation and invasiveness. Following Amot knockdown in MCF-7 cells, the expression of YAP, YAP/TAZ and LATS1 was decreased. In particular, the expression of YAP was markedly reduced in the nucleoprotein. The results suggested that Amot was highly expressed in breast cancer tissues and was important in the

promotion of breast cancer cell proliferation and invasion. In addition, there was a more intimate connection between Amot and Hippo-YAP pathway.

## Introduction

Breast cancer is a common malignant tumor that occurs in women. Approximately 1.2 million women suffer from, and ~500,000 succumb to breast cancer annually. According to the American Cancer Society (ACS), the incidence and mortality of breast cancer ranked the first and second of all female cancer types in America in 2014, accounting for 29 and 15%, respectively (1). Approximately 50% of the breast cancer cases and 60% of the deaths are estimated to occur in economically developing countries (2). A significant reduction in the mortality of breast cancer patients worldwide has been observed in the past 20 years. This reduction has been largely due to improvement in the early detection and development of more effective adjuvant therapies (3). In addition, therapies have been developed to be specifically tailored to targeting each molecular subtype of breast cancer. These therapies include human epidermal growth factor receptor-2 (HER2)-targeting agents for HER2-overexpressing tumors, aromatase inhibitors, third-generation hormonal therapies for hormone-sensitive disease, and poly(ADP-ribose) polymerase (PARP) inhibitors for BRCA1-deficient and triple-negative breast cancers (4). Nevertheless, a large number of women with breast cancer experience relapse. Therefore, identification of more effective treatment targets is needed in breast cancer research.

Angiomotin (Amot) was first identified from its ability to bind to angiostatin using a yeast two-hybrid screen (5). Amot is characterized by a conserved coiled-coil domain and a C-terminal PDZ-binding motif (6) and is expressed as two different isoforms, p80-Amot and p130-Amot. Compared with p80-Amot, p130-Amot contains an extended N-terminal domain (7,8). It was previously reported that Amot regulates endothelial cell migration, tube formation and is important in angiogenesis (6,9-13). Amot is also involved in regulating permeability and the movement of epithelial cells in tight junctions (6,9,10). Using RT-PCR Jiang *et al* (14) found that Amot transcript was significantly highly expressed in human breast cancer tissues, particularly in highly invasive and metastatic tumor tissues, when compared with its expression in normal mammary tissues. However, the role and mechanism of the abnormal expression of Amot in breast cancer remain to be elucidated.

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**Key words:** angiomotin, breast cancer, proliferation, invasion, Hippo-YAP

To evaluate the function and mechanism of Amot, the expression and location of Amot in breast cancer, adjacent non-cancerous tissues, breast cancer and breast epithelium cell lines, were first determined using western blotting, RT-PCR and immunofluorescence. shRNA was applied to block and silence the expression of Amot in the breast cancer cell line and the effect of the silenced Amot on the biological behavior of breast cancer cell line was studied. The results showed that breast cancer tissues had a significantly increased Amot protein level, when compared with adjacent non-cancerous tissues and the expression level of Amot was closely correlated with the expression level of Ki-67 ( $P < 0.01$ ). We also observed that Amot downregulation resulted in a significant decrease in cell proliferation, cell invasiveness and migration *in vitro* and was closely associated with the Hippo-YAP pathway which regulates cell proliferation during development, tissue regeneration and carcinogenesis. Thus, Amot acts as a potential tumor promoter.

## Materials and methods

**Patients and tissue samples.** A total of 242 breast cancer tissue samples and 92 adjacent non-cancerous tissue samples were obtained from the First Affiliated Hospital of Xi'an Jiaotong University College of Medicine and Shanghai Outdo Biotech Co. Ltd. (Shanghai, China). The clinicopathological tumor-node-metastasis (TNM) staging was stage I for 16 cases, stage II for 139 cases and stage III-IV for 81 cases. Clinically relevant parameters are presented in Table I. The pathological types of all the specimens were confirmed by independent pathologists. No patients received radiotherapy or chemotherapy prior to surgery. TNM stages were assigned using the 2010 Union for International Cancer Control (UICC) criteria. The study was approved by the Human Ethics Committee of the First Affiliated Hospital, College of Medicine of Xi'an Jiaotong University. Informed consent was obtained from each patient. All of the specimens were fixed in 10% buffered formalin solution and embedded in paraffin.

**Immunohistochemistry.** Fixed tumor tissue samples were sectioned, deparaffinized, rehydrated and subjected to heat-induced antigen retrieval in EDTA buffer (1.0 mM, pH 8.0) for 10 min in a microwave oven. The samples were blocked with 3% hydrogen peroxide. After being blocked with 1% bovine serum albumin (BSA), the sections were incubated overnight at 4°C with a primary antibody specific for Amot (Genemed Synthesis Inc., San Antonio, TX, USA). Control sections were incubated with an isotype-matched polypeptide control antibody and phosphate-buffered saline (PBS). Subsequently, the sections were incubated with HRP-conjugated secondary antibody for 30 min. The sections were stained with 3,3'-diaminobenzidine, and then counterstained with hematoxylin and examined under a microscope (Olympus CX21; Tokyo, Japan). To evaluate Amot protein expression, the staining intensity was graded as 0 for no staining; 1 for weak staining; 2 for moderate staining; or 3 for strong staining. The extent of staining was scored according to the percentage of positively stained cells as follows: 0 ( $\leq 5\%$ ), 1 (6-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%). The number of positively stained cells was determined by counting

Table I. Amot expression in breast cancer and adjacent non-cancerous tissues.

Group	Negative expression (%)	Weak expression (%)	Moderate expression (%)	Strong expression (%)
Cancer tissues	37 (15.3)	54 (22.3)	106 (43.8)	45 (18.6)
Non-cancerous tissues	80 (87)	8 (8.6)	4 (4.3)	0 (0)
Total	117	62	110	45
H	129.4			
P	0.000 <sup>a</sup>			

<sup>a</sup> $P < 0.001$ . Amot, Angiomotin.

cells from 10 random fields at x400 magnification. The final immunohistochemical staining score was obtained by multiplying the staining intensity and the extent of staining (negative expression, scores 0-2; weak expression, scores 3-5; moderate expression, scores 6-9; and strong expression, scores 10-12).

**Antibodies.** The Amot antibody was produced by Genemed Synthesis Inc. The synthetic peptide sequence (C+LVKSSSKREALEKAMR and C-KTPIQLGQEPDAEMVEYLI) was conjugated to keyhole limpet hemocyanin (KLH) for immunizations. GAPDH and rabbit flag were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). YAP, YAP/TAZ, LATS1, MOB, MST1 and SAV1 were purchased from Cell Signaling Technology (Danvers, MA, USA).

**Cell culture and lentiviral transfection.** The MCF-7, T-47D, BT-474, MDA-MB-453, MDA-MB-231 breast cancer cell lines and MCF-10A breast epithelial cell line were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). MCF-10A cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 5% horse serum (HyClone, Logan, UT, USA), 1% penicillin/streptomycin, 0.5  $\mu\text{g/ml}$  hydrocortisone, 10  $\mu\text{g/ml}$  insulin (both from Sigma, Santa Clara, CA, USA) and 20 ng/ml recombinant human EGF (Invitrogen). MCF-7, T47D and MDA-MB-435 breast cancer cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (both from HyClone). BT474 was cultured in RPMI-1640 medium supplemented with 10% FBS (both from HyClone). All the cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

Lentivirus vectors for human Amot small hairpin RNA (shRNA) encoding a green fluorescent protein (GFP) and a puromycin-resistant gene were constructed, packed and purified by GeneChem Co., Ltd. (Shanghai, China). The RNA interference sequences used were: shAMOT (8489-1R), 5'-TGCAGAGATGGTGAATAT-3'; shAMOT (8491-2R), 5'-ACACATCGAAATCCGAGAT-3'; negative control: shNC, 5'-TTCTCCGAATGTGTCACGT-3'.

MCF-7 cells were transfected with lentivirus according to the manufacturer's instructions (GeneChem Co., Ltd.). For transfection, the lentiviruses mixed with medium containing Polybrene were added to the cells at the confluence of 30-40%. After 8 h of transfection, the medium was replaced by fresh DMEM medium containing 10% FBS. Seventy-two hours after transfection, the cells were selected with 3.5  $\mu\text{g/ml}$  puromycin for 2 weeks. Amot knockdown was verified by western blot analysis. The cells were divided into three groups: CON (control; the uninfected breast cancer cells); KD (knockdown; cells transfected with the Amot shRNA lentivirus); NC (negative control; cells transfected with the mock control lentivirus).

**Real-time polymerase chain reaction analysis.** Total mRNA was extracted using the Fast 200 reagent (Pioneer Biotechnology Inc., Shaanxi, China) and reverse transcription was performed using an RT-PCR kit (Takara, Dalian, China). Complementary DNA synthesis was conducted using a SYBR ExScript RT-PCR kit (Takara) according to the manufacturer's instructions. RT-PCR was conducted using the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and SYBR Premix Ex Taq™ II (Takara). The primer sequences used for amplification were: Amot 5'-CCAGAATATCCCTTCAAG-3' and 5'-GAGTTCCTGGCTGACAAT-3', GAPDH: 5'-CTCCTCCACCTTTGACGCTG-3' and 5'-TCCTCTTGCTCTTGCTGG-3'. GAPDH was applied as the internal housekeeping gene control. Each reaction was performed in a final volume of 10  $\mu\text{l}$  containing 1.0  $\mu\text{l}$  of appropriately diluted cDNA, 0.4  $\mu\text{l}$  (10  $\mu\text{M}$ ) of forward and reverse primers specific for human Amot or GAPDH, 5  $\mu\text{l}$  of SYBR Premix Ex Taq and 3.2  $\mu\text{l}$  of water. The PCR consisted of 1 min at 95°C followed by 40 cycles of denaturation for 15 sec at 95°C, annealing for 15 sec at 55°C and a primer extension for 45 sec at 72°C. The  $\Delta\Delta\text{Ct}$  method was used for the relative quantification of Amot expression.

**Western blotting.** Cells were lysed with RIPA buffer [50 mmol/l Tris (pH 7.5), 100 mmol/l NaCl, 1 mmol/l EDTA, 0.5% NP40, 0.5% Triton X-100, 2.5 mmol/l sodium orthovanadate, 10  $\mu\text{l/ml}$  protease inhibitor cocktail and 1 mmol/l PMSF] by incubating for 20 min at 4°C. Protein concentrations were determined by a BCA assay (Pierce, Rockford, IL, USA). Equal amounts of the cell lysate protein were subjected to 10% SDS-PAGE, transferred to nitrocellulose membranes (Millipore, Boston, MA, USA), blocked with 5% non-fat dry milk in Tris-buffered saline with Tween-20 for 1 h, and incubated with the indicated antibody overnight at 4°C. The reactive bands were developed by chemiluminescence with the luminol reagent (Millipore). The blots were re-probed with GAPDH antibody as a loading control.

**Immunofluorescent staining.** MCF-7, BT-474 and MDA-MB-231 cells were cultured on coverslips to the appropriate densities. The cells were fixed with 4% paraformaldehyde solution for 10 min at room temperature, washed three times with PBST and then permeabilized with 0.1% Triton X-100 for 10 min. The slides were blocked with 5% BSA and 10% horse serum in PBST for 1 h at room temperature and incubated with antibodies against Amot (1:100) overnight at 4°C. After being washed with PBS, cells were incubated with IgG-HRP

secondary antibody (1:100; ZhongShan Jinqiao Biological Company, Peking, China) for 1 h at RT. The cells were then washed three times and visualized using a laser scanning confocal microscope (Leica, Germany).

**Cell proliferation assay.** Cells were seeded in 1% gelatin-coated 96-well plates with  $5 \times 10^3$  cells/well. Relative cell numbers were quantified each day using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay. The absorbance was measured at 492 nm using a multifunction microplate reader (POLARstar OPTIMA, Germany).

**Plate colony formation assay.** The cells were trypsinized and resuspended in DMEM containing 10% FBS. One hundred cells were cultured in 6-well plates until visible cell colonies were formed. After the cells were fixed and stained, the number of cell colonies was counted under a microscope (Leica, Germany).

**5-Bromodeoxyuridine (BrdU) incorporation assay.** BrdU assay was used to detect the variation of the S phase of breast cancer cells and evaluate the effect of Amot downregulation on cell proliferation. BrdU (10  $\mu\text{mol/l}$ ) was added to the medium, cultured in a constant-temperature incubator for 40 min, and fixed by 35% ethanol at 4°C for 1-2 h. Then cells were resuspended in 2N HCl and 0.1 M sodium borate successively. After centrifugation at 800 x g for 5 min, the supernatant was decanted and the cells were cultured in 5  $\mu\text{l}$  anti-BrdU antibody (BD Biosciences, Bedford, MA, USA) and 45  $\mu\text{l}$  PBS (containing 0.5% Tween-20 + 0.5% BSA) at room temperature for 30 min away from light. The S phase of the cells were observed by flow cytometry (BD Biosciences).

**Wound-healing assay.** The cells were seeded in 6-well plates and cultured with DMEM containing 10% FBS until the cells reached subconfluence. Following removal of the culture medium, a monolayer of the sub-confluent cells was scratched with a 200  $\mu\text{l}$  pipette tip to create a wound area. The wounded monolayer was washed with PBS twice and cultured in FBS-free medium or 2% FBS medium for 48 h. Cell migration into the wound area was monitored by inverted microscopy, and photographed at the indicated time points until the wound was completely closed.

**Cell migration and invasion assays.** Migration and invasion assays were performed using the BioCoat cell migration chamber (BD Biosciences), which consists of a 24-well companion plate with cell culture inserts containing a filter with 8- $\mu\text{m}$ -diameter pores. The Transwell for the invasion assay was coated with Matrigel (1:3 dilution with DMEM free of serum; BD Biosciences). The cells were trypsinized and suspended with DMEM without FBS at  $2 \times 10^5/\text{ml}$  for the migration assay and with DMEM without FBS at  $2 \times 10^6/\text{ml}$  for the invasion assay. Cell suspension (100  $\mu\text{l}$ ) was added to the upper well and 600  $\mu\text{l}$  DMEM medium containing 10% FBS was added to the lower well. Cells in the wells were incubated in 5% CO<sub>2</sub> at 37°C for 24 h for the migration assay and 48 h for the invasion assay. After incubation, cells in the upper wells were gently removed by scrubbing, fixed in 95% ethanol for 15 min and stained with 0.4% crystal violet for

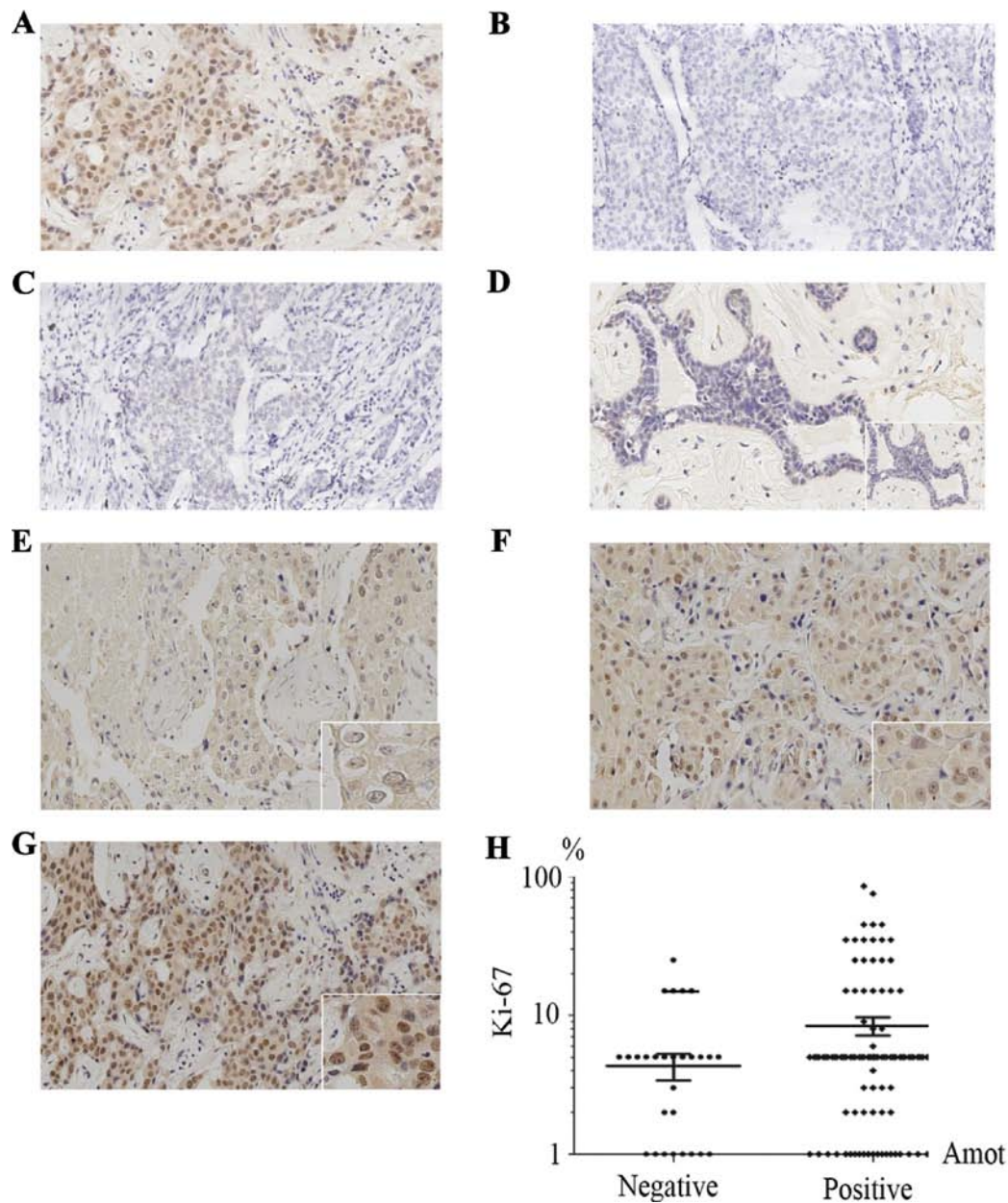


Figure 1. Expression and localization of Angiotensin II Type 1 Receptor (Amot) in breast cancer tissues. (A-C) Detection of antibody specificity in breast cancer tissues by immunohistochemistry (magnification, x200): (A) Amot antibody group; (B) Amot antibody mixed with enclosed peptide group; (C) PBS (negative control) group. (D-G) Immunohistochemical staining of Amot in adjacent non-cancerous tissues (D), and breast cancer tissues (E-G) (magnification, x200): (D) negative expression (scores 0-2); (E) weak expression (scores 3-5); (F) moderate expression (scores 6-9); (G) strong expression (scores 10-12). Each inset showed images captured at a magnification of x400. (H) Association of Amot and Ki-67. Amot-positive tumors have higher Ki-67.

30 min. Invasive or migrated cells were subsequently photographed with a microscope.

**Statistical analysis.** Statistical analysis was performed using SPSS 13.0 (Chicago, IL, USA). Data were presented as the mean  $\pm$  SD for at least three replicates for each group. Statistical differences between groups were determined using the ANOVA, Student's t-test, Wilcoxon and Chi-square tests.  $P < 0.05$  was considered to indicate a statistically significant result.

## Results

**Amot expression and localization in breast cancer and adjacent non-cancerous tissues.** To verify the specificity of the

Amot antibody, immunohistochemistry of breast cancer tissues was performed using Amot antibody, polypeptide-enclosed Amot antibody and PBS (negative control), respectively. The results showed that Amot was highly expressed in breast cancer tissues with Amot antibody, and negatively expressed in breast cancer tissues with polypeptide-enclosed Amot antibody or PBS (Fig. 1A-C). The results suggested that Amot antibody was specific and sensitive.

Immunohistochemistry was performed on 242 breast cancer and 92 adjacent non-cancerous tissues, to assess the expression and localization of Amot. The results showed that Amot was expressed in 205 breast cancer and 12 adjacent non-cancerous tissues. Amot was highly expressed in breast cancer tissues, but weakly expressed in adjacent non-cancerous

tissues. The difference was statistically significant ( $P < 0.001$ ; Table I). Notably, Amot expression was observed in the nucleus and cytoplasm of breast cancer tissues. In particular, Amot was strongly positively expressed in the nucleus. However, Amot showed a weak positive expression in the cytoplasm of adjacent non-cancerous tissues (Fig. 1D-G). We examined whether the strong positive expression of Amot in breast cancer tissues was connected with the clinically relevant parameters including age, tumor size, clinical stage, pathological grade, local lymph node status, the expression of ER, PR, Her-2 and Ki-67 (Table II). We found that the expression level of Amot was increased in specimens from patients with a high level of Ki-67, which is an immunohistochemical proliferation marker in many types of cancer (Fig. 1H; Table II;  $P < 0.01$ ). This finding indicated that Amot was significantly correlated with cell proliferation and invasion.

**Amot expression and localization in breast cancer cell lines.** The expression of Amot in MCF-7, T-47D, BT-474, MDA-MB-453 and MDA-MB-231 breast cancer cell lines and the MCF-10A breast epithelial cell line was detected by western blotting and RT-PCR. Our findings showed that Amot mRNA and protein were expressed in all the breast cancer cell lines, while the Amot expression level was significantly higher in MCF-7 cells than in the remaining cell lines (Fig. 2A and C). The specificity of the Amot antibody was verified by western blotting (Fig. 2B). Notably, Amot was expressed in the nucleus and cytoplasm of breast cancer cells. In particular, a strong positive Amot expression was observed in the nucleus. The localization of Amot in breast cancer cell lines was similar to those in breast cancer tissues (Fig. 2D).

**Amot downregulation decreased proliferative, invasive and metastatic capacity of MCF-7 cells in vitro.** The Amot protein expression was effectively suppressed in MCF-7 cells by the shAmot lentivirus (Fig. 3A). We then conducted MTT assay, plate colony formation and BrdU incorporation assay to estimate the effect of Amot silencing on cell proliferation. MTT assay showed that compared to CON and NC cells, the cells in the Amot knockdown group grew gradually, with decreased cell viability (Fig. 3B). The plate colony formation assay demonstrated that in MCF-7 Amot KD cells, the number of colonies were reduced significantly (Fig. 3C and D;  $P < 0.001$ ). BrdU incorporation assay further verified the above findings. The Amot knockdown cells exhibited a significant decrease in the percentage of S-phase cells, when compared with the CON and NC cells (Fig. 3E and F;  $P < 0.001$ ). The above results consistently suggested that Amot downregulation inhibited MCF-7 cell proliferation.

The effect of Amot downregulation on invasion and metastasis was explored using wound healing and Transwell assays. In the absence of the serum, cell migration showed no significant change between the Amot KD and NC groups. Of note, the NC cells exhibited characteristic morphological changes of apoptosis after the serum was decanted 24 h, while the Amot knockdown cells were allowed to grow in the serum-free medium. In the presence of 2% FBS, the migration of the Amot knockdown cells was slower than that of the NC cells (Fig. 4A). Transwell assay was conducted to further confirm the abovementioned results. It was found that

Table II. The relationship between the expression of Amot protein and clinicopathological factors.

Variables	N	Expression level		$\chi^2$	P-value
		Neg.	Pos.		
Age (years)				0.000	1.000
<35	11	2	9		
≥35	229	36	19		
Missing <sup>a</sup>	2				
Histological grade				2.424	0.298
1	18	5	13		
2	184	26	158		
3	40	7	33		
Missing <sup>a</sup>	0				
Clinical stage				0.497	0.781
I	16	3	13		
II	139	24	115		
III-IV	81	11	70		
Missing <sup>a</sup>	6				
Size (cm)				1.158	0.560
<2	41	7	34		
≥2	199	31	168		
Missing <sup>a</sup>	2				
Lymph node				0.057	0.811
Negative	93	14	79		
Positive	141	22	117		
Missing <sup>a</sup>	8				
ER stage				1.518	0.218
ER (-)	91	17	74		
ER (+)	134	17	117		
Missing <sup>a</sup>	17				
PR stage				0.033	0.857
PR (-)	122	19	103		
PR (+)	102	15	87		
Missing <sup>a</sup>	18				
Her-2 stage				2.741	0.098
Her-2 (-)	165	21	144		
Her-2 (+)	60	13	47		
Missing <sup>a</sup>	17				
Ki-67 stage				6.790	0.009 <sup>b</sup>
<14%	114	20	94		
≥14%	46	1	45		
Missing <sup>a</sup>	82				

<sup>a</sup>Missing, samples were missing some medical records. <sup>b</sup> $P < 0.01$ . Neg., negative; Pos., positive. Amot, Angiomotin.

the number of invading and migrating Amot knockdown cells were significantly reduced, when compared to those of NC cells (Fig. 4B and C), suggesting that Amot downregulation



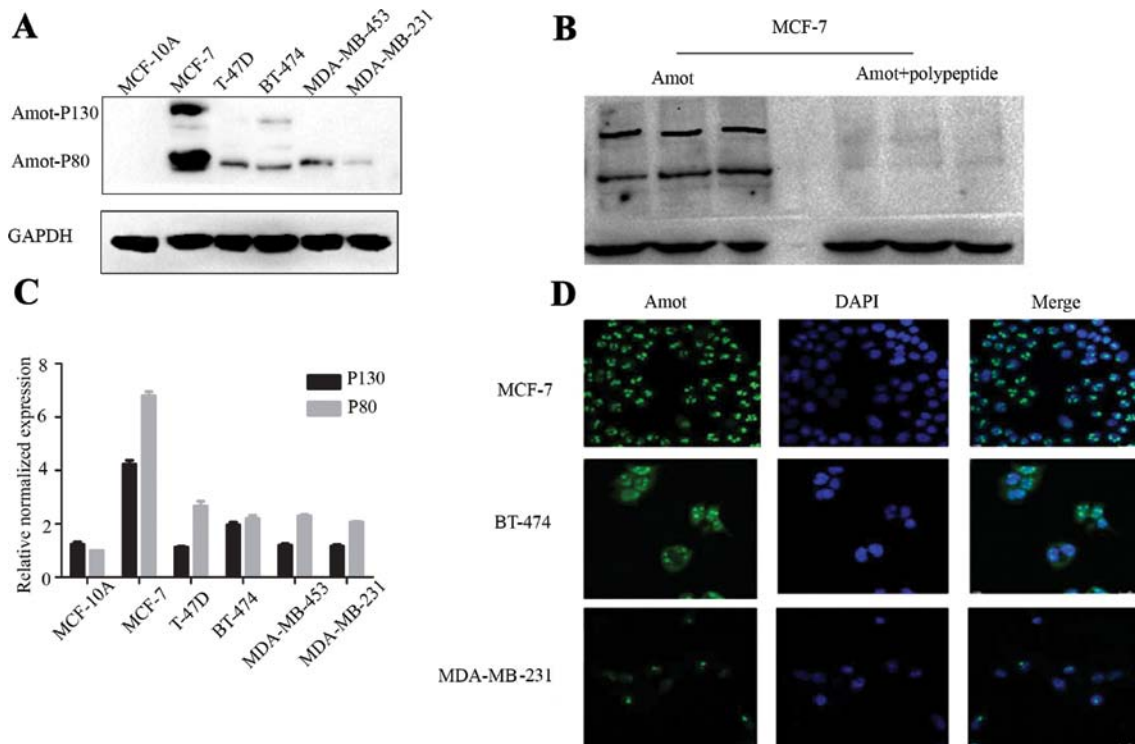


Figure 2. Expression and localization of Angiotensin (Amot) in breast cancer cells. (A) Western blot analysis showed Amot expression in breast cancer cells and mammary epithelial cells. GAPDH was used as an internal control. (B) Western blotting detected the antibody specificity in MCF-7 cells. The expression of Amot and Amot with polypeptide in MCF-7 cells. GAPDH was used as an internal control. (C) RT-PCR analyses showed Amot expression in breast cancer cells and mammary epithelial cell. The comparative CT method was used to quantify the relative levels of Amot mRNA. (D) Immunofluorescence assay shows Amot positively located in the nucleus in breast cancer cells.

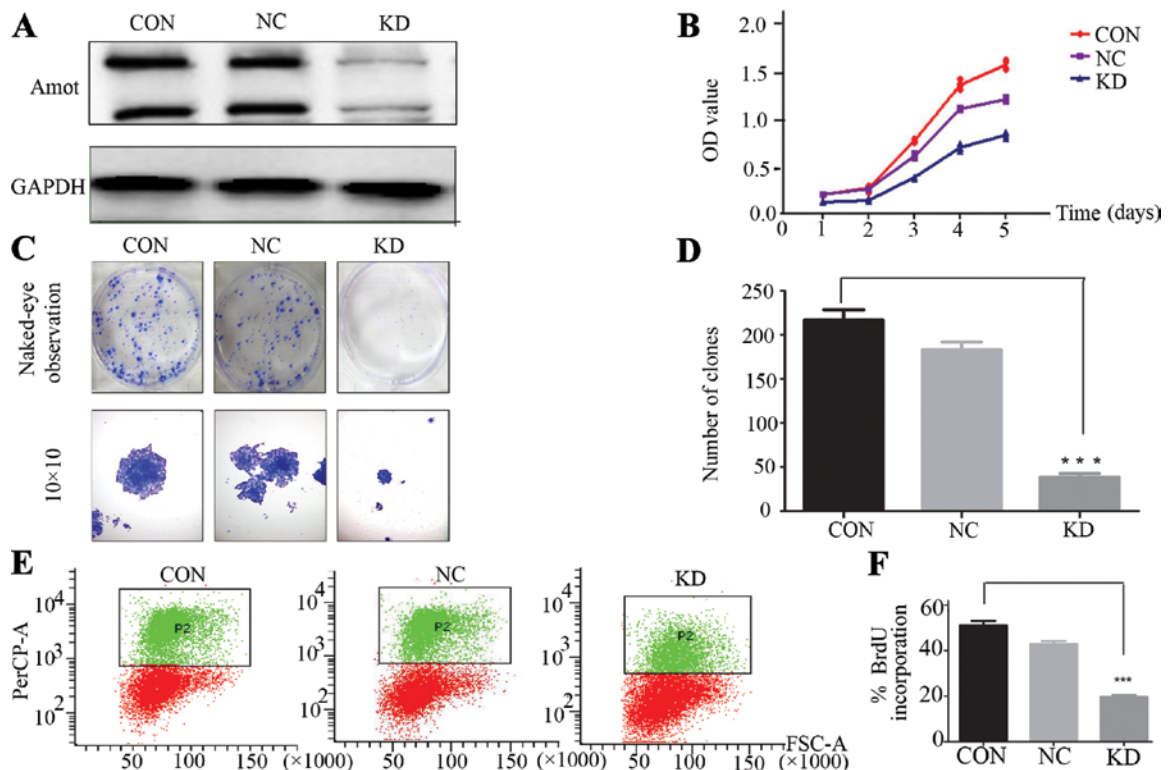


Figure 3. Downregulation of Angiotensin (Amot) decelerated the proliferation of MCF-7 breast cancer cell. CON (control), the uninfected breast cancer cells; KD (knockdown), the cells infected with the Amot shRNA lentivirus; NC (negative control), the mock control lentivirus. (A) Western blotting verificate knock-down efficiency of Amot in MCF-7 cells. GAPDH was used as an internal control. (B) MTT assays were performed to quantify relative numbers of MCF-7 cells at the indicated time points (1-5 days). (C) Plate clone formation assay detected the ability of MCF-7 cells forming clone *in vitro* by naked-eye observation or at 10x10 magnification. (D) The number of clones was quantified by counting cells in 10 random fields. (E) BrdU (5-bromodeoxyuridine) incorporation assay. Flow cytometry tested the number of BrdU incorporation in MCF-7 cells. (F) The percentage of BrdU incorporation in MCF-7 cells was quantitated to value the influence of Amot silence on the proliferation of breast cancer cells. The data represent the means  $\pm$  SD. \*\*\* $P < 0.001$ , compared to KD groups.

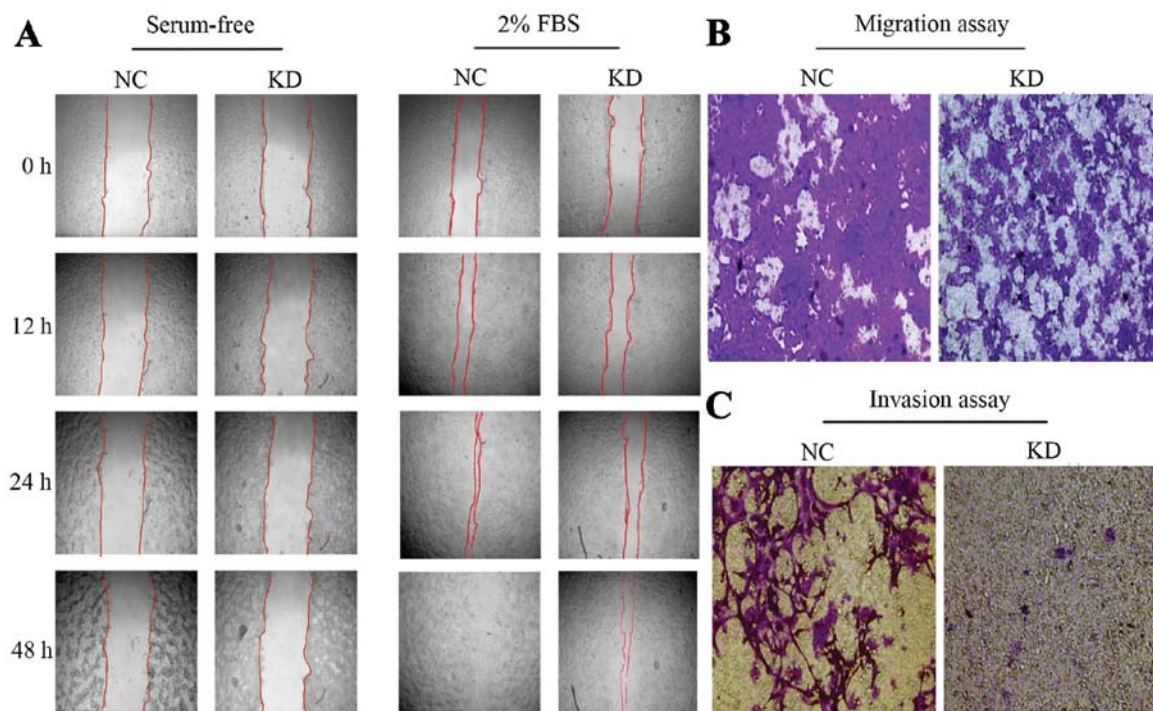


Figure 4. Downregulation of Angiomin (Amot) decreased the capacity of invasion and metastasis of MCF-7 cells *in vitro*. CON (control), the uninfected breast cancer cells; KD (knockdown), the cells infected with the Amot shRNA lentivirus and; NC (negative control), the mock control lentivirus. (A) Wound-healing assay measured the effect of Amot downregulation on migration in MCF-7 cells. Cell migration into the wound area was photographed at indicated time points (0-48 h). (B) The migration assay measured the effect of Amot downregulation on migration in MCF-7 cells. Migration cells on the bottom surface of the filter were stained and photographed. (C) Invasion assay measured the effect of Amot downregulation on invasion in MCF-7 cells. Invading cells on the bottom surface of the filter were stained and photographed.

significantly decelerated the invasion and migration of MCF-7 cells *in vitro*.

Our results revealed that Amot knockdown significantly decreased the proliferative, invasive and metastatic capacity of MCF-7 cells *in vitro*.

**Association of Amot and Hippo-YAP pathway.** Amot has been shown to participate in the activities of the Hippo-YAP pathway (15). To elucidate the relationship of Amot and Hippo-YAP pathway, we examined the expression of YAP, YAP/TAZ, LATS1, MOB, MST1 and SAV1 in MCF-7 cells following the downregulation of Amot using western blot analysis. The results showed that the expression of YAP, YAP/TAZ and LATS1 was significantly decreased in MCF-7 cells following the knockdown of Amot (Fig. 5A). However, the expression of MOB, MST1 and SAV1 did not exhibit any notable change. Additionally, the expression of YAP was obviously decreased in the nucleoprotein (Fig. 5B and C).

## Discussion

Amot is an angiostatin-binding protein that promotes endothelial cell migration and angiogenesis (5) and is expressed as the protein isoforms, p80-Amot and p130-Amot. The angiostatin-responsive migration-promoting function has been observed in the Amot p80 splicing variant, but not in the YAP-binding p130 variant (11). YAP-binding p130 Amot has been found to be involved in tumorigenesis. It was observed for the first time in 2011 that AmotL2 knockdown can activate YAP and induce cell transformation of MDCK epithelial

cells, suggesting that Amot family proteins may play tumor suppressive roles (15). However, using RT-PCR Jiang *et al* (14) found that breast cancer tissues expressed significantly higher levels of Amot transcript, compared with normal mammary tissues. The Amot expression was significantly increased with the increasing degree of invasion and metastasis. Amot expression showed close relationships with VE-cadherin and PECAM-1. Findings of Jiang *et al* suggested that Amot was closely related to angiogenesis, invasiveness and poor survival of breast cancer (14). In 2008, Levchenko found that a DNA vaccine targeting Amot induced an antibody response and significantly inhibited angiogenesis and tumor growth (16). In addition, it has been reported that Amot expression likely promotes cell growth by prolonging the activation of MAPK signaling. Amot expression enhanced the proliferation rate of MCF-7 cells and induced MCF-10A cells to form large, disorganized spheroids in Matrigel. On the other hand, a reduced expression of Amot resulted in decreased ERK1/2-associated growth of MDA-MB-468 and SKBR3 cells (17). The above studies have indicated that Amot acts as a potential tumor promoter, which was well supported by our results.

In the present study, Amot was highly expressed in breast cancer tissues and cells, but weakly expressed in normal controls, while the expression level of Amot was increased in specimens from patients with a high level of Ki-67. The results indicated that Amot may be involved in breast cancer proliferation and invasion. Amot knockdown, not only retarded growth and viability of MCF-7 cells, but also significantly decreased the percentage of S-phase cells. In addition, lentiviral-mediated Amot silencing significantly reduced the

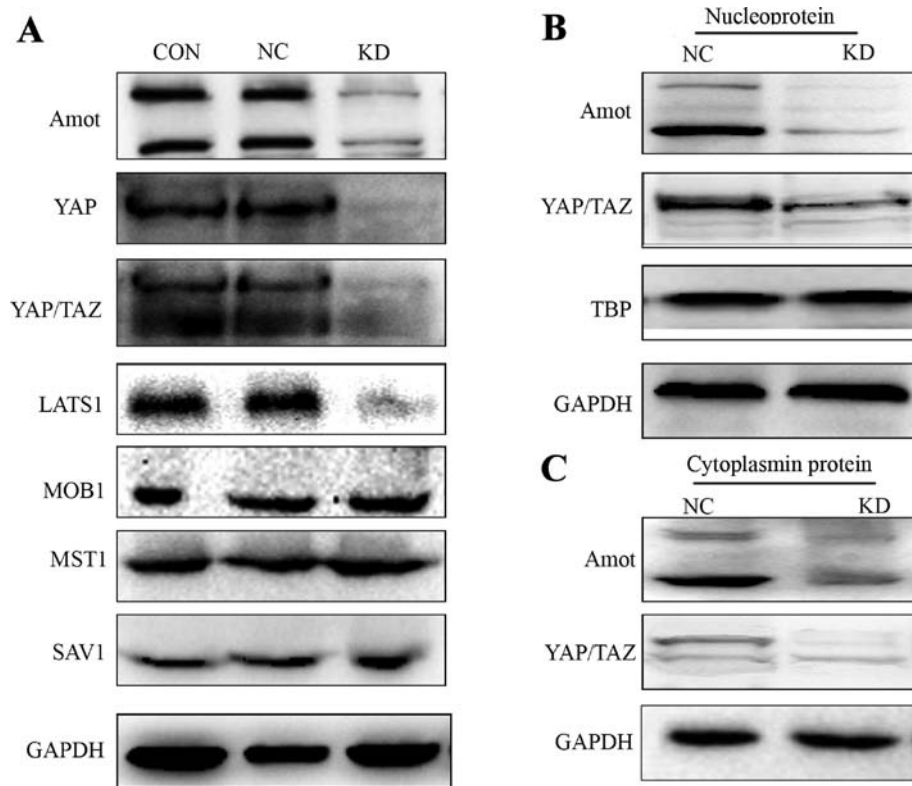


Figure 5. Preliminary study on Angiomotin (Amot) gene expression with Hippo-YAP signaling pathway. CON (control), the uninfected breast cancer cells; KD (knockdown), the cells infected with the Amot shRNA lentivirus; NC (negative control), the mock control lentivirus. (A) Western blotting detected the expression of Hippo-YAP signaling pathway protein (YAP, YAP/TAZ, LATS1, MOB, MST1 and SAV1) following the downregulation of Amot in MCF-7 cells. GAPDH was used as an internal control. (B) Western blotting was applied to detect the expression of Amot and YAP/TAZ in the nucleus after the downregulation of Amot in MCF-7 cells. GAPDH was used as an internal control and TBP was used as a nuclear protein marker. (C) Western blotting was applied to detect the expression of Amot and YAP/TAZ in the cytoplasm after the downregulation of Amot in MCF-7 cells. GAPDH was used as an internal control.

number of colonies formed by MCF-7 cells. Our results show that Amot downregulation inhibited MCF-7 cell proliferation. We also found that Amot knockdown in cells retarded the migration and invasion of MCF-7 cells *in vitro*. Taken together, Amot downregulation decreased the proliferative, invasive and metastatic capacity of MCF-7 cells, indicating that Amot is a potential tumor promoter in breast cancer.

The Hippo-YAP signaling pathway regulates cell proliferation during development, tissue regeneration and carcinogenesis. Amot family proteins have been recently identified as negative regulators of YAP by promoting YAP phosphorylation to preventing their nuclear translocations (16-21). However, it has been shown that Amot-p130 may promote nuclear translocation of YAP and act as a transcriptional cofactor of the YAP-TEAD complex to facilitate biliary epithelial cell proliferation and liver cancer development either in response to tissue injury or in the absence of the tumor suppressor Merlin (22,23). The different results were obtained probably since different organs and cells were used in those studies. In the present study, Amot knockdown significantly decreased the YAP and LATS1 expression in MCF-7 cells. Notably, the expression of YAP was obviously decreased in the nucleoprotein. The results suggest that Amot is involved in regulation of the proliferation of MCF-7 cells by modulating the nucleoprotein expression of YAP in the Hippo-YAP

pathway. Future studies are needed to reveal the mechanism for a clear role of Amot in the Hippo-YAP pathway.

The respective roles of p80-Amot and p130-Amot were not studied in the present study since we did not make a clear distinction between p80 and p130 splicing variants of the Amot antibody and shRNA. Future studies are to focus mainly on the specific mechanisms of Amot and Hippo-YAP pathway, including the expression changes of total YAP, phosphorylated YAP, p80-Amot, and p130-Amot in the nucleus and cytoplasm, the activity of the downstream transcription factor TEAD, as well as the relationship between Amot and other signaling pathways associated with cell proliferation, invasion and metastasis.

In conclusion, our results have shown that Amot was highly expressed in breast cancer tissues and played an important role in promoting breast cancer cell proliferation and invasion. In addition, there was a more intimate connection between Amot and the Hippo-YAP pathway. Further studies are needed to reveal the mechanisms underlying the effect of Amot-induced Hippo-YAP pathway on breast cancer growth and invasion.

#### Acknowledgements

This study was supported by the National Natural Science Fund of China (no. 81172171).



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