



Cell adhesion molecule 2 (CADM2) promotes brain metastasis by inducing epithelial-mesenchymal transition (EMT) in human non-small cell lung cancer

Lu Dai^{1,2,3}, Jian Zhao³, Jun Yin³, Wenfan Fu³, Gang Chen^{1,2}

¹The Second Clinical Medical College, Southern Medical University, Guangzhou 510515, China; ²Department of Thoracic Surgery, Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangzhou 510080, China; ³Department of Thoracic Surgery, Affiliated Cancer Hospital & Institute of Guangzhou Medical University, Guangzhou 510095, China

Contributions: (I) Conception and design: G Chen, L Dai; (II) Administrative support: J Zhao; (III) Provision of study materials or patients: L Dai; (IV) Collection and assembly of data: W Fu; (V) Data analysis and interpretation: J Yin; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Gang Chen. Department of Thoracic Surgery, Guangdong General Hospital, Guangdong Academy of Medical Sciences, No. 106, Zhongshan 2nd Road, Guangzhou 510080, China. Email: tgzy2019@outlook.com.

Background: To investigate the effect of CADM2 on brain metastasis in non-small cell lung cancer (NSCLC).

Methods: Human transcriptome-wide microarray analysis was used to identify gene expression in lung tissue of NSCLC patients with or without brain metastasis, which indicated that CADM2 was significantly up-regulated. Quantitative real-time PCR (qRT-PCR) was used to confirm the CADM2 up-regulation further. SiRNA was used to knock down the expression of CADM2 in NSCLC cell lines and a Transwell assay was performed to determine the effects of CADM2 knockdown on cell migration and invasion. The expressions of Vimentin and E-cadherin were detected by western blot assay.

Results: The result of microarray analysis and qRT-PCR showed that CADM2 was significantly up-regulated in NSCLC patients with brain metastasis than in those without brain metastasis. The result of the Transwell assay showed that the migration and invasion abilities of NSCLC cells were inhibited after CADM2 knockdown. Also, the expression of Vimentin was reduced while E-cadherin was increased, followed by CADM2 knockdown.

Conclusions: The results showed that CADM2 might promote brain metastasis by inducing epithelial-mesenchymal transition (EMT) in human NSCLC. We propose that CADM2 can be used as a novel molecular target for the prevention and treatment in NSCLC with brain metastasis patients.

Keywords: Non-small cell lung cancer (NSCLC); brain metastasis; CADM2; epithelial-mesenchymal transition (EMT)

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Introduction

Brain metastasis is the most feared complication of cancer and the most common intracranial tumor in adults (1). Lung cancer is one of the most common tumors among men and women worldwide, and 85% of them are non-small cell lung cancer (NSCLC) (2,3). Up to 50% of NSCLC patients

are expected to experience brain metastasis at some point (4). The mechanism of tumor brain metastasis is still unclear, and due to the migration and invasiveness of NSCLC, the existing treatment for NSCLC in patients with brain metastasis is generally ineffective, the prognosis is reduced, and the median survival time is only a few months (5).

Finding molecular targets, understanding the mechanisms of brain metastasis, and discovering new diagnostic markers or therapeutic targets are particularly urgent.

In advanced NSCLC, localized large mediastinal lymph nodes (>2 cm) (6), non-squamous cell carcinoma (7), and age ≤60 years were found to be associated with high brain metastasis rates. However, we currently know little about the genetic and genomic changes that occur in NSCLC brain metastasis. Through tumor genomics and proteomics methods, we can now study the above changes as a whole to comprehensively understand the changes leading to brain metastasis in transcription levels, exploring the molecular mechanism of NSCLC with brain metastasis, and possibly identifying new therapeutic targets.

In this study, we found that cell adhesion molecule 2 (CADM2) is up-regulated in NSCLC patients with brain metastasis compared to NSCLC patients without brain metastasis. CADM2 is a member of the synaptic cell adhesion molecule one family that is involved in homologous and heterophilic interactions with other members of the Nectin-like family, leading to cell aggregation (8,9). In renal cell carcinoma (10) and prostate cancer (11), CADM2 acts as a tumor suppressor. Low CADM2 expression in patients with hepatocellular carcinoma predicts a higher risk of recurrence after hepatectomy (12). However, we found that silencing of CADM2 inhibited cell migration, invasion, and epithelial-mesenchymal transition (EMT) in NSCLC cell lines A549 and H322, indicating that CADM2 may be a poor prognostic factor for NSCLC patients with brain metastasis.

Methods

Subjects

Tissues used in this study were obtained from the Affiliated Cancer Hospital & Institute of Guangzhou Medical University, including samples of NSCLC with [n=26, brain metastasis (BM) group] or without (n=36, nonBM group) brain metastasis. We received ethical approval from the local research ethics committee, and all subjects received written informed consent for sample collection and research use. All diagnoses of NSCLC were confirmed by radiographic imaging and other methods, of those cases that brain metastases were observed.

Microarray and data analysis

Trizol/Chloroform was used to extract total RNA from samples, and Affymetrix Human Transcriptome Array 2.0 analysis (Affymetrix, Santa Clara, CA, USA) was performed by Gene Tech Company Limited (Shanghai, China). Briefly, after RNA measurement using the Nanodrop ND-1000 and denaturing gel electrophoresis, the samples were amplified and labeled with biotin by terminal deoxynucleotidyl transferase (TdT). Labeled DNA was then hybridized with the Affymetrix GeneChip™ Human Transcriptome Assay 2.0 (Cat No. 902309). Slides were carefully washed and scanned with Affymetrix® GeneChip Command Console (AGCC) which installed in GeneChip® Scanner 3000 7G. The data were analyzed with Robust Multichip Analysis (RMA) algorithm. Normalized data were further analyzed using moderated *t*-test. Differentially expressed genes were named through fold-change (fold change >2, P<0.05) screening.

Quantitative real-time PCR (qRT-PCR)

Total RNA from lung tissue was extracted using E.Z.N.A.™ Fast Protocol for FFPE Tissue (OMEGA, Guangzhou, China), and total RNA from cell samples was extracted using TRIzol Reagent (Invitrogen) following the manufacturer's protocols. Then the extracted RNA was reverse-transcribed into cDNA, using the Prime Script™ RT reagent Kit (TaKaRa, Shiga, Japan). qRT-PCR was performed using Premix EX Taq™ (Probe qPCR) (TaKaRa). The primers used are as follows:

Forward primer: 5'- GCTCTGGGCCTCATGGTTT-3'

Reverse primer: 5'-CAGCTGAGCAGAGGCAACTTT-3'

Probe primer: 5'- FAM-TTCTCTTGGAGCCTTT CATTACAGA-BHQ1-3'.

Amplification was performed using the following PCR profile: preheating at 94 °C for 3 min, followed by 40 cycles of 94 °C for 3 min, 94 °C for 5 s, and 60 °C for 30 s. PCR reactions were performed on an ABI PRISM® 7300 Sequence Detection System (Foster City, CA, USA). Gene expression was measured in triplicate, quantified by the $2^{-\Delta\Delta Ct}$ method (13), and normalized using GAPDH as the internal control.

Cell culture and siRNA transfection

NSCLC cell lines A549 and H322 were bought from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT, USA)

supplemented with 10% fetal bovine serum (FBS, Hyclone). The negative control siRNA (si-NC) and siRNAs targeting CADM2 (si-CADM2) were bought from GenePharma (Suzhou, China). Cells were plated to 50% confluency and transfected with 10, 50, and 100 nM si-NC or si-CADM2, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The sequences of si-CADM2 are:

5'-UUCAACAACGGUACAUUCUG-3' (sense)

5'-GAAUGUAACCGUUGUUGAAGG-3' (antisense)

Transwell assays

Transwell assay was carried out to detect the migration and invasion abilities of A549 and H322 after transfection with si-NC or si-CADM2. For migration, transfected A549 and H322 cells were harvested, and 1×10^5 cells in 100 μ L of 0.1% serum medium inserted in the upper chamber (pore size, 8 μ m) (Becton Dickinson Labware). The lower chamber was filled with 600 μ L DMEM (Hyclone) supplemented with 10% FBS (Hyclone). For invasion, the same density of cells was placed into the upper chambers, which were pre-coated with Matrigel (BD Biosciences). After incubation for 24 hours, the cells in the upper chamber of the filter are removed with a cotton swab, the cells on the underside were fixed with 4% paraformaldehyde for 15 min, stained with 0.1% crystal blue for 10 min. The migrated or invasive cells were counted by photographing at 200 \times magnification with LEICA Microscope (Tokyo, Japan) in five independent fields for each well. The assays were performed in triplicate.

Western blot analysis

Total protein from cell samples was extracted using RIPA Lysis Buffer (Beyotime Biotechnology, Shanghai, China). Thirty micrograms of protein were separated by 10% SDS polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Membranes were then blocked with 5% non-fat milk for 1 h at 37 $^{\circ}$ C. Subsequently, membranes were incubated in 5% non-fat milk for one h at 37 $^{\circ}$ C with anti-CADM2, anti-Vimentin, anti-E-cadherin, and anti-GAPDH primary antibodies. After washing with TBST (TBS holding 0.5% Tween 20), membranes were incubated at 37 $^{\circ}$ C for 40 min with an HRP-conjugated secondary antibody. Membranes were then re-washed with TBST and assayed via enhanced chemiluminescence (ECL) and recorded on X-ray films.

Statistical analysis

Statistical analysis was conducted using SPSS 19.0 software package (SPSS Inc., Chicago, IL, USA). Student's *t*-test was used for comparisons between two groups, and one-way ANOVA was used for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

Results

Expression of CADM2 in NSCLC with or without brain metastasis patients

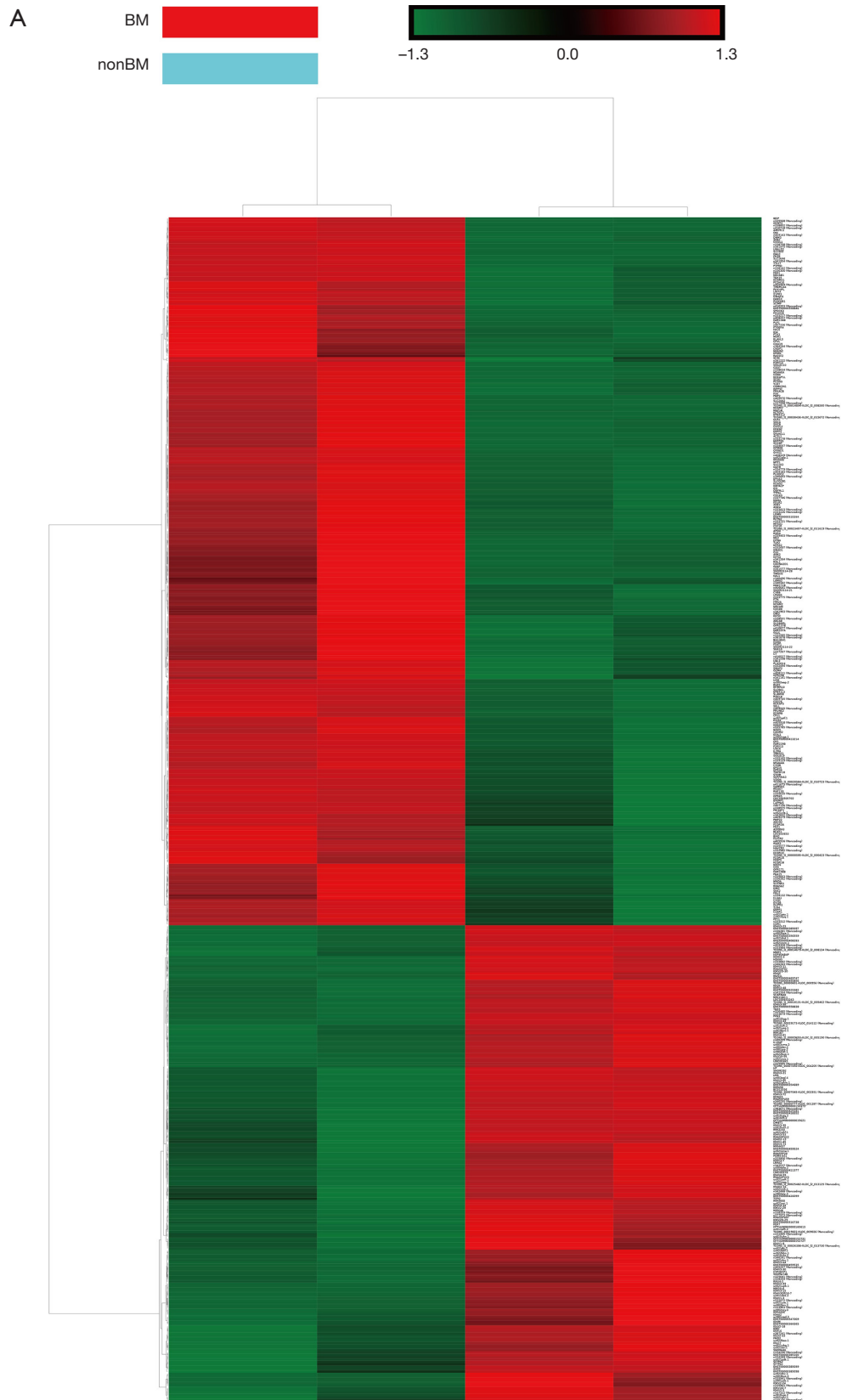
Human transcriptome array analysis and qRT-PCR were performed to determine the expression of CADM2 in NSCLC with or without brain metastasis patients. Compared with NSCLC patients without brain metastasis (nonBM), 513 up-regulated, and 438 down-regulated genes were found in NSCLC plus BM patients, data were shown in <http://fp.amegroups.cn/cms/cd0b98a53c8f6fa0f12bc5c789fd6322/atm.2020.03.85-1.pdf>. *Figure 1A* was the heatmap of the up-regulated and down-regulated genes. From this, we found that CADM2 was up-regulated in BM patients compared to nonBM patients. The gene expression level of CADM2 was verified in 26 BM samples and 36 nonBM samples using qRT-PCR assays. *Table 1* shows the baseline characteristics of the test subjects. As a result, shown, CADM2 was up-regulated 2.05-fold in BM patients compared to nonBM patients (*Figure 1B*). And there was a good match between the result of microarray and qRT-PCR (*Figure 1C*).

Silencing of CADM2 by siRNAs

To reduce the expression of CADM2, we synthesized NC and three active siRNAs targeting different CADM2 sequences and then transfected A549 and H322 cells. qRT-PCR and western blot assays were used to determine the efficiency of the silencing. In the siRNAs evaluated, the lowest expression of CADM2 mRNA in A549 and H322 cells was observed by the transfection of si-CADM2-3 (*Figure 2A,B*) and it also proved to be able to successfully knockdown CADM2 protein (*Figure 2C,D*).

Silencing of CADM2 inhibited cell migration and invasion in NSCLC cells

Transwell assay was performed following the transfection of si-CADM2 or si-NC to prove the role of CADM2 in regulating cell migration and invasion in NSCLC cells. The results showed that the number of cells entering the lower chamber through the membrane of si-CADM2-



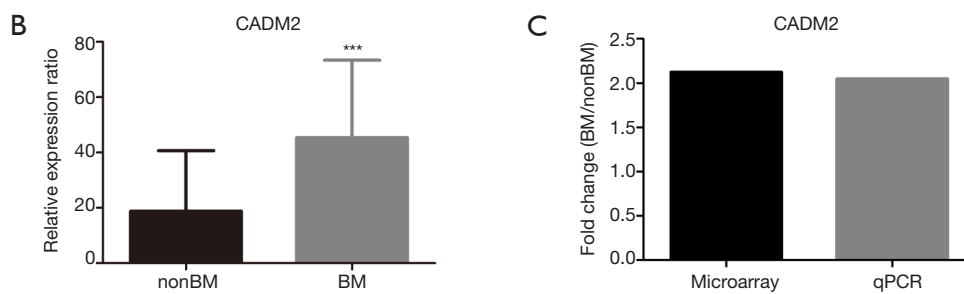


Figure 1 Expression of CADM2 in NSCLC patients. (A) Heat map showing expression profiles of genes. Two cases of NSCLC patients with or without brain metastasis were analyzed by microarray. BM represent NSCLC with brain metastasis; nonBM represent NSCLC without brain metastasis. The intensity increases from green to red. Each column is one sample, and each row shows a gene. (B) qRT-PCR assay was used to determine the relative expression level of CADM2 in 26 cases of BM patients and 36 cases of nonBM patients. The result was quantified using the $2^{-\Delta\Delta CT}$ method. (C) Comparison between microarray data and qRT-PCR data revealing a good correlation of such two methods. ***, $P < 0.001$. CADM2, cell adhesion molecule 2; NSCLC, non-small cell lung cancer; BM, brain metastasis; qRT-PCR, quantitative real-time PCR.

Table 1 Baseline characteristics of NSCLC with or without brain metastasis patients

Characteristics	BM (n=26)	nonBM (n=36)
Age	57.2±8.2	59.1±9.2
Sex		
Male	17	26
Female	9	10
Location		
Left lung	10	12
Right lung	16	24
Histological types		
Adenocarcinoma	25	26
Squamous carcinoma	1	10

NSCLC, non-small cell lung cancer; BM, NSCLC with brain metastasis; nonBM, NSCLC without brain metastasis.

transfected A549 and H322 cells was significantly lower than that of si-NC transfected cells (Figure 3A,B), indicating that silencing of CADM2 inhibited cell migration in NSCLC cells. Besides, the number of cells that passed through the matrigel-coated membrane into the lower chamber was significantly lower in the si-CADM2 transfected A549 and H322 cells than in the si-NC transfected A549 and H322 cells (Figure 3C,D), indicating that silencing of CADM2 inhibited cell invasion in NSCLC cells.

Silencing of CADM2 inhibited EMT in NSCLC cells

The protein expression levels of Vimentin and E-cadherin were detected after transfection with si-CADM2 or si-NC to prove the role of CADM2 in regulating NSCLC cell EMT. The results showed that CADM2 knockdown significantly decreased the level of Vimentin (mesenchymal marker) (Figure 4A,B), and correspondingly increased the protein level of the E-cadherin (epithelial marker) in both A549 and H322 cells (Figure 4C,D).

Discussion

Brain metastasis is a common complication of NSCLC, which is associated with severe morbidity and mortality. Understanding the cellular and molecular mechanisms of lung cancer-related brain metastasis will supply hope for effective treatment and improve the prognosis of the disease. Preventive treatment may prevent or delay brain metastasis; showing high-risk patients for brain metastasis may be a better option for those who are at high risk. It is reported that some proteins or signaling pathways take part in brain metastasis of lung cancer. The expressions of CDH2 (N-cadherin), KIFC1, and FALZ, are highly predictive for brain metastasis in early and advanced lung cancer and can be used to identify brain metastasis high-risk patients (14). Also, the ability of lung adenocarcinoma cells to metastasize to the brain was enhanced by Wnt/Tef signal program of LEF1 and HOXB9 (15), and recently, Claudin-5 (16), ACTN4 (17)

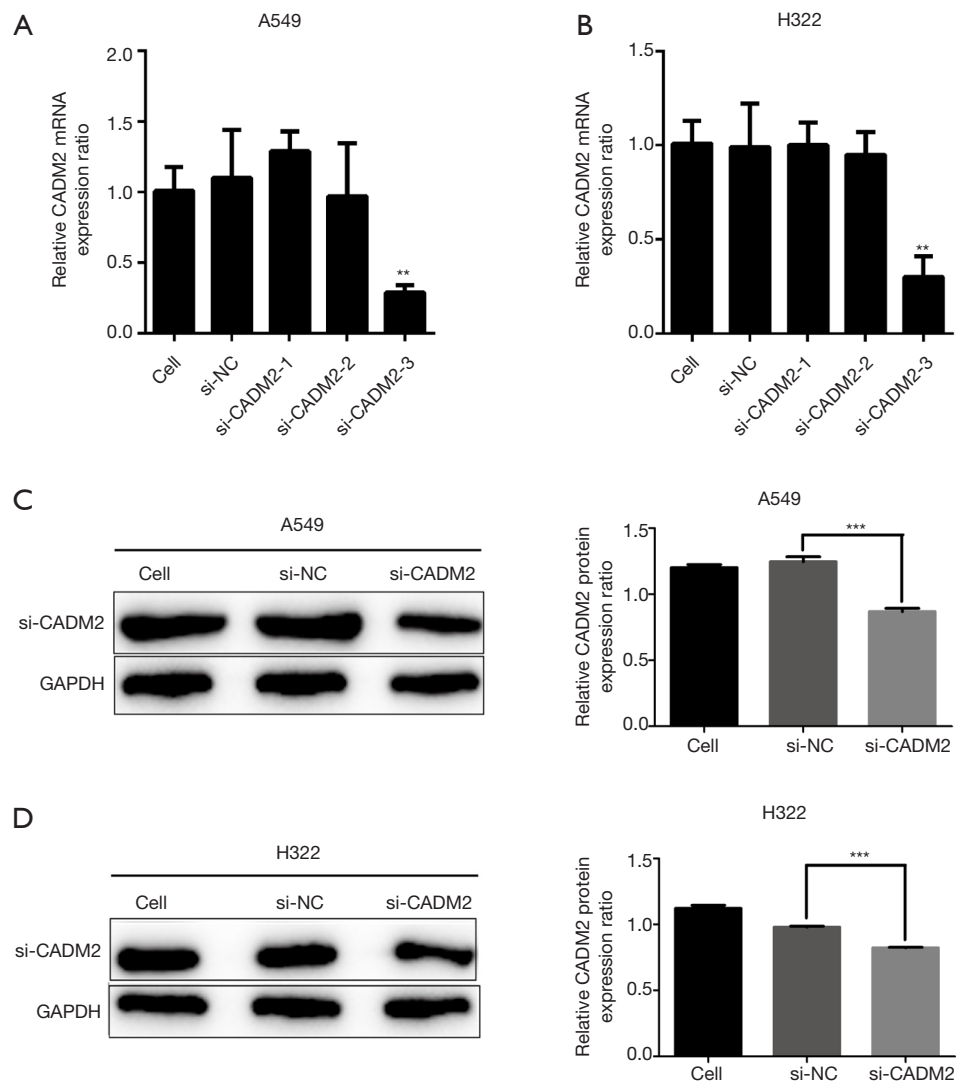


Figure 2 Silencing of CADM2 by siRNAs. (A,B) Expression of CADM2 mRNA in A549 (A) and H322 (B) cells after transfection with si-CADM2-1, si-CADM2-2, si-CADM2-3 or si-NC detected by qRT-PCR. The results of qRT-PCR were quantified using the $2^{-\Delta\Delta CT}$ method. Results are represented as mean \pm SD. (C,D) Expression of CADM2 protein in A549 (C) and H322 (D) cells after transfection with si-CADM2 or si-NC detected by western blot. **, $P < 0.01$; ***, $P < 0.001$, si-CADM2 vs. si-NC. CADM2, cell adhesion molecule 2; qRT-PCR, quantitative real-time PCR; SD, standard deviation.

and ADAM9 (18) have been proved to be associated with lung cancer metastasis. However, the mechanism of brain metastasis of NSCLC is still unclear, so more research is needed. In the current study, we use human genome array analysis to study the gene profiles of NSCLC patients with or without brain metastasis, to identify genes that contribute to brain metastasis of NSCLC patients. Our research supplies a comprehensive understanding of the changes in transcriptional levels of brain metastasis in NSCLC, and we also supply information on the potential cellular and

molecular mechanisms.

To our knowledge, this is the first study investigating CADM2 in NSCLC with brain metastasis. Based on microarray analysis and qRT-PCR, it was showed that CADM2 was up-regulated more than two folds in NSCLC patients with brain metastasis compared to those without brain metastasis. Further study showed that the silence of CADM2 inhibited the migration and invasion of NSCLC cell lines A549 and H322. These results showed that CADM2 might play a key role in preventing brain

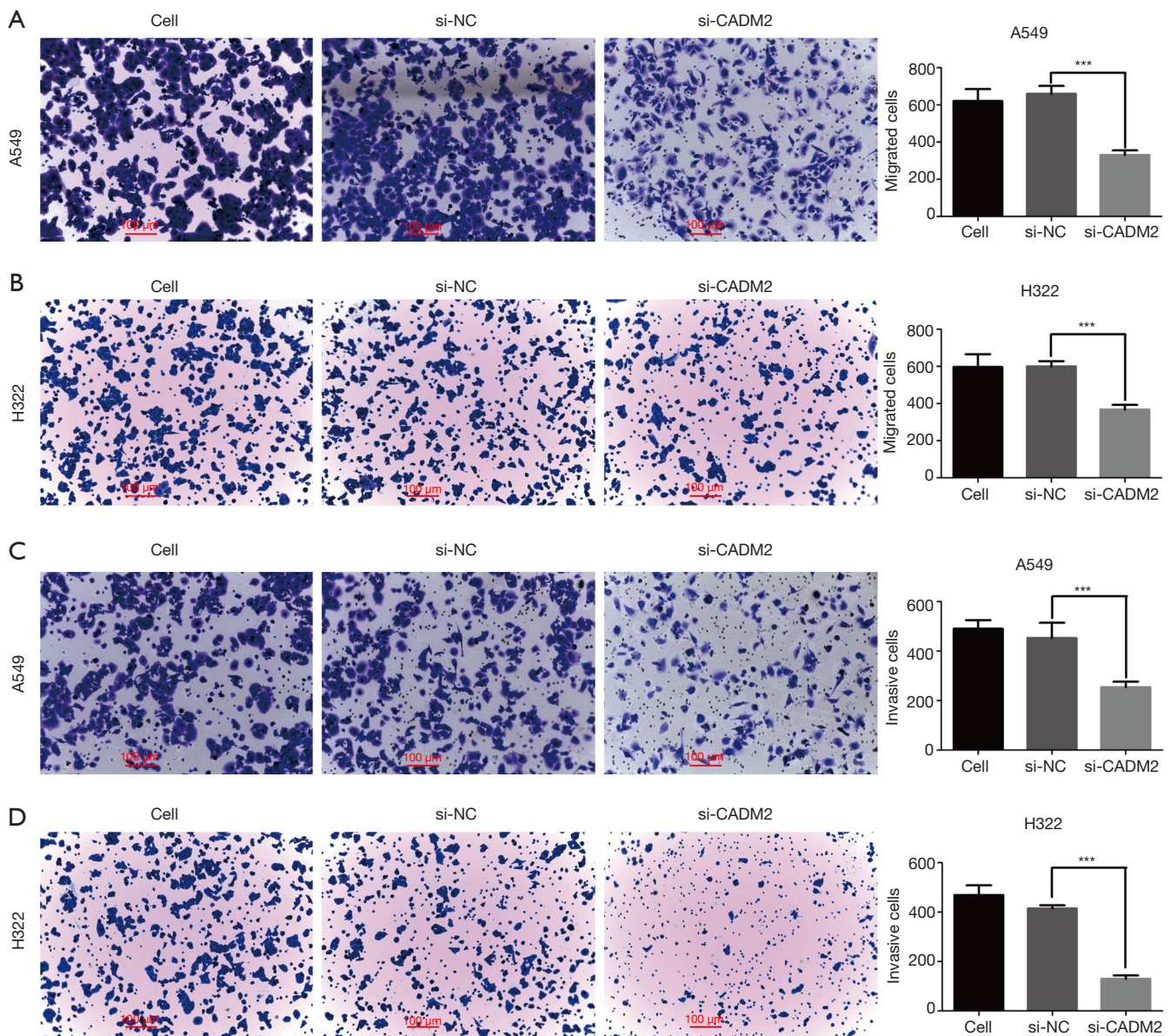


Figure 3 The effect of CADM2 silencing on the cell migration and invasion ability of A549 and H322 cells. A549 and H322 cells were transfected with si-CADM2 or si-NC, and later cultivated for 24 h. Then, Transwell assay (A,B) were performed to detect the cell migration ability of A549 and H322 cells and matrigel-Transwell assay (C,D) were performed to detect the cell invasion ability of A549 and H322 cells (200 \times). Representative graphs for each group are presented on the left. The graphs on the right show the results of analysis of five randomly selected fields. ***, $P < 0.001$, si-CADM2 *vs.* si-NC. CADM2, cell adhesion molecule 2.

metastasis in NSCLC. However, it has been reported that overexpression of CADM2 may inhibit cell proliferation in the prostate, renal, and endometrial cancers (7,10,11). Also, the overexpression of CADM2 could promote apoptosis and inhibit cell invasion in renal and endometrial cancer cells (10). We must do more research to clarify this opposite

effect of CADM2 in different cancers.

Furthermore, we found that CADM2 knockdown markedly reduced the level of Vimentin, a mesenchymal marker, and increased the level of E-cadherin, an epithelial marker, suggesting that CADM2 could promote EMT in NSCLC. EMT is a conservative evolutionary process

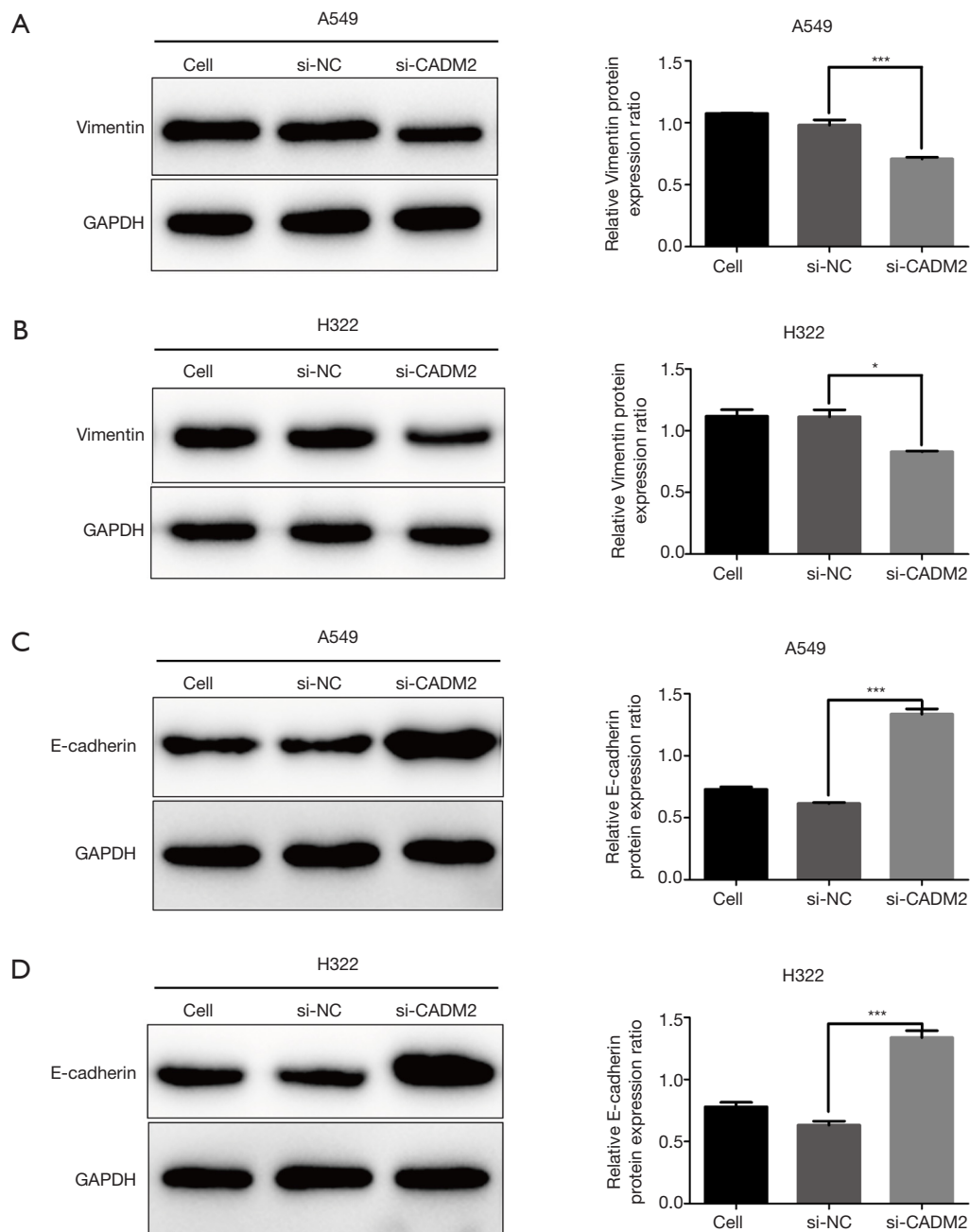


Figure 4 The protein expression levels of mesenchymal marker Vimentin (A,B) and epithelial marker E-cadherin (C,D) after transfection with si-CADM2 or si-NC in A549 and H322 cells detected by western blot. *, $P < 0.05$; ***, $P < 0.001$, si-CADM2 vs. si-NC. CADM2, cell adhesion molecule 2.

and is considered necessary for normal embryonic development (6). EMT is an essential biological process in the development of malignant tumors, such as enhancing the invasion and metastasis of cancer cells (19-21). Also, increased studies have shown that EMT (22-24) can

regulate the ability of cancer cells to migrate and invade. Based on these reports, we speculate that CADM2 promotes brain metastasis by inducing EMT in human NSCLC.

Our study focused on the mechanism of CADM2 in NSCLC with brain metastasis patients and showed that

CADM2 might promote brain metastasis in NSCLC patients by inducing EMT. In conclusion, CADM2 may be a new target for the prevention and treatment of brain metastasis in NSCLC soon.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/atm.2020.03.85>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. We received ethical approval from the local research ethics committee, and all subjects received written informed consent for sample collection and research use.

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