ADAM9 promotes lung cancer metastases to brain by a plasminogen activator-based pathway

Authors:
Chen-Yuan Lin1,4, Hung-Jen Chen5, Cheng-Chung Huang3, Liang-Chuan Lai7, Tzu-Pin Lu8, Guan-Chin Tseng6, Ting-Ting Kuo3, Qian-Yu Kuok3, Jennifer L. Hsu3,9,10, Shian-Ying Sung11, Mien-Chie Hung2,3,9,10,* and Yuh-Pyng Sher1,3,*

Affiliations:
1Graduate Institute of Clinical Medical Science, 2Graduate Institute for Cancer Biology, China Medical University, Taichung 404, Taiwan
3Center for Molecular Medicine, 4Division of Hematology and Oncology, 5Department of Internal Medicine, 6Department of Pathology, China Medical University Hospital, Taichung 404, Taiwan
7Graduate Institute of Physiology, National Taiwan University, Taipei 106, Taiwan
8YongLin Biomedical Engineering Center, National Taiwan University, Taipei 106, Taiwan
9Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas 77030, USA
10Department of Biotechnology, Asia University, Taichung 413, Taiwan
11PhD Program for Translational Medicine, College of Medical Science and Technology, Taipei Medical University, Taipei 110, Taiwan
*Correspondence should be addressed to Mien-Chie Hung, Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030. Phone: 713-792-3668; Fax: 713-794-0209. E-mail: mhung@mdanderson.org or to Yuh-Pyng Sher, Graduate Institute of Clinical Medical Science and Center for Molecular Medicine, China Medical University & Hospital, Taichung 404, Taiwan. Phone: 886-4-22052121 ext. 7819; Fax: 886-4-2233-3496. E-mail: ypsher@mail.cmu.edu.tw

Conflicts of interest
All authors have no conflicts of interest.

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Abbreviations: ADAM9, a disintegrin and metalloprotease domain 9; CDCP1, CUB domain-containing protein-1; tPA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor type I.
ABSTRACT

The transmembrane cell adhesion protein ADAM9 has been implicated in cancer cell migration and lung cancer metastasis to the brain, but the underpinning mechanisms are unclear and clinical support has been lacking. Here we demonstrate that ADAM9 enhances the ability of tissue plasminogen activator (tPA) to cleave and stimulate the function of the pro-migratory protein CDCP1 to promote lung metastasis. Blocking this mechanism of cancer cell migration prolonged survival in tumor-bearing mice and cooperated with dexamethasone and dasatinib (a dual Src/Abl kinase inhibitor) treatment to enhance cytotoxic treatment. In clinical specimens, high levels of ADAM9 and CDCP1 correlated with poor prognosis and high risk of mortality in lung cancer patients. Moreover, ADAM9 levels in brain metastases derived from lung tumors were relatively higher than the levels observed in primary lung tumors. Our results show how ADAM9 regulates lung cancer metastasis to the brain by facilitating tPA-mediated cleavage of CDCP1, with potential implications to target this network as a strategy to prevent or treat brain metastatic disease.
**Introduction**

Lung cancer is the leading cause of cancer deaths worldwide among both men and women, with 159,480 deaths estimated for 2013 in the United States (1). Brain metastases from lung are found in 20-50% of patients with non-small-cell lung cancer (NSCLC) and cause the patients a worse prognosis (2). Lung cancer accounts for the majority of brain metastases. In contrast to metastases from other primary cancer sites such as breast cancer, colorectal cancer, and melanoma that can develop over one year after diagnosis, brain metastases from lung cancer can occur within months after or even at the time of diagnosis of the primary (3).

Once cancer cells intravasate from the primary site into the blood and before extravasation and colonization in the distant organ, the first step for cancer cells to complete the process of metastasis is their adhesion to vascular endothelial cells (4). Several genes related to lung cancer metastatic to the brain that play a role in cancer cells’ adhesion to brain endothelial cells include N-cadherin, integrin, and a disintegrin and metalloproteinase domain 9 (ADAM9) (5, 6). ADAM9 belongs to the ADAM family of type I transmembrane proteins, which are involved in several biological processes, including neurogenesis, angiogenesis, and cell adhesion and migration (7). The disintegrin domain of ADAM9 adheres to cells by binding to integrins (8) or heparin sulfate proteoglycans (9), and the metalloproteinase domain functions in ectodomain shedding to release a variety of cell-surface proteins, including growth factors, cytokines, cell adhesion molecules, and receptors (10).

Overexpression of ADAM9 has been consistently observed in pancreatic, breast, and prostate cancers (11-13). Using an artificial overexpression system, investigators showed that ectopic expression of ADAM9 in lung cancer cells correlated with metastases to the brain through the regulation of integrin α3 and β1 (6). To further understand the mechanisms by which
ADAM9 promotes lung cancer metastatic to the brain in addition to ADAM9’s role in cancer cells’ adhesion to brain endothelial cells, we analyzed the differential gene expression between control cells and ADAM9 knockdown lung cancer cells metastatic to the brain and investigated the ADAM9-related pathways required for lung cancer metastasis to the brain. We also analyzed tissue samples from lung adenocarcinoma patients to investigate the clinical relevance of ADAM9.

Materials and Methods

Cell lines and animal model

Human lung adenocarcinoma cell lines CL1-0 (low invasiveness) and F4 (high invasiveness) from the same lung cancer origin (14) and F4-luc cells with stable expression of high levels of luciferase were established as previously described (15). Experiments were carried out within 6 months of acquiring the cells from the established cell bank to ensure that cells maintained their ability to form lung cancer tumors in SCID mice. All cell lines were tested to be free of Mycoplasma and authenticated by short tandem repeat (STR) DNA typing (Genelabs Life science, Taipei, Taiwan) in November 2013. To generate cell lines of lung cancer metastatic to the brain, parental F4-luc cells (5x10^4 cells) were injected intracardially into 8-week-old SCID mice (n=5) (BioLASCO, Taiwan) and imaged weekly by IVIS (In Vivo Imaging System) Spectrum Imaging system (Xenogen, Hopkinton, MA, USA) for one month under SPF condition. When brain metastases were detected by IVIS, the whole brain was dissected, minced, treated with collagenase type IA (0.2 mg/ml, Sigma) for 1 hr, and then washed and cultured in DME/F12 plus 10% FBS media to get clones of the lung cancer cells metastatic to the brain such as Bm2 and Bm7. Bm7 cells were injected intracardially into SCID mice again, and the same
process was repeated to verify the cells’ ability to metastasize to the brain. Bm7brm cells were obtained from the second cycle (Fig. 1 A). To avoid increasing the risk of genetic mutation during the prolonged period in which cells were generated, Bm7 cells were used as brain-metastatic cells in this study. All animal experiments were carried out under protocols approved by the Institutional Animal Care and Use Committee of China Medical University and Hospital.

**Microarray processing and survival analyses**

Three public microarray datasets were analyzed: two were downloaded from the Gene Expression Omnibus (16), including GSE8894 (61 patients in South Korea, two adenocarcinoma patients were excluded because age was unavailable) (17) and GSE11969 (90 patients in Japan) (18), and the third dataset, composed entirely of lung adenocarcinoma patients, was obtained from the Shedden et al. study (469 patients in the United States) (19). For our analysis, only lung adenocarcinoma patients from each of the datasets were examined. We expanded the number of patient for the survival curve analysis by combining the GSE8894 and GSE11969 datasets (referred to as Asia datasets). After retrieving the raw data, a quantile normalization method was performed for each dataset to remove the potential systematic biases. Survival analyses were performed based on the procedures in our previous studies (20, 21). Briefly, for each gene, samples were divided into two groups according to the following criteria: (a) a high group, in which gene expression levels of the specific gene were higher than the median in all samples, and (b) a low group, in which gene expression levels of the specific gene were lower than the median in all samples. Kaplan-Meier survival curves were used to assess the differences between these two groups. Finally, a Cox hazard regression model was used to evaluate the quantitative risk of the gene expression grouping in patients with lung adenocarcinoma.
Immunohistochemistry Staining

The primary and metastatic specimens were obtained from the files of the Department of Pathology of China Medical University Hospital in compliance with protocols approved by the CMUH IRB (DMR99-IRB-131). Group I (n=18), primary lung tumor from patient with early-stage disease without relapse for more than 4 years; Group II (n=24), primary lung tumor from patients with early-stage disease that relapsed in 1-3 years; Group III (n=21), primary lung tumor from patients with late-stage disease metastatic to brain or bone at diagnosis; Group IV (n=21), paired lung tumor metastatic to brain or bone from patients in Group III. Asian lung cancer tissue array was purchased from US Biomax (LC1006, Rockville, MD). ADAM9, CDCP1, and tPA expression in clinical specimens from lung cancer patients were detected by immunohistochemical staining as previously described (15). Briefly, goat anti-mouse ADAM9 antibody (AF949, Abcam) (13), goat anti-human CDCP1 antibody (AB1377, Abcam) and rabbit anti-human tPA (sc-5241, Santa Cruz Biotechnology) were used to perform IHC staining using horseradish peroxidase-conjugated avidin biotin complex (ABC) from the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) and AEC chromogen (Vector Laboratories). The sections were counterstained with hematoxylin and mounted. All stainings were evaluated by experienced histologists.

Results

ADAM9 expression is associated with lung cancer metastasis to the brain

In a series of established human lung cancer cell sublines metastatic to the brain (see Materials and Methods; left, Fig. 1A), ADAM9 protein expression in sublines Bm7 and Bm7brm
was higher than ADAM9 expression in the low metastatic CL1-0 and high metastatic F4 cells (Fig. 1A; right). The Bm7 cells showed stronger metastatic ability \textit{in vitro} and were more drug resistant than the F4-luc parental cells were (Supplementary Fig. S1A and B), a finding consistent with clinical observations in lung cancer patients with brain metastases (22). Furthermore, Bm7 cells maintained the luciferase activity of the original F4-luc cells for establishing an animal model of lung cancer metastatic to the brain (Fig. S1C).

We generated two Bm7 ADAM9 knockdown cell lines (shADAM9-C and shADAM9-E) by lentivirus expressing shRNAs targeting different regions of ADAM9. The expression of ADAM9 was more strongly inhibited in shADAM9-E than in shADAM9-C cells (Fig. 1B; top). Bm7 cells with ADAM9 knockdown (Bm7-shADAM9-C and -E) had a significantly weaker migration rate than shGFP control cells had (Fig. 1B; bottom) but had a similar proliferation rate to controls within 2 days (Supplementary Fig. S1D). Results from the \textit{in vitro} blood-brain-barrier assay indicated that there were more trans-migrated cells in Bm7 than in CL1-0 or F4 cells (Supplementary Fig. S1E) but the increase in trans-migrated cells was attenuated in Bm7 ADAM9 knockdown cells (Fig. 1C). However, adhesion of control and ADAM9 knockdown Bm7 cells to human brain endothelial cells (HBMEC) was not altered (Fig. 1D). While these data indicated that adhesion to HBMEC required more than ADAM9 alone, all lung cancer cells metastatic to the brain demonstrated stronger adhesion than parental F4-luc did, further validating that lung cancer cells metastatic to the brain have a high ability to adhere to brain endothelial cells.

To study the metastatic effect of ADAM9 \textit{in vivo}, control and Bm7-shADAM9 cells were introduced by intracardiac injection into SCID mice and monitored for brain metastasis, as this approach has been previously established for a preclinical metastasis model for studying
metastasis to brain or bone (23). Twenty days after injection, mice that received Bm7-shADAM9 cells had lower ability of lung cancer cells to metastasize to brain and/or bone than did mice in the control (Bm7-shGFP) group, as indicated by the loss of luciferase signals under IVIS imaging. In the Bm7-shGFP group, all mice developed tumor metastasis to brain, bone and lung whereas metastasis developed in 80% and 25% of mice in the Bm7-shADAM9-C and Bm7-shADAM9-E groups, respectively (Fig. 1E). Further histological examination of brain sections confirmed tumor formation in the brains of mice in the Bm7-shGFP group (Fig. 1F). Taken together, these data indicate that cells with higher levels of ADAM9 are also more metastatic and that depletion of ADAM9 significantly reduces cancer metastasis.

**ADAM9 enhances CDCP1 expression and activation**

While ectopic expression of ADAM9 in lung cancer cells was reported to correlate with brain metastasis through upregulation of integrin α3 and β1 (6), Bm7-shADAM9 cells did not significantly suppress surface integrin β1 compared with suppression in parental or control cells when we detected total, active, and inactive integrin β1, except for CL1-0 cells, which have low integrin β1 expression (Supplementary Fig. S2). These findings support our previous results showing that Bm7-shADAM9 cells did not affect cell adhesion (Fig. 1D). To investigate how ADAM9 affects lung cancer metastatic to the brain and to determine which genes are regulated by ADAM9 to promote metastasis, we analyzed differential gene transcription profile by comparing the control cells and ADAM9 knockdown cells in two cell sublines of lung cancer metastatic to brain, Bm2 and Bm7, as well as comparing Bm2 and Bm7 with parental F4-luc cells. Genes with more than two-fold change were considered as likely to be involved in the ADAM9-associated lung cancer metastatic to the brain. A total of 182 differentially-expressed
genes identified by comparing control with ADAM9 knockdown and brain-metastatic cell sublines with parental cells (Supplementary Table S1) was used in hierarchical clustering analysis (Fig. 2A). Using the Ingenuity Pathway Analysis (IPA) network generation algorithm to determine highly interconnected pathways representing fundamental biological functions, we noticed that ADAM9 and CUB-domain-containing protein 1 (CDCP1) were shown in one interaction network involved in cellular movement (Fig. 2B). The protein levels of ADAM9 and total CDCP1 (both full-length and cleavage) were increased in cells from low metastatic (CL1-0) to high metastatic cell lines (Fig. 2C). Notably, the cleaved CDCP1 was higher in the brain metastatic cells (Bm7) than in the F4 cells. We observed similar results (higher cleaved CDCP1) in the brain metastatic cell subline MDA-MB-231-brm (24) than in the parental MDA-MB-231 breast cancer cell line (Fig. 2C). To further determine whether the metalloproteinase activity of ADAM9 proteins affects the expression level of CDCP1, we treated lung cancer Bm7 cells with a broad-spectrum metalloproteinase inhibitor BB-94. ADAM9 expression was suppressed under BB-94 treatment (Fig. 2D). In addition, the levels of full-length CDCP1 proteins were strongly decreased at 160 μM BB-94 treatment whereas the levels of cleaved CDCP1 were slightly decreased (Fig. 2D). CDCP1 expression was slightly decreased in A549 cells treated with BB-94 (Fig. 2E). These results suggest that the metalloproteinase activity of ADAM9 mainly affects the expression of full-length CDCP1 but not of CDCP1 cleavage as the latter may be regulated by other factors.

CDCP1 is a transmembrane protein involved in cell-cell interaction and has been shown to function as a regulator of anoikis resistance (which bypasses apoptosis induced by anchorage-dependent cells detaching from the surrounding extracellular matrix) in lung adenocarcinomas (25). CDCP1 is overexpressed in leukemia, metastatic colon cancer, and breast cancer (26). De
novo expression of CDCP1 in HeLa cells dramatically increased their potential to colonize lungs, brain, and ovaries *in vivo* (27). However, the molecules that regulate CDCP1 expression and activation have not yet been fully identified. To further demonstrate that ADAM9 regulates CDCP1 expression, 293 cells with high transfection efficiency and low ADAM9 expression were used to overexpress ADAM9. Ectopic expression of ADAM9 in 293 cells increased the full-length CDCP1 but not the cleaved form; transient transfection of ADAM9 in Bm7 cells increased the full-length and cleaved CDCP1 proteins (Fig. 3A), suggesting ADAM9 increases the level of full-length CDCP1 but does not directly affect the level of CDCP1 cleavage. The full-length CDCP1 ranges from 130 to 140 kDa, which is larger than calculated size of 90 kDa due to extensive glycosylation and is cleaved by a serine protease, e.g., matriptase, plasmin, or trypsin (28, 29), through an unclear mechanism to generate a 70-kDa C-terminal fragment. Proteolysis of CDCP1 induces tyrosine phosphorylation of its C-terminal 70-kDa fragment and recruitment of Src and PKCδ to initiate signaling pathways (30). In Bm7-shGFP cells, two forms of CDCP1 proteins were detected, and the levels of the cleaved form were lower in Bm7-shADAM9-E cells (Fig. 3B). The levels of tyrosine-phosphorylated-CDCP1-interacting signaling molecules (phospho-Src and phospho-PKCδ) were also decreased in Bm7-shADAM9-E cells but only slightly affected in Bm7-shADAM9-C cells; that difference was associated with ADAM9 expression (Fig. 3B). To further determine whether ADAM9-enhanced CDCP1-interacting signaling molecules was due to the more stable cleaved CDCP1 rather than to the full-length CDCP1, the degradation rate of two forms of CDCP1 was determined. Under cycloheximide treatment to block protein neosynthesis, the levels of full-length CDCP1 decreased after 6 hours whereas the levels of cleaved CDCP1 remained high for 24 hours in control cells. The calculated degradation rate of full-length CDCP1 was 2.9% per hour whereas
almost no degradation of the cleaved form was observed, indicating that cleaved CDCP1 is more stable than the full-length form (Fig. 3C). Thus, ADAM9 likely enhances the CDCP1 signaling as more cleaved CDCP1 is present in the control cells.

Both forms of CDCP1 were tyrosine phosphorylated, an event required to overcome anoikis (25), and no difference between the phospho-CDCP1 signal and total CDCP1 proteins in control or shADAM9-E cells was observed, suggesting that both forms (full-length and cleavage) of CDCP1 are functionally active (Fig. 3D). To determine whether suppression of CDCP1 cleavage and CDCP1-interacting signaling molecules in ADAM9 knockdown cells influences the function of CDCP1 in anoikis resistance, we performed a soft agar assay and found that ADAM9 knockdown cells formed lower number of colonies than did the parental or control cells (Fig. 3E). In addition, the number of apoptotic cells was higher in the Bm7-shADAM9-E than in the control group in an anchorage-free culture condition (Fig. 3F, upper, and supplementary Fig. S3A).

To further investigate the function of the ADAM9-CDCP1 axis, we knocked down CDCP1 in Bm7 cells and found that the levels of CDCP1-interacting signaling molecules were attenuated but not those of ADAM9 (Supplementary Fig. S3B). A similar trend of increasing number of apoptotic cells in an anchorage-free culture condition was observed in CDCP1 knockdown cells (Fig. 3F, lower, and supplementary Fig. S3A and C), consistent with the known function of CDCP1 in anoikis resistance. Ectopic expression of CDCP1 in Bm7-shADAM9-E cells rescued the cells’ migration ability to a level comparable to that of Bm7-shGFP (Fig. 3G). Thus, ADAM9 increases CDCP1 expression and the function of CDCP1 in anoikis resistance and metastasis.
**ADAM9 enhances CDCP1 cleavage through tPA activation**

Several proteases, including matriptase and plasmin, have been shown to cleave CDCP1. Tissue plasminogen activator (tPA), a serine protease responsible for the degradation of blood clots by cleaving plasminogen to become plasmin, is necessary and sufficient to induce opening of the BBB that causes BBB breakdown in a plasmin-dependent or –independent manner (31, 32). No matriptase protein in Bm7 cells (Supplementary Fig. S4A) and a near background level of plasminogen RNA from transcriptome analysis in lung cancer cells were detected. Notably, from the transcriptome analysis list (Supplementary Table S1), high tPA (PLAT) expression and low PAI-1 (plasminogen activator inhibitor 1, SERPINE1) expression were observed in cancer cells metastatic to the brain (Fig. 2A). We further investigated whether ADAM9-regulated CDCP1 cleavage requires tPA activation by examining both tPA and PAI-1 expression. Since the ADAM9 levels in Bm7-shADAM9-C and -E remained high in the pooled population, we selected the stable clones with low ADAM9 expression for further experiments. The reduced levels of ADAM9 level in ADAM9 knockdown cells substantially decreased expression of full-length and cleaved CDCP1 (Fig. 4B). In addition, the mRNA and protein levels were lower for tPA and higher for PAI-1 ADAM9 knockdown stable clones than in the control cells (Fig. 4A and B). The catalytic activity of tPA for plasminogen to plasmin conversion was also suppressed in ADAM9 knockdown cells (Fig. 4C).

To investigate whether the activity of tPA correlates with ADAM9-regulated CDCP1 cleavage, tPA and PAI-1 knockdown cells (Supplementary Fig. S4B) were used to investigate the correlation of CDCP1 cleavage with tPA activity. We measured the ratio of cleaved CDCP1 over the full-length CDCP1 and found that CDCP1 cleavage was attenuated in tPA knockdown but increased in PAI-1 knockdown cells compared with control cells (Supplementary Fig. S4C).
The activity of secreted tPA in the cultured media of these cells for plasminogen to plasmin conversion was measured (Fig. 4C and Supplementary Fig. S4D), and further analysis showed a high correlation between tPA activity and the ratio of CDCP1 cleavage to full-length CDCP1 ($R = 0.89$) among these cancer cells (Fig. 4D). The percentage of apoptotic cells in an anchorage-free culture condition was increased in tPA knockdown cells over the percentage in the control cells (Fig. 4E and Supplementary Fig. S3A), which is consistent with our observation in ADAM9 and CDCP1 knockdown cells and indicates that tPA plays a role in anoikis resistance.

To test whether tPA addition could enhance CDCP1 cleavage, Bm7-shADAM9-E cells were treated with recombinant tPA proteins. In 10% serum culture media, cleaved CDCP1 was slightly increased at a higher dose of tPA treatment. Interestingly, CDCP1 cleavage in Bm7-shADAM9-E cells treated with 100 nM tPA was also enhanced to a level comparable with that in the control under serum-free conditions (Fig. 4F); this finding indicated that CDCP1 is likely a substrate of tPA.

Next, to investigate whether tPA directly cleaves CDCP1, full-length CDCP1 was isolated and treated with tPA and then analyzed by Western blotting. Plasmin and trypsin, which are known to cleave CDCP1, were included as the control. The conversion of full-length to cleaved CDCP1 was increased in a dose-dependent manner with tPA treatment similar to plasmin and trypsin treatment, indicating that tPA can directly cleave CDCP1, albeit at a high concentration (Fig. 4G). These results suggest that tPA activation mediates ADAM9-enhanced CDCP1 cleavage.

**Blocking the ADAM9-CDCP1-tPA axis in lung cancer cells prolongs animal survival time**
Next, we detected the migration rate of Bm7 cells by silencing genes at the ADAM9-CDCP1-tPA axis. We showed that control cells migrated farther and in a wider range of direction than Bm7-shADAM9, Bm7-shCDCP1, and Bm7-shtPA cells did, whereas Bm7-shPAI-1 rescued the migration ability (Fig. 5A; top). Quantitation of cellular movement confirmed that the loss of gene expression at ADAM9-CDCP1-tPA axis significantly inhibited cell migration (Fig. 5A; bottom). These cells were further investigated for their metastatic ability in animal models by intracardiac injection in mice. Kaplan-Meier analysis indicated that mice inoculated with Bm7-shADAM9 and Bm7-shtPA cells had a significantly longer overall survival than did mice inoculated with the control cells. Notably, mice in the Bm7-shCDCP1 group had only moderately prolonged survival, but mice in the Bm7-shtPA group had the longest survival time (Fig. 5B). When these cells were cultured in anchorage-free conditions, the sub-G1 percentage, which measures the apoptotic cell population, was higher in the Bm7-shtPA, moderately higher in the Bm7-shADAM9, and slightly higher in the Bm7-shCDCP1 cells than in the control cells in anchorage-free conditions (Fig. 5C). These results provide a partial explanation for the longer survival time in the Bm7-shtPA group.

Next, we examined whether targeting the ADAM9-CDCP1-tPA axis could benefit lung cancer treatment using clinically available drugs such as dexamethasone and dasatinib. Dexamethasone has been reported to inhibit CDCP1 cleavage through upregulation of PAI-1 (33). Dasatinib is a dual Src/Abl kinase inhibitor that also targets several other kinases (34) with potent anti-proliferative activity against hematologic malignancies and cancer cell metastasis (35). We found that dexamethasone enhanced PAI-1 expression in Bm7-shGFP cells and enhanced PAI-1 expression even more strongly in Bm7-shADAM9 cells and that dasatinib significantly inhibited the levels of pSFK and pPKCδ in both cells (Fig. 5D). Moreover, the two-
drug treatment combination acted synergistically in Bm7 cells and induced a dose-dependent cytotoxic effect (Fig. 5E; upper panel) (CI < 1; Fig. 5E; bottom panel). These findings suggest that blocking the ADAM9-CDCP1-tPA axis inhibits lung cancer cell growth and metastasis.

**Overexpression of ADAM9 and CDCP1 in primary lung adenocarcinoma is associated with a poor prognosis**

From above, we demonstrated that ADAM9 enhances CDCP1 activation through tPA for anchorage-free survival of cancer cells; this finding raises the question of whether activation of the ADAM9-CDCP1 axis in lung adenocarcinoma patients has clinical relevance owing to its association with poor prognosis. In the Asia datasets, patients with high ADAM9 (ADAM9\(^{\text{High}}\)) expression had significantly shorter survival times than did patients with low ADAM9 (ADAM9\(^{\text{Low}}\)) expression (p = 0.0008). Similar results were observed in patients with high CDCP1 (CDCP1\(^{\text{High}}\)) expression, who also had significantly shorter survival times than did those with low CDCP1 (CDCP1\(^{\text{Low}}\)) expression (p = 0.015; Fig. 6A; top). Conversely, in the Western datasets (Shedden), ADAM9 expression level in patients did not correlate with patient prognosis (p = 0.174), whereas patients with CDCP1\(^{\text{High}}\) expression had significantly shorter survival times than did those with CDCP1\(^{\text{Low}}\) expression (p = 0.0002; Fig. 6A; bottom). However, when we combined ADAM9 and CDCP1 as a predictor, patients with ADAM9\(^{\text{High}}\)/CDCP1\(^{\text{High}}\) expression had a significantly worse prognosis than did patients with ADAM9\(^{\text{Low}}\)/CDCP1\(^{\text{Low}}\) in the Asia and Western datasets (Fig. 6A).

Cox hazard regression model analysis also showed that patients with ADAM9\(^{\text{High}}\)/CDCP1\(^{\text{High}}\) expression have a significantly higher risk of lung cancer mortality than do patients with ADAM9\(^{\text{Low}}\)/CDCP1\(^{\text{Low}}\) expression in both the Asia and Western datasets.
(hazard ratios of 5.97 and 1.80, respectively; Fig. 6B). To rule out statistical bias as the reason for these findings, we compared the predictability of ADAM9 versus CDCP1, and selected the more predictive of these two genes; the selected gene in random combination with one of other genes was then used to generate a profile that illustrates the predictability for the different pairs of genes. This gene predictability profile indicated that the predictor of ADAM9 plus CDCP1 ranked among the top 3.3% (GSE8894), 7.9% (GSE11969), and 18.6% (Shedden datasets) of random combination (Figure 6C). A number that is less than 20% indicated that our finding in prognosis analysis is not from statistical bias; thus, lung adenocarcinoma patients with ADAM9\textsuperscript{High}/CDCP1\textsuperscript{High} expression indeed had a higher risk of mortality than those with ADAM9\textsuperscript{Low}/CDCP1\textsuperscript{Low} expression. Moreover, Fisher’s exact test indicated that the percentage of patients with high expression of tPA was significantly higher (67%) in the ADAM9\textsuperscript{High}/CDCP1\textsuperscript{High} group than those in the ADAM9\textsuperscript{Low}/CDCP1\textsuperscript{Low} group in the Asia datasets (37%) (Fig. 6D) and Western (Fig. 6E) datasets (37%) (p < 0.05). These results support the clinical relevance of ADAM9\textsuperscript{High}/CDCP1\textsuperscript{High} in patients via its association with poor prognosis and imply that ADAM9\textsuperscript{High}/CDCP1\textsuperscript{High} expression in tumors is associated with cancer metastasis.

**High expression of ADAM9, CDCP1, and tPA in metastatic lung adenocarcinoma**

To assess the clinical relevance of ADAM9 protein expression in lung cancer patients, we performed ADAM9 immunohistochemical (IHC) staining in a lung cancer tissue array from the Asian patients. ADAM9 expression was high in lung adenocarcinoma tissue but remained low expression in matched adjacent normal lung tissue (p = 0.012; Fig. 7A). Most of the lung lesions metastatic to the brain had elevated ADAM9 mRNA expression compared with expression in bone metastatic lesions or normal lung tissues (p = 0.01; Fig. 7B). To evaluate whether activation
of the ADAM9-CDCP1 axis contributes to lung cancer metastasis, we performed IHC staining of four groups of clinical paraffin block tissue specimens from lung adenocarcinoma patients at the China Medical University Hospital (see Materials and Methods). Comparison of ADAM9 expression in these specimens showed that the rate of positive ADAM9 detection increased almost two-fold in Groups II and III (54% and 52%) and reached a three-fold increase in Group IV (81%) compared with Group I (28%; Fig. 7C). The rate of positive CDCP1 detection remained high in all four groups (from 67% to 71%) (Fig. 7C). The rate of positive tPA detection increased from 22% to 81% (Fig. 7C). The distribution of ADAM9 staining significantly correlated with the tPA staining results ($p = 0.034$, Fig. 7D). On comparing the IHC staining scores in paired tumor specimens (primary and metastatic) from the same patient, the trend toward higher ADAM9 expression in metastatic tumor tissue was significantly increased only in patients with brain metastasis ($p = 0.03$) but not in patients with bone metastasis ($p = 0.53$, Fig. 7E). The trend was absent in CDCP1 or tPA staining in patients with metastasis to brain or bone (Fig. 7E). By setting the IHC score $\geq 1+$ as positive staining, the triple-gene-positive (ADAM9-CDCP1-tPA positive staining) detection rate was low in patients without metastasis (Group I) but increased in patients with metastasis (Groups II and III) and even reached 50% in tumor specimens metastatic to brain or bone (Group IV; Fig. 7F). In contrast, the triple-gene-negative (ADAM9-CDCP1-tPA negative staining) detection rate decreased from 22% in the non-metastatic group to 5% in the metastatic groups and even to zero in patients with tumor metastatic to brain or bone. Taken together, these clinical results indicate that activation of the ADAM9-CDCP1-tPA axis contributes to lung cancer metastasis, especially to the brain.

**Discussion**
Here, we address the potential implications of ADAM9 in lung cancer metastatic to the brain and reveal a novel connection between ADAM9 and CDCP1 by which ADAM9 increases the cleavage form of CDCP1 through activation of tPA by enhancing tPA and suppressing PAI-1 expression. The contribution of the ADAM9-CDCP1-tPA axis to lung cancer metastasis was validated in the cell model, the animal model and clinical samples. On the basis of our results, blocking this axis in cancer cells is expected to provide a therapeutic benefit, as knockdown of each one of the genes in the axis reduced cancer cell migration and prolonged survival time in mouse tumor models. In addition, targeting tPA activity and tyrosine-phosphorylated-CDCP1-interacting signaling molecules, phospho-Src with dexamethasone and dasatinib showed a synergistic cytotoxic effect in lung cancer cells.

Although several studies have focused on ADAM9 as a new anti-cancer drug target (36), clinical evidence of ADAM9 expression in lung cancer is still lacking (6). Our analysis of three lung cancer datasets and clinical samples suggested that ADAM9 alone is a good prognostic marker in the Asia datasets but not in the U.S. datasets. However, the combination of ADAM9 and CDCP1 appears to be a better prognostic marker for patient outcome, implying these genes play important roles in cancer metastasis. Such clinical evidence and results of our study do not indicate that ADAM9 is related specifically to metastases to the brain. Rather, they support that ADAM9 enhances the ability of cancer cells to establish distant metastases such as to the brain. By investigating the paired clinical tumor specimens from the tumor’s primary site and distant site, albeit in limited samples, we found that ADAM9 protein expression was significantly higher in lung tumor tissue metastatic to the brain than in the primary lung tumor but was not higher in lung tumor tissue metastatic to bone. While high levels of ADAM9 in cancer cells are essential for metastasis to the brain, these levels may not be specific to or only limited to the brain. In
addition, enhanced transmigration of the BBB by cancer cells appears to be a result of the enhanced expression of ADAM9 that triggers the activity of tPA.

The catalytic unit of tPA is known to function autonomously (37), and there is no report showing ADAM9 cleaves tPA. While we did not provide sufficient evidence to demonstrate the role of ADAM9 protease activity in regulating the CDCP1-tPA axis, knocking down ADAM9 not only downregulated the transcription of tPA but also enhanced transcription of PAI-1, which suppressed the catalytic activity of tPA for plasminogen conversion to plasmin (Fig. 4C). This suggests that ADAM9 indirectly regulates the tPA activity.

The mechanism by which ADAM9 increases the expression of CDCP1 is still unclear. Heparin-binding EGF-like growth factor (HB-EGF), a member of EGF family, is overexpressed and associated with carcinogenesis in hepatoma and pancreatic cancer (38, 39). The transmembrane domain of HB-EGF (pro-HB-EGF) can be cleaved by metalloproteases such as ADAM9, yielding a secreted form that activates EGFR (40). Activation of EGFR signaling has been reported to upregulate CDCP1 mRNA and protein expression (41). Interestingly, we also observed increased mRNA levels of HB-EGF in the brain-metastatic Bm7 cells compared with parental cells. Therefore, it is plausible that ADAM9 increases the expression of CDCP1 via ADAM9-dependent shedding of HB-EGF, leading to EGFR activation and subsequent upregulation of CDCP1 protein and mRNA expression. In addition, EGF has also been reported to increase tPA mRNA in HeLa cells (42) and enhance PAI-1 transcription via sequential activation of c-Src and PKCζ in glioma cells (43). However, we observed downregulation of tPA and upregulation of of PAI-1 in ADAM9 knockdown cells, suggesting other molecules besides EGF are involved in their regulation. PAI-1 transcription can be regulated by many transcription factors such as hypoxia-inducible factor-1α (HIF-1α) and early growth response protein 1.
(EGR1). Although EGR1 is quickly activated under hypoxia, it can induce transcription and expression of PAI-1 independently of HIF-1α in murine macrophages (44). From the transcriptome analysis (Supplementary Table S1), we notice that EGR1 mRNA levels were 10-fold higher in ADAM9 knockdown cells than in control cells, suggesting that ADAM9 may influence expression of PAI-1 through activation of EGR1, although more studies will be required to validate this.

The biological behavior and function of PAI-1 seems paradoxical. PAI-1 is thought to inhibit activation of tPA or uPA, an event that correlates with poor clinical outcomes in cancer (45, 46); however PAI-1 also serves as an indicator of poor prognosis in breast cancer (47). These findings may be attributed to differences in cell types and experimental models. Our data indicate that PAI-1 knockdown increases tPA activity, which results in more CDCP1 cleavage and cell migration ability, implying that PAI-1 inhibits lung cancer metastasis. More studies are warranted to clarify the role of PAI-1 in metastasis.

Here, our findings indicate that ADAM9 regulates a complicated network in lung cancer metastatic to the brain through tPA-mediated CDCP1 cleavage, especially in Asian patients, and establish a potentially important association for predicting or preventing cancer metastasis to the brain.

Disclosure of Potential Conflicts of Interest

All authors have no conflicts of interest.

Authors' Contributions

Conception and design: Y.P. Sher, M.C. Hung
Development of methodology: Y.P. Sher

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.Y. Lin, H.J. Chen, G.C. Tseng

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.C. Huang, L.C. Liang, T.P. Lu, T.T. Kuo, Q.Y. Kuok

Writing, review, and/or revision of the manuscript: C.Y. Lin, J.L. Hsu, M.C. Hung, Y.P. Sher

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.Y. Sung

Study supervision: Y.P. Sher

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References


46. Borgfeldt C, Bendahl PO, Ferno M, Casslen B. High preoperative plasma concentration of tissue plasminogen activator (tPA) is an independent marker for shorter overall survival in patients with ovarian cancer. Gynecol Oncol 2003;91:112-7.


Figure Legends

Figure 1. Knockdown of ADAM9 decreases lung cancer metastatic to the brain. A, flowchart for establishing cell sublines of lung cancer metastatic to the brain. F4-luc cells were injected intracardially into SCID mice and cultured from dissected brain tissue. The cycle was repeated twice to establish the brain-metastatic sublines Bm7 and Bm7brm. Western blot of ADAM9
expression is shown. ADAM9 proteins were detected in non-reducing gels using the antibody from R&D (MAB939, R&D Systems). B, migration ability of ADAM9 knockdown (Bm7-shADAM9) and control (Bm7-shGFP) cells was evaluated by wound-healing assay. Western blot analysis of ADAM9 protein expression is shown at the top. EF1α served as protein-loading control. C, trans-BBB migration assay of ADAM9 knockdown and control cells in vitro. Top, the images of trans-migrated cells. Bottom, quantitation of the number of trans-migrated cells from three independent experiments. *p < 0.05 and ** p < 0.01. D, relative binding activity of each indicated cell line to human brain endothelial cells (HBMEC) at 0.5 hr and 2 hr. The binding activity of Bm7-shGFP at 2 hr was set as 100% for comparison between cell lines. ** p < 0.01. E, Bm7-shGFP, Bm7-shADAM9-C, or Bm7-shADAM9-E cells were injected intracardially into SCID mice and monitored under IVIS detection. The number of mice in which the result was detected out of the total mice in each group is shown in parenthesis. F, the metastatic lung tumor from the brain of mice bearing Bm7-shGFP cells was confirmed by pathological hematoxylin and eosin staining.

Figure 2. ADAM9-regulated genes and associated networks in lung cancer cells metastatic to brain identified from transcriptome microarray analysis. A, hierarchical clustering of the 182 differentially expressed genes by comparing control versus ADAM9 knockdown and brain-metastatic cell sublines (Bm2 and Bm7) versus parental lung cancer cells. Red indicates genes that were upregulated; green indicates downregulated genes. B, Ingenuity Pathway Analysis (IPA) showing the gene network in cellular movement, molecular transport, and neurological disease. C, Western blot analysis of ADAM9 and CDCP1 in parental cells and brain-metastatic cell sublines (left, lung cancer cells; right, breast cancer cells). The numbers below the middle
panel represent the levels of full-length and cleaved CDCP1 in F4 and Bm7 cells relative to CL1-0 cells. D, Western blot analysis of ADAM9 and CDCP1 in Bm7 cells treated with BB-94, a broad-spectrum inhibitor of metalloproteinases. The numbers below the top panel represent the levels of ADAM9 expression in BB-94-treated relative to DMSO-treated Bm7 cells. The numbers below the middle panel represent the levels of full-length and cleaved CDCP1 in BB-94-treated relative to DMSO-treated Bm7 cells. E, Western blot analysis of ADAM9 and CDCP1 in A549 cells treated with BB-94. The numbers below the top panel represent the levels of ADAM9 expression in BB-94-treated relative to DMSO-treated A549 cells. The numbers below the middle panel represent the levels of full-length and cleaved CDCP1 in BB-94-treated relative to DMSO-treated A549 cells. Western blots shown in panels C, D, and E were probed with anti-ADAM9 antibody (#2099, Cell Signaling) under reducing conditions. EF1α served as protein-loading control.

Figure 3. ADAM9 enhances cleaved CDCP1 protein level and the function of CDCP1 in anoikis resistance. A, Western blot analysis of ADAM9 and CDCP1 in 293 cells transfected with ADAM9 expression or control vector. B, Western blot analysis of expression of CDCP1 and its downstream proteins in control and ADAM9 knockdown cells. P/T indicates the quantified ratio of phosphorylated proteins to total proteins. ADAM9 proteins were detected in non-reducing gels using the antibody from R&D (MAB939, R&D Systems). C, protein stability of ADAM9 and CDCP1 in ADAM9 knockdown cells treated with cycloheximide at different incubation times was analyzed by Western blot (top) and quantified (bottom). The intensity of the band at 0 hr was used as a control (100%). D, detection of CDCP1 phosphotyrosine levels. Total CDCP1 proteins were immunoprecipitated from control and ADAM9 knockdown cells and detected
using anti-phosphotyrosine antibody. E, colony formation of ADAM9 knockdown cells by soft agar assay. Representative results showing the number of colonies (each one > 200 μm in diameter) from stained soft agar plates after 4 weeks’ growth. F, the percentage of apoptosis in Bm7-shGFP, Bm7-shADAM9E, Bm7-shCDCP1-A1, and Bm7-shCDCP1-B2 cells was determined by flow cytometry using Annexin-V (high) and PI staining (low) under anchorage-free culture conditions at different time points. Each experiment was repeated independently and showed a similar trend. Raw data are shown in Fig. S3A. G, plasmid mixture of CDCP1 and GFP (molar ratios = 5:1) was transiently transfected into Bm7-shADAM9-E cells. After 24 hr, the 16 hr-migration distance (motile activity) of Bm7-shADAM9-E cells was measured by time-lapse video microscopy in each group (left) and quantified (right). Transfected Bm7-shADAM9-E cells with high GFP signal represents CDCP1-transfected group whereas low GFP signal represents control cells. Error bars, SD; **, p < 0.01. Western blot analysis of CDCP1 is shown at the top.

Figure 4. The tPA activity correlates with conversion of full-length CDCP1 to the cleavage form. A, RT-qPCR of tPA (top) and PAI-1 (bottom) in control and ADAM9 knockdown cells. B, Western blot of ADAM9, CDCP1, tPA, and PAI-1 expression in control and ADAM9 knockdown cells. C, activity of tPA in different conditioned media from the indicated cell cultures. D, activity of tPA versus the ratio of CDCP1 cleavage over the full-length form; R = 0.89. E, the percentage of apoptotic cells was determined by flow cytometry under anchorage-free culture conditions at different time points. Raw data are shown in Supplementary Fig. S3A. F, Bm7-shADAM9-E cells were cultured with or without serum and treated with different concentrations of tPA (Boehringer Ingelheim, Ingelheim, Germany). The numbers below the
CDCP1 blot represent the levels of CDCP1 cleavage in cells treated with tPA treatment relative to cells without tPA treatment in serum-containing or -free culture condition. G, Western blot analysis of immunoprecipitated CDCP1 proteins in ADAM9 knockdown stable cells treated with indicated proteases (top). The numbers below the panel represent the ratio of CDCP1 cleavage over full-length and were plotted against various concentrations of protease treatment (bottom). Stable clones of ADAM9 knockdown cells were used instead of the pooled population.

Figure 5. Blocking the ADAM9-CDCP1-tPA axis in lung cancer cells prolongs animal survival time. A, the migration distance (motile activity) for ADAM9, CDCP1, tPA and PAI-1 knockdown cells was measured by time-lapse video microscopy (top), and quantified (bottom). Error bars, SD from three independent experiments; *, p < 0.05; **, p < 0.01. B, Bm7 cells with indicated shRNA knockdown were intracardially injected into SCID mice and mice survival time was monitored for 50 days. The number (n) of mice shown in each group was from two independent experiments. C, time-course analysis of sub-G1 fraction from the cell cycle. Knockdown cells as indicated were cultured in anchorage-free conditions and subjected to cell cycle analysis. Sub-G1 fraction values at 0, 24, and 48 h are shown. D, Western blot analysis of indicated proteins in Bm7 cells after dexamethasone (Dexa) or dasatinib (Dasa) treatment for 24 hr. Media-alone treatment served as negative control. E, cytotoxic effects of dexamethasone and/or dasatinib after 72-hr treatment. Dose-dependent cytotoxic fraction (top); combination index (CI) summary (bottom) at 50, 75, and 90% inhibition (ED50, ED75, and ED90) for two cell lines treatments with a combination of dexamethasone and dasatinib are shown. A CI value < 1 indicates synergism.
Figure 6. High expression of ADAM9 and CDCP1 in primary tumors predicts mortality in lung adenocarcinoma patients. A, Kaplan-Meier survival analyses of lung adenocarcinoma patients in the indicated groups calculated by dividing the lung adenocarcinoma patients into different groups via the gene expression level in three independent datasets. H indicates high expression and L indicates low expression above or below the median, respectively. DH, SH, and DL indicate dual-high, single-high, and dual-low expression of ADAM9 and CDCP1, respectively. The number of patients (n) in each group is shown. B, Cox proportional hazard regression analyses of overall survival of lung adenocarcinoma patients with ADAM9 or CDCP1 expression. C, ranking of the p value of ADAM9 plus CDCP1 among the null p-value baseline obtained by combining a fixed predictor with the other genes randomly selected from the original gene pool. Fixed predictor: ADAM9 in the GSE11969 dataset and CDCP1 in the GSE8894 and Shedden datasets. p value of ADAM9\textsuperscript{High}/CDCP1\textsuperscript{High} versus ADAM9\textsuperscript{Low}/CDCP1\textsuperscript{Low} is marked by the red line. D, comparison of tPA expression in DH, SH, and DL patient groups in combined GSE8894 and GSE11969 datasets using Fisher’s exact test. E, comparison of tPA expression in DH, SH, and DL patient groups in the Shedden dataset using Fisher’s exact test.

Figure 7. Detection of ADAM9, CDCP1, and tPA protein expression in human lung cancer specimens. A, IHC analysis of ADAM9 in lung cancer tissue array scored by staining intensity from 0 to 3+ (0, negative; 1, weak; 2, moderate; 3, strong) by histologists. Matched lung adenocarcinoma tissue and adjacent normal lung tissue from the same patients were analyzed for the distribution of ADAM9 staining by the McNemar method. Representative staining for ADAM9 is shown below the table. B, RT-qPCR analysis of ADAM9 mRNA expression in lung cancer cells and metastatic lung cancer specimens from different patients. HPRT was served as
internal control. Abbreviations: N, normal part; mets, metastatic tumor. C, IHC analysis of ADAM9/CDCP1/tPA expression in 84 clinical paraffin block specimens from four groups of lung adenocarcinoma patients. A score > 1+ indicates positive detection. D, correlation of ADAM9 and tPA expression in all 84 clinical paraffin block specimens. E, Trend-change plots of staining scores in paired primary lung (Group III) and metastatic lung tumors (Group IV). Each line indicates the trend change between a primary and metastatic lung tumor. Red: increasing trend; blue: decreasing trend; black: no changes in the trend; p value was calculated by Wilcoxon Signed Ranks Test. F, the indicated detection rate of ADAM9/CDCP1/tPA in all four groups as defined in (C).
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Table 1: Liver cancer tissue array (AR2).

<table>
<thead>
<tr>
<th>Tumor</th>
<th>( \leq 1^+ )</th>
<th>( \geq 2^+ )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
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<td>10 (59%)</td>
</tr>
<tr>
<td></td>
<td>1 (6%)</td>
<td>1 (6%)</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>11</td>
</tr>
</tbody>
</table>

\( P = 0.012 \) (McNemar, two-tailed)

![High ADAM9 stain](image1)
![Low ADAM9 stain](image2)

Figure 7.
ADAM9 promotes lung cancer metastases to brain by a plasminogen activator-based pathway


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