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FAST TRACK

ADAM28 is overexpressed in human non-small cell lung carcinomas and correlates with cell proliferation and lymph node metastasis

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ADAM (a disintegrin and metalloproteinases) are a recently discovered gene family of proteins with sequence similarity to the reprolysin family of snake venom metalloproteinases, and about one-third of the family members have the catalytic site consensus sequence in their metalloproteinase domains. We screened the mRNA expression of 11 different ADAM species with putative metalloproteinase activity in human non-small cell lung carcinomas by RT-PCR, and found that prototype membrane-anchored ADAM28 (ADAM28m) and secreted ADAM28 (ADAM28s) are predominantly expressed in the carcinoma tissues. Real-time quantitative PCR demonstrated that the expression levels of ADAM28m and ADAM28s are significantly 16.8-fold and 9.0-fold higher in the carcinomas than in the non-carcinoma tissues, respectively. In addition, the expression levels of ADAM28m and ADAM28s were significantly higher in the carcinomas with >30 mm in diameter than in those ≦30 mm. The expression levels were also significantly higher in the carcinomas with lymph node metastasis than in those without metastasis. MIB1-positive cell index of the carcinomas had a direct correlation with the expression levels of ADAM28m and ADAM28s ($r = 0.667$, $p < 0.001$ and $r = 0.535$, $p < 0.01$, respectively). *In situ* hybridization and immunohistochemistry demonstrated that ADAM28 is expressed predominantly in the carcinoma cells. Immunoblot analysis showed the activated form of ADAM28 in the carcinoma tissues. These data demonstrate for the first time that ADAM28 is overexpressed and activated in human non-small cell lung carcinomas, and suggest the possibility that ADAM28 plays a role in cell proliferation and progression of the human lung carcinomas.

Key words: ADAM; MMP; lung cancer; proliferation; metastasis

ADAM (a disintegrin and metalloproteinases) are a gene family that have significant sequence similarity to the reprolysin/adamalysin family of snake venom metalloproteinases.[1] ADAM are composed of several domains including propeptide, metalloproteinase, disintegrin, cysteine-rich, epidermal growth factor (EGF)-like, transmembrane and cytoplasmic tail domains. More than 30 members of the ADAM gene family have been identified in a variety of animal species (see http://www.people.virginia.edu/ jw7g/Table_of_the_ADAMs.html). Although the specific biological functions of ADAM are not clear, they may be involved in shedding of various membrane-anchored receptors and proteins, degradation of extracellular matrix (ECM), and cell adhesion and migration.[2,3] About one-third of the ADAM members have the catalytic consensus sequence (HEXGHXXGXXHD) in their metalloproteinase domains, and are predicted to have catalytic activity. ADAM10 is reported to digest myelin basic protein[4] and type IV collagen.[5] ADAM9 degrades insulin B chain,[6] and ADAM28 cleaves myelin basic protein[7] and insulin-like growth factor binding protein-3 (IGFBP-3).[8] In addition, the precursor of tumor necrosis factor-α (proTNF-α) is processed to the mature form by ADAM10 and ADAM17.[9] CD23 is shed by ADAM8, ADAM15 and ADAM28.[10] Furthermore, ADAM9 and ADAM12 are involved in shedding of heparin binding-EGF (HB-EGF).[11,12] We have reported recently that in human glioblastomas, ADAM[12] plays a key role in glioma proliferation through shedding of HB-EGF.[13] These data on the proteinase-type ADAM species suggest that they may be implicated in tumor cell growth and progression through processing of cytokines and growth factors, degradation of various proteins including ECM, or modulation of cell-cell interactions. Only limited information about the expression and localization of ADAM in human cancer tissues is available so far.

Lung carcinoma is one of the most common cancers and a major cause of mortality in many industrialized countries.[14] Non-small cell lung carcinoma represents about 80% of all lung cancers, and survival rate of the patients with non-small cell lung carcinoma has not been improved significantly in the past 2 decades mainly because of the invasion and metastases.[15] Accumulated lines of evidence have indicated that matrix metalloproteinases (MMP), especially membrane type-1 MMP (MT1-MMP) and MMP-2, play a central role in invasion and metastases of various human carcinomas including non-small cell lung carcinomas through degradation of ECM macromolecules such as type IV collagen.[16,17] Catalytic site of the metalloproteinase domains of MMP and ADAM has high sequence homology, although overall homology between the 2 families is <10%. It is expected that like MMP, ADAM are involved in the invasion and metastases of human lung carcinomas, if they are expressed and activated within the carcinoma tissues.

We examined the mRNA expression of 11 different ADAM with putative metalloproteinase activity in non-small cell lung carcinomas, correlation of prototype membrane-anchored and secreted short forms of ADAM28 (ADAM28m and ADAM28s, respectively) with clinicopathological factors, their tissue localization by *in situ* hybridization and immunohistochemistry and protein bands by immunoblotting. Our results demonstrate that ADAM28m and ADAM28s are overexpressed selectively in the non-small cell lung carcinomas, and suggest the possibility that ADAM28 is involved in cell proliferation and progression of the lung carcinomas.

Material and methods

Tissue samples and histology

Fresh tissue samples were obtained from 36 patients with non-small cell lung carcinoma who underwent surgery in the Keio University Hospital, Tokyo, Japan. Ages of the patients with carcinoma ranged from 43–88 year of age (65.3 ± 9.4 years old; mean ± SD). Carcinoma tissues (36 cases) and non-carcinoma lung tissues (16 cases) remote from the tumor were divided into 2 parts,

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TABLE I – CHARACTERISTICS OF PATIENTS AND RELATIONSHIPS BETWEEN ADAM28 EXPRESSION AND CLINICOPATHOLOGICAL FACTORS

Characteristics	No. of patients (n = 36)	ADAM28m (mean ± SD)	p	ADAM28s (mean ± SD)	p
Gender					
Male	22	0.75 ± 2.20		1.25 ± 2.02	
Female	14	0.98 ± 2.07	0.845	3.63 ± 6.71	0.626
Histological type					
Squamous cell carcinoma	19	1.02 ± 2.68		2.52 ± 5.10	
Adenocarcinoma	17	0.64 ± 1.30	0.084	1.78 ± 3.83	0.739
Tumor cell differentiation					
Well differentiated	7	1.22 ± 1.95		3.49 ± 5.75	
Moderately differentiated	23	0.86 ± 2.45		2.18 ± 4.73	
Poorly differentiated	6	0.33 ± 0.22	0.065	0.61 ± 0.57	0.564
Tumor size					
≤ 30 mm	17	0.18 ± 0.28		0.56 ± 0.72	
> 30 mm	19	1.43 ± 2.81	0.027[1]	3.39 ± 5.93	0.018[1]
Lymph node status					
N0	20	0.19 ± 0.25		0.47 ± 0.63	
N+	16	1.65 ± 3.02	0.021[1]	4.29 ± 6.23	0.001[1]

[1]Significant difference.

TABLE II – OLIGONUCLEOTIDE PRIMERS USED IN REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTIONS

	Primer sequences (Forward and reverse primers)	PCR product size (bp)
ADAM9	GCTGTCTTGCCACAGACCCGGTATGTGGAG AGGGGCGTTGGCGAGGCACACCGACTGCGG	604
ADAM10	ATCCAGTCATGTTAAAGCGATTGATACAATTTAC TCCAAAGTTATGTCCAACTTCGTGAGCAAAAGTAA	434
ADAM12	GAGACCCTCAAGGCAACTAAGTATGTGGAG CGGCAGGTTAAAACAGGCACACCCCCATTCC	627
ADAM15	CTGGGACAGCGCCACATTCGCCGGAGGCGG TCCGCAGAAAGCAGCCATAGGGGGTAGGCT	688
ADAM17	AGAGCTGACCCAGATCCCATGAAGAACACG GCGTTCTTGAAAACACTCCTGGCCTTACT	777
ADAM19	TGTGGGAAGATCCAGTGTCA AGAGCTGAGGGCTTGAGTTG	500
ADAM20	AAAATAGCACACCAGATGGAGTTGCAATTG ATTCCCACAGTACTTCAGTCTAAATATATT	702
ADAM21	TCTGGCTTGGGGTATTTTTG TTGGCGTGCTACTTCCTTCT	500
ADAM28m	GCTGTGATGCTAAGACATGT TGAACAGCCTTTACCATCTG	871
ADAM28s	GCTGTGATGCTAAGACATGT GTTTATGATCTTAGTAGGGTTGCC	544
ADAM30	AACCAGGTGCCAACTGTAGC CCCATGGGTTTCATGGATAG	496
β-actin	TGACGGGGTCACCCACACTGTGCCCATCTA CTAGAAGCATTTGCGGTGGACGATGGAGGG	661

which were subjected to histopathological diagnosis and experiments for RNA extraction and immunoblotting. Surgical specimens were fixed in 4% buffered formalin and paraffin sections were stained with hematoxylin and eosin. Histological typing of the tumors was carried out according to the WHO classification,[18] and pathological stages determined by the current TNM classification.[19] All 36 cases of carcinomas were non-small cell lung carcinomas, which consisted of squamous cell carcinomas (19 cases) and adenocarcinomas (17 cases). The profiles of the 36 patients are summarized in Table I. Half of each fresh tissue sample was snap-frozen in liquid nitrogen immediately after surgery and stored at −80°C before used for extraction of RNA and proteins. None of the patients had been subjected to chemotherapy or radiation therapy before surgery. For the experimental use of the surgical specimens, informed consent was obtained from the patients according to the hospital ethical guidelines.

RNA extraction and RT-PCR

Total RNA was extracted from the tissues by Isogen (Nippon Gene CO. LTD., Toyama, Japan), and evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) according to the manufacturer's protocol. The tissue samples, the 28S/18S ribosomal RNA ratios of which was larger than 1.0, were used for further study. RNA (2 μg) was reverse transcribed with SuperScript II (Life Technologies Inc., Rockville, MD) using a random oligonucleotide hexamer (Takara Bio Inc., Shiga, Japan). Reverse transcription (RT) reaction was carried out at 42°C for 50 min, followed by heating at 70°C for 15 min. cDNA was amplified by PCR with primers specific for ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, ADAM19, ADAM20, ADAM21, ADAM28 (ADAM28m and ADAM28s), ADAM30 and housekeeping gene β-actin. The sequences of oligonucleotides used in PCR are shown in Table II. Each reaction mixture (20 μl) contained 800 nM of each primer, 220 μM of dNTP, 1 U of ExTaq DNA polymerase (Takara Bio Inc.) and 0.5 μl of cDNA solution. PCR amplification was carried out after predenaturing at 94°C for 2 min on a thermal cycler by running 30 cycles under the following condition: denaturation for 1 min at 94°C; annealing for 1 min at 62°C (ADAM9, ADAM10, ADAM12, ADAM17, ADAM19, ADAM20, ADAM21, ADAM28m, ADAM28s and ADAM30) or 67°C (ADAM 15); and extension for 3 min at 72°C. As for positive controls, cDNA prepared from a human lung adenocarcinoma cell line, A549 cells, was used for ADAM9, ADAM10, ADAM12, ADAM15, ADAM17,

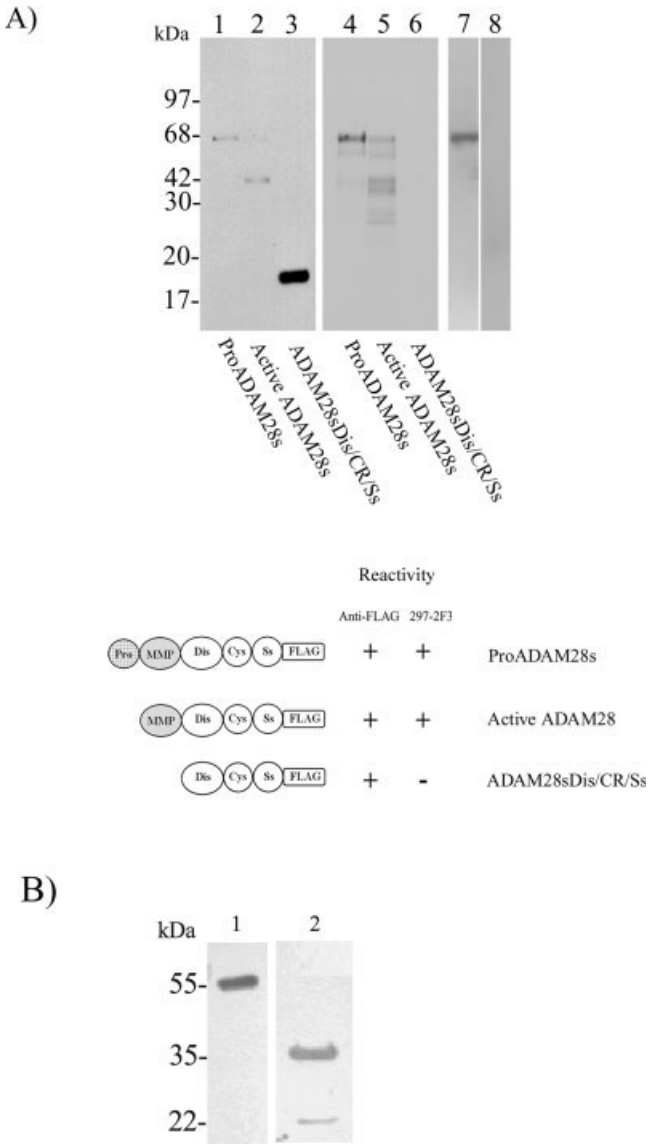

FIGURE 1 – Characterization of monoclonal and polyclonal anti-ADAM28 antibodies by immunoblotting. (*a*) Proteins of proADAM28s (*lanes 1*,*4*), active ADAM28s (*lanes 2*,*5*) and ADAM28sDis/CR/Ss (*lanes 3*,*6*) were subjected to SDS-PAGE, and immunoblotting was carried out with anti-FLAG antibody (*lanes 1–3*) and anti-ADAM28 antibody (297-2F3) (*lanes 4–6*) as described in Material and Methods. Note that anti-ADAM28 antibody detects the bands of 65-kDa proADAM28s (*lane 4*) and ADAM28s forms including 42-kDa active ADAM28s (*lane 5*), but not ADAM28sDis/CR/Ss (*lane 6*). A single band of 65-kDa proADAM28s is selectively detected in the media of Sf9 cells infected with baculovirus particles encoding proADAM28s with the 297-2F3 antibody (*lane 7*) but not with non-immune IgG (*lane 8*). The reactivity of the antibody is illustrated. (*b*) Immunoblotting data of sheep anti-ADAM28 polyclonal antibodies. The antibodies recognize the 55-kDa ADAM28 fragment of the propeptide and catalytic domains in the inclusion bodies (*lane 1*) and 35-kDa and 22-kDa fragments (*lane 2*) in the culture media of Chinese hamster ovary cell transfectants (*lane 1*).

ADAM19, ADAM20, ADAM21, ADAM28m and ADAM28s, and that from SVGp12 human fetal glial cells was used for ADAM30, because our preliminary study showed that these human cell lines expressed the corresponding ADAM species. PCR was also undertaken using non-reverse transcribed RNA as a substrate to confirm the absence of PCR amplicons and genomic DNA (negative control). After amplification, PCR products were separated by 2% agarose gel electrophoresis and visualized by UV light. The specific PCR amplification from the target mRNA was confirmed by sequencing the PCR products using DYEnamic ET terminator cycle sequencing kit and MegaBACE 1000 sequencer (Amersham Bioscience Corp., Piscataway, NJ).

Real-time quantitative PCR

For quantitative analysis of ADAM28m and ADAM28s expression, cDNA was used as template in a TaqMan real-time PCR assay (ABI Prism7000 Sequence Detection System; Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Cycling conditions were 50°C for 2 min, 95°C for 10 min, and then 40 cycles at 95°C for 15 sec and 60°C for 1 min. The primers and TaqMan probe for ADAM28m and ADAM28s were chosen with the assistance of the computer programs Primer Express (Applied Biosystems) as follows: forward primer 5′-CCAGTGTCAAGTGAGGAAGGA-3′, reverse primer 5′-AACAGCACCCCAACCACAAT-3′ and TaqMan probe FAM-ACTGCGTGACTCCTCAGTGGTCTTCCA-TAMRA for ADAM28m; forward primer 5′-AGCAACCCTAAAACCAACCTCA-3′, reverse primer 5′-CCTTTCCCCTGTGCATGTG-3′ and TaqMan probe FAM-AACCTTTCCATGATTTTGAGTTCGAGCT-TAMRA for ADAM28s. Sample data were normalized by 18S ribosomal RNA, which was selected as endogenous control using TaqMan Human Endogenous Control Plate (Applied Biosystems) according to the manufacturer's protocols. The total gene specificity of the nucleotide sequences chosen for the primers and probe and the absence of DNA polymorphisms were ascertained by BLASTN and Entrez on web sites (http://www.ncbi.nlm.nih.gov/).

In situ *hybridization*

To identify the cells expressing ADAM28 mRNA, the carcinoma samples that showed ADAM28 expression by RT-PCR were used for *in situ* hybridization. *In situ* hybridization was carried out as described previously.[13] Briefly, the cDNA fragments encoding ADAM28 nucleotides 952–1132 (181 bp) was subcloned into pGEM-11Zf(+) Vector (Promega Biotec, Oakland, CA), and sense and antisense digoxigenin-labeled RNA probes were prepared with T7 or SP6 RNA polymerases using DIG RNA labeling kit (Boehringer-Mannheim, Mannheim, Germany). BLASTN searches were carried out to confirm the specificity of the probe. Paraffin sections of the tissues were hybridized with the digoxigenin-labeled RNA antisense or sense probes and then subjected to immunohistochemistry using mouse anti-digoxigenin antibody (1/750 dilution; Boehringer Mannheim), followed by the peroxidase-labeled avidin-biotin-complex method (ABC method) (1/100 dilution; DAKO, Glostrup, Denmark). After the reaction, the sections were counterstained with hematoxylin.

Preparation of recombinant ADAM28s and its deletion mutant ADAM28sDis/CR/Ss

Recombinant full-length ADAM28s and ADAM28sDis/CR/Ss, which contains disintegrin-like domain, cysteine-rich domain and secretory specific domain but lacks propeptide domain and metalloproteinase domain, were expressed and purified according to our previous methods.[8] The cDNA fragment encoding ADAM28sDis/CR/Ss with the ADAM28 signal sequence and FLAG tag was prepared from the full-length cDNA for ADAM28s[8] by using PCR forward primer 5′-ACGCAGATCTATTTGTGGGAACCAGTTG-G-3′ and reverse primer 5′-ACGCGTCGACTCTGAAATGAT-TTTCCTTCGC-3′, and cloning into pCMV-Tag4a vector. The cDNA fragment was cloned into pFASTBac1 vector (Invitrogen, Tokyo, Japan), generating pFASTBac1/ADAM28sDis/CR/Ss. For the expression and purification of ADAM28sDis/CR/Ss, pFASTBac1/ADAM28sDis/CR/Ss vectors were transfected to DH10Bac cells (Invitrogen), and the recombinant bacmids were purified. The bacmids encoding ADAM28s or ADAM28sDis/CR/Ss were transfected to generate baculovirus particles in Sf9 insect cells as

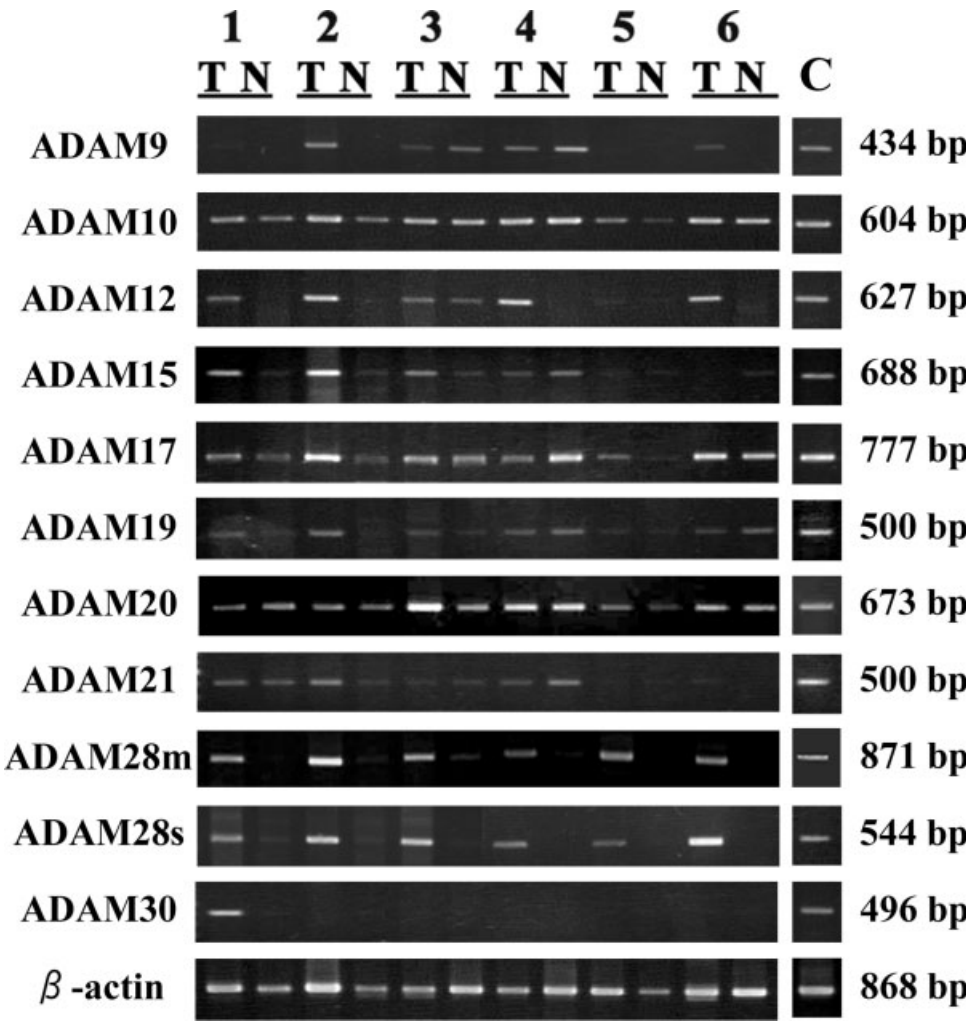

FIGURE 2 – mRNA expression of ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, ADAM19, ADAM20, ADAM21, ADAM28m, ADAM28s, ADAM30 and β-actin in representative 6 cases of human non-small lung carcinoma (T) and control non-carcinoma lung tissues (N) by RT-PCR. Total RNA was extracted from the lungs with squamous cell carcinoma (*lanes 1–3*) or adenocarcinoma (*lanes 4–6*), reverse-transcribed into cDNA and followed by PCR as described in Materials and Methods. Each amplification of ADAM species and β-actin was carried out at least in triplicate. C, positive controls carried out as described in Material and Methods.

described by the manufacturer, and then the insect cells (about 1.0×10^6/ml) were infected with baculovirus particles. They were cultured in serum-free media, and conditioned media were harvested after a 3-day culture. The media were applied to the immunoaffinity column of anti-FLAG M2-agarose affinity gels (Sigma-Aldrich, Inc., St. Louis, MO), and the proteins bound were eluted with 6 M urea in 50 mM Tris-HCl, pH 7.5, 3 M NaCl, 10 mM $CaCl_2$, 0.01% Brij 35. The eluates were dialyzed extensively against 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM CaCl2, 0.01% Brij 35 at 4°C. Purified ADAM28s and ADAM28sDis/CR/Ss showed protein bands of 65 kDa and 42 kDa on the silver stained gel, respectively (data not shown).

Preparation of antibodies against ADAM28

Monoclonal antibody to ADAM28 was developed by using purified recombinant ADAM28s[8] as an antigen according to our previous methods.[20] Five clones were screened initially by enzyme-liked immunosorbent assay with the recombinant ADAM28s, and clone 297-2F3 was selected as a candidate for the antibody against ADAM28. Monospecificity of the monoclonal antibody (297-2F3) was determined by immunoblotting of recombinant precursor of ADAM28s (proADAM28s), active ADAM28s without propeptide 8 and ADAM28sDis/CR/Ss (Fig. 1*a*). These proteins (15 μg/lane) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (10% total acrylamide) under reducing conditions and transferred to polyvinylidene difluoride membranes, which were then incubated with monoclonal antibody (297-2F3; 5 μg/ml) or anti-FLAG antibody (Sigma-Aldrich, Inc.; 5 μg/ml) after blocking nonspecific reaction with 5% skim milk. The membranes were incubated with secondary antibody (1:5,000; Amersham Pharmacia Biotech, Buckinghamshire, UK), and immunoreactive protein bands were detected with ECL immunoblotting reagents (Amersham Pharmacia Biotech). As shown in Figure1*a*, monoclonal antibody (297-2F3) detected proADAM28s of 65 kDa and processed ADAM28s species including a major active form of 42 kDa (*lanes 4* and *5*), both of which were also recognized with anti-FLAG antibody (*lanes 1* and *2*). Although anti-FLAG antibody detected the recombinant ADAM28sDis/CR/Ss protein, it was not recognized with the 297-2F3 antibody (Fig. 1*A*, *lanes 3* and *6*). When culture media of Sf9 cells infected with baculovirus particles encoding ADAM28s were subjected to immunoblotting with the 297-2F3 antibody or non-immune mouse IgG, a single band of 65 kDa was detected only with the 297-2F3 antibody (Fig. 1*A*, *lanes 7* and *8*). These results demonstrate that the monoclonal antibody specifically recognizes the metalloproteinase domain of ADAM28 protein (see schematic representation in Fig. 1*a*).

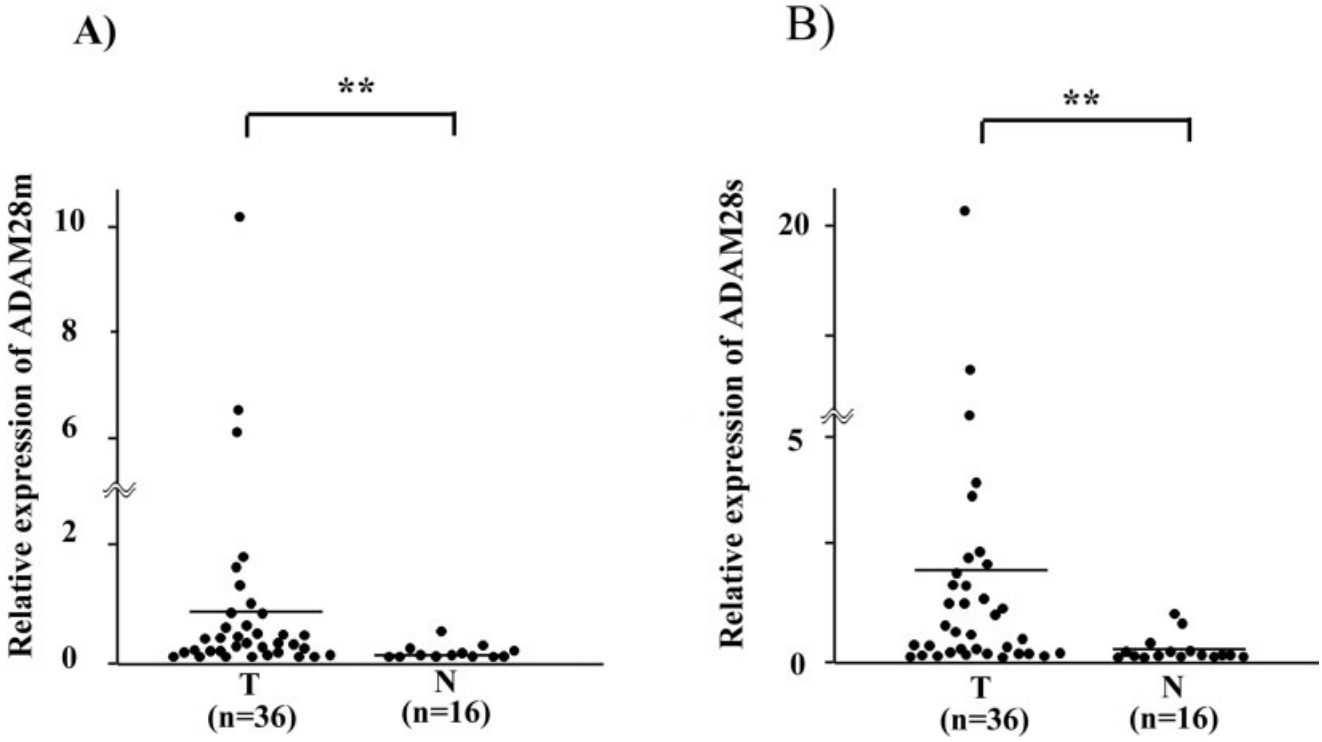

Figure 3 – The mRNA expression levels of ADAM28m and ADAM28s in human non-small lung carcinoma (T) and control non-carcinoma lung tissues (N). Relative mRNA expression levels of ADAM28m (*a*) and ADAM28s (*b*) were analyzed by real-time quantitative PCR. The results were standardized for sample-to-sample variations using 18S ribosomal RNA in each sample as described in Material and Methods. Scale bars = mean values. $^{**}p < 0.01$.

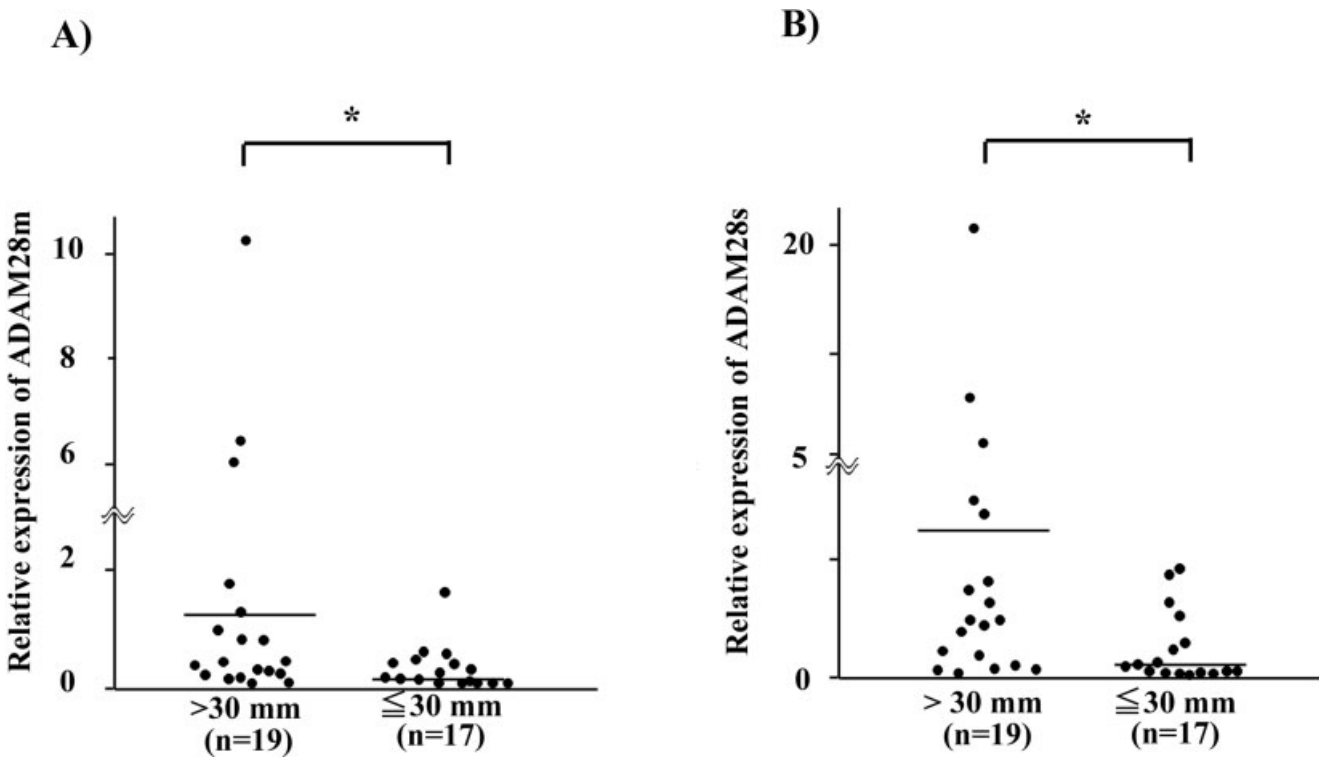

Figure 4 – The mRNA expression levels of ADAM28m (*a*) and ADAM28s (*b*) in the carcinomas with a tumor size of >30 mm or ≦30 mm in diameter. $^{*}p < 0.05$.

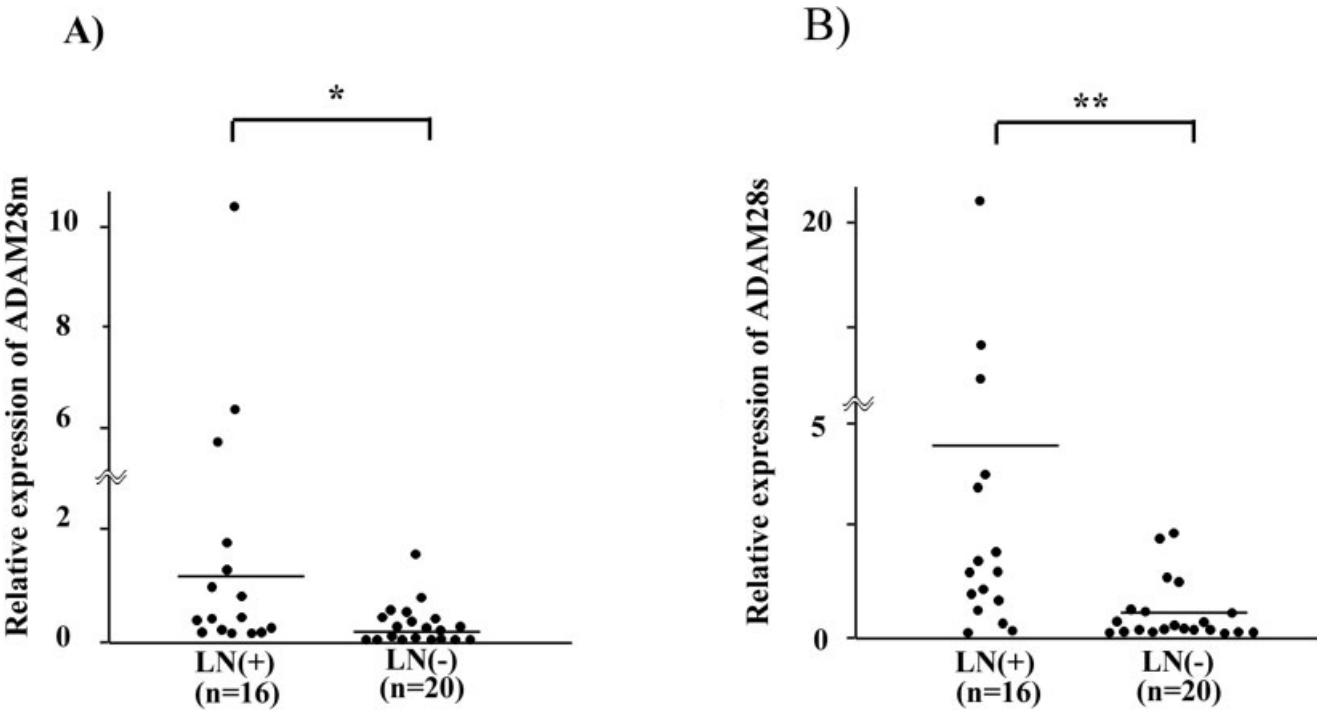

FIGURE 5 – The mRNA expression levels of ADAM28m (*a*) and ADAM28s (*b*) in the carcinomas with or without lymph node metastasis. LN(+) and LN(−), carcinoma samples with and without lymph node metastasis, respectively. *$p < 0.05$; **$p < 0.01$.

Polyclonal antibodies were prepared as follows. A cDNA fragment encoding the propeptide and catalytic domains of ADAM28 was isolated from the human THP1 cell line as described previously.[21] The coding sequence was then ligated into the vectors pRSETB and pEE14. The pRSETB construct was further truncated by removing a fragment encoding the NH_2-terminal half of the propeptide domain by NheI digestion followed by re-ligation. The truncated propeptide and catalytic domains were expressed in *E. coli*, purified from the inclusion bodies, and used to immunize sheep. Anti-ADAM28 antibodies were purified with the antigen as described previously.[22] As shown in Figure 1*b*, polyclonal antibodies recognized the 55-kDa ADAM28 fragment containing the propeptide and catalytic domains in the inclusion bodies and the 35-kDa and 22-kDa fragments in the culture media of Chinese hamster ovary cell transfectants. Non-immune IgG did not detect any protein bands (data not shown).

Immunohistochemistry of ADAM28 and Ki-67

Paraffin sections were dewaxed, and treated with 0.3% hydrogen peroxide and 10% normal serum to block endogenous peroxidase and non-specific binding, respectively. They were incubated with mouse monoclonal antibody (297-2F3; 10 μg/ml) or sheep polyclonal antibodies (5 μg/ml) against ADAM28 at 4°C overnight. Subsequently, the specimens were incubated at room temperature for 30 min with biotinylated horse antibodies to mouse IgG (1/200 dilution; Vector Laboratories Inc., Burlingame, CA) or rabbit antibodies to sheep IgG (1/200 dilution; Vector Laboratories Inc.). Color was developed by the ABC method as described above. As for a control, sections were reacted by replacing the first antibodies with non-immune mouse IgG or sheep IgG (DAKO). After immunohistochemistry, the sections were counterstained with hematoxylin.

For immunostaining of Ki-67, the paraffin sections were treated in a microwave oven for 5 min at 500 W using a citrate buffer (pH 6.0). After blocking non-specific binding with 10% goat serum, they were incubated with mouse monoclonal antibody against Ki-67 (MIB1; 1/50 dilution; DakoCytomation Norden A/S). Subsequently, the specimens were incubated with biotinylated horse antibodies against mouse IgG (1/200 dilution; Vector Laboratories Inc.) followed by the incubation with goat antibodies against mouse IgG conjugated to horseradish peroxidase-labeled dextran polymer (no dilution; EnVision+ Peroxidase Mouse; DakoCytomation, California Inc., Carpinteria, CA). After immunostaining, the sections were counterstained with hematoxylin. To assess proliferative activity of non-small cell lung carcinomas, MIB1-positive cell index was determined by counting nuclei immunoreactive with MIB1 antibody in 5 different fields at a magnification of ×200 without knowledge of clinical data. Any nuclear staining, regardless of intensity, was considered positive for MIB1. The MIB1-positive cell index was expressed as a percentage of immunoreactive tumor cells to the total counted tumor cells.

Immunoblotting of lung carcinoma tissues with anti-ADAM28 monoclonal antibody

Carcinoma and non-carcinoma tissues were homogenized on ice in 1 ml of 50 mM Tris-HCl buffer (pH 7.5), 150 mM NaCl, 10 mM $CaCl_2$ and 0.05% Brij35 containing a cocktail of proteinase inhibitors (Roche Diagnostics, Mannheim, Germany). Supernatants of the homogenates were subjected to SDS-PAGE (10% total acrylamide) under reducing conditions after determining protein concentrations by the dye-binding method (Proteostain Protein Quantification Kit Wide Range; Dojindo Laboratories, Kumamoto, Japan). The resolved proteins were transferred to polyvinylidene difluoride membranes with a semidry blotter. The membranes were then blotted with monoclonal antibody against ADAM28 (297-2F3; 5 μg/ml) and immunoreactive protein bands were detected with ECL Western blotting reagents (Amersham Pharmacia Biotech) as described above.

Statistical analyses

The difference in the mRNA expression of ADAM28 between the 2 independent groups was determined by the Mann-Whitney

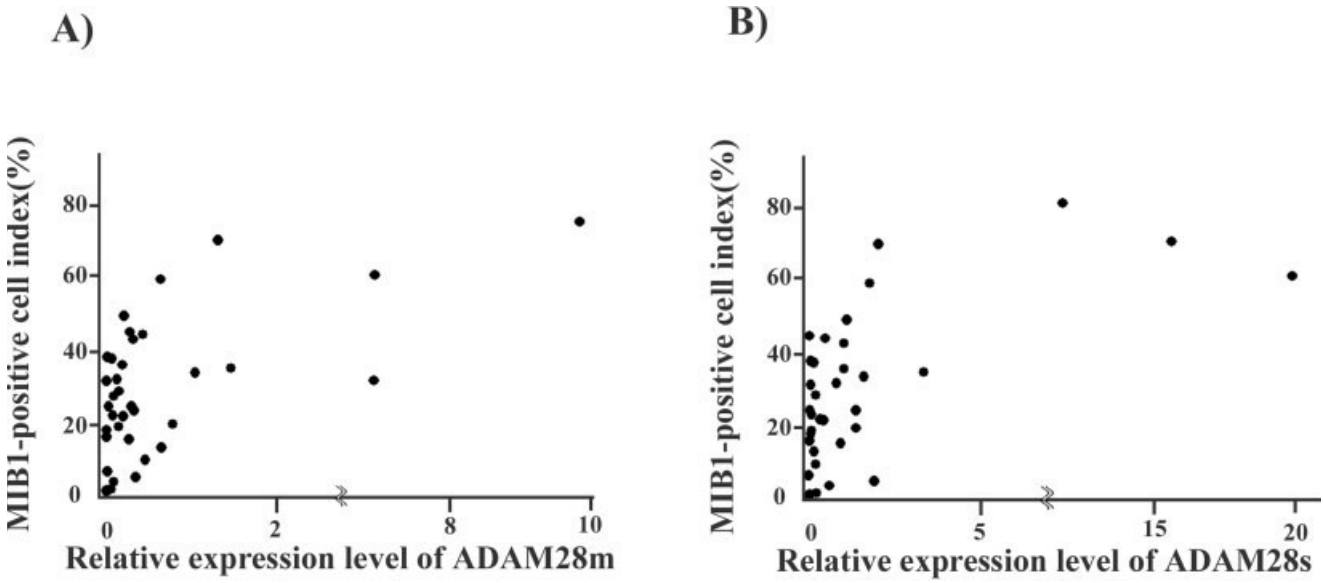

Figure 6 – Correlations of the relative expression levels of ADAM28m and ADAM28s with the MIB1-positive cell index of non-small cell lung carcinomas. The relative mRNA expression levels of ADAM28m (*a*) and ADAM28s (*b*) in the lung carcinomas were plotted against MIB1-positive cell index. Note direct correlations of the ADAM28m expression levels ($r = 0.667$, $p < 0.01$; $n = 36$) and the ADAM28s levels ($r = 0.535$, $p < 0.01$; $n = 36$) with the MIB1-positive cell index.

U-test. Comparisons among more than 3 groups were determined by the Kruskal-Wallis test. The correlations between the mRNA expression levels of ADAM28 species and the MIB1-positive cell index were studied using the Pearson correlation coefficient. *p*-Values <0.05 were considered significant.

Results

mRNA expression of proteinase-type ADAM in non-small cell lung carcinomas

The mRNA expression of ADAM9, ADAM10, ADAM12, ADAM15, ADAM17, ADAM19, ADAM20, ADAM21, ADAM28m, ADAM28s and ADAM30 in 12 matched carcinoma and control non-carcinoma lung tissues was analyzed by RT-PCR. As shown in Figure 2, ADAM10, ADAM17 and ADAM20 were constitutively expressed in all the carcinoma and control lung tissues. ADAM9, ADAM15, ADAM19 and ADAM21 were expressed in >66% of the carcinomas, but the expression was also observed in >33% of the control tissues. ADAM12 was expressed in 83% of the carcinomas, but it was also expressed in 17% of the control tissues. ADAM30 was expressed selectively in the carcinomas, but the expression rate was only 17%. ADAM28m and ADAM28s were expressed in all the carcinoma tissues, and negligible expression was observed in the control tissues. The difference of the expression between carcinoma and control non-carcinoma lung tissues was most prominent in ADAM28m and ADAM28s.

Real-time quantitative PCR analysis of ADAM28m and ADAM28s expression and its correlation with clinicopathological parameters

Because ADAM28m and ADAM28s were expressed predominantly in lung carcinoma tissues, we further examined their mRNA expression levels by real-time quantitative PCR. As shown in Figure 3*a*, the relative expression level of ADAM28m was significantly higher in the carcinomas (0.84 ± 2.12; mean ± SD) than in the control non-carcinoma lung tissues (0.05 ± 0.78) ($p < 0.01$). The ADAM28s expression level was also significantly higher in the carcinomas (2.17 ± 4.53) than in the control lung tissues (0.24 ± 0.31) ($p < 0.01$) (Fig. 3*b*). When the mRNA expression levels in the carcinoma tissues were compared to the clinicopathological parameters, the levels of ADAM28m and ADAM28s in the carcinomas with a tumor size of >30 mm in diameter (1.43 ± 2.81 and 3.39 ± 5.93 for ADAM28m and ADAM28s, respectively) were significantly higher than those in the carcinomas of ≦30 mm in diameter (0.18 ± 0.28 and 0.56 ± 0.72 for ADAM28m and ADAM28s, respectively) ($p < 0.05$ for each group) (Fig. 4*a*,*b*). In addition, the levels of ADAM28m (1.65 ± 3.02) and ADAM28s (4.29 ± 6.23) in the carcinomas with lymph node metastasis were significantly higher than those in the carcinomas without metastasis (0.19 ± 0.25 for ADAM28m and 0.47 ± 0.63 for ADAM28s) ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 5*a*,*b*). No significant correlations were obtained between the mRNA expression levels and other clinicopathological parameters including gender, histological type and tumor cell differentiation (Table I).

Correlations between ADAM28 expression levels and MIB1-positive cell index

To further study correlations of the mRNA expression levels of ADAM28m and ADAM28s with proliferation of lung carcinoma cells, MIB1-positive index in the carcinoma tissues ($n = 36$) was determined by immunostaining for Ki-67 using MIB1 antibody. MIB1-positive cell index for the lung carcinoma cells was 29.1 ± 17.8%, and no staining was observed in the normal lung tissues or in the lung carcinoma tissues stained with non-immune mouse IgG (data not shown). When the mRNA expression levels of ADAM28m was plotted against the MIB1-positive cell index of lung carcinomas in each case, there was a direct correlation between the expression levels and the index ($r = 0.667$, $p < 0.01$; $n = 36$) (Fig. 6*a*). A similar correlation was also obtained between the ADAM28s expression and the index ($r = 0.535$, $p < 0.01$; $n = 36$) (Fig. 6*b*).

In situ *hybridization*

Cells expressing ADAM28 mRNA in the lung carcinoma tissues were identified by *in situ* hybridization. With the antisense probe, the signals for ADAM28 were observed predominantly in the carcinoma cells of both squamous cell carcinomas (Fig. 7*a*) and adenocarcinomas (Fig. 7*c*), although some lymphocytes infiltrated in the carcinoma tissues were also weakly positive. The

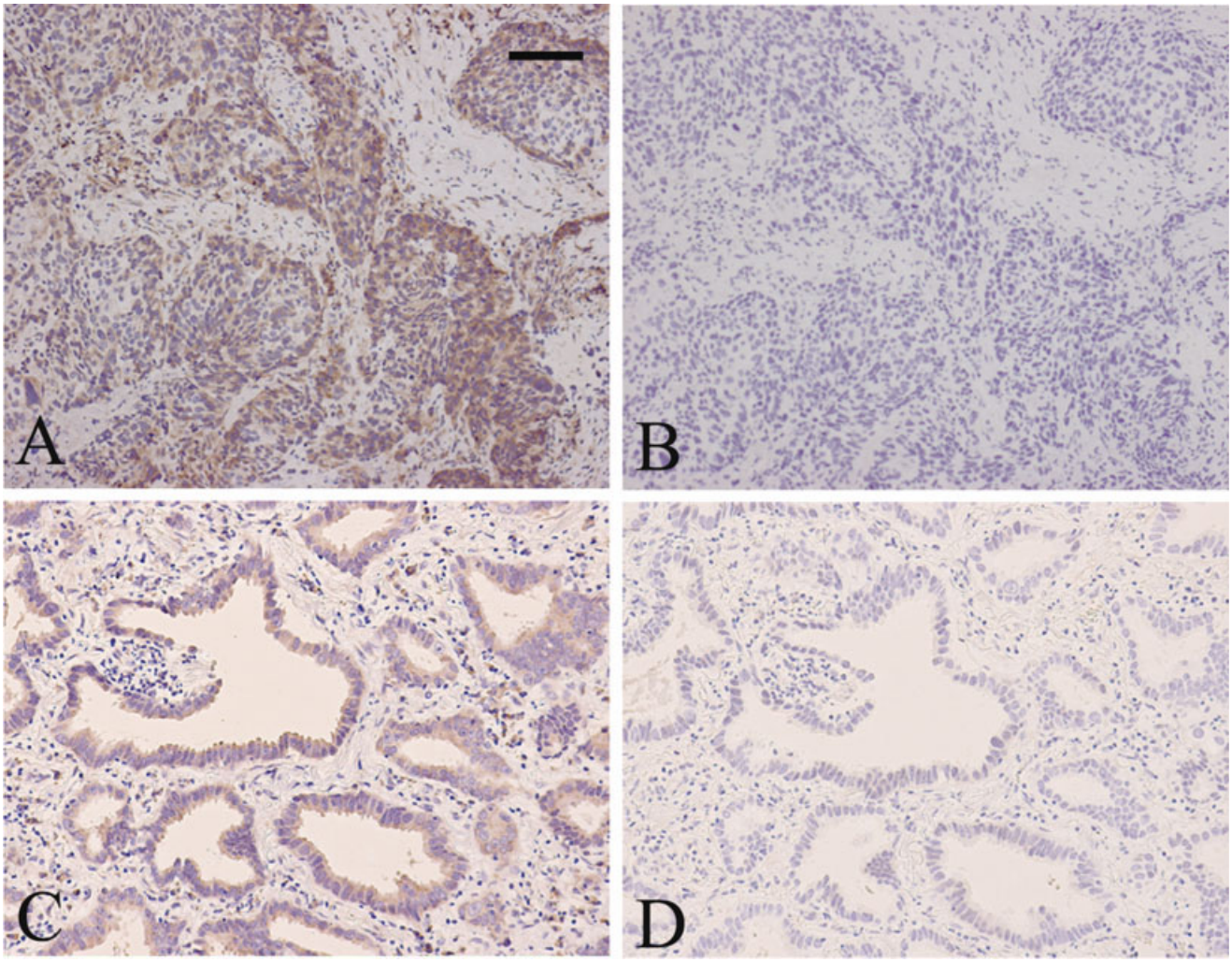

FIGURE 7 – *In situ* hybridization for ADAM28 in the lung carcinoma tissues. In situ hybridization was carried out as described in Materials and Methods. Note that positive signals for ADAM28 in the carcinoma cells with the antisense probe (*a*,*c*). The sense probe gives only negligible signals in the carcinoma tissues (*b*,*d*). (*a*,*b*) Squamous cell carcinoma. (*c*,*d*) Adenocarcinoma. Scale bar = 50 μm.

sense probe gave negligible background signals in the carcinoma tissues (Fig. 7*b*,*d*).

Immunohistochemistry and immunoblotting of ADAM28

By immunohistochemistry using monoclonal antibody to ADAM28 (297-2F3), ADAM28 was localized predominantly to the carcinoma cells of both squamous cell carcinomas (Fig. 8*a*) and adenocarcinomas (Fig. 8*c*) in all the carcinoma samples examined (36 of 36 cases). A similar pattern of immunolocalization in the carcinoma cells was obtained with sheep polyclonal antibodies to ADAM28 (Fig. 8*e*,*g*). No staining was observed with non-immune mouse IgG (Fig. 8*b*,*d*) or sheep IgG (Fig. 8*f*,*h*). By immunoblotting using monoclonal antibody against ADAM28, a protein band of 42 kDa was identified in the carcinoma tissues, whereas no such species was recognized in the control non-carcinoma tissues (Fig. 9).

Discussion

We have demonstrated for the first time that among the 11 different ADAM species with putative metalloproteinase activity, ADAM28m and ADAM28s are selectively overexpressed in human non-small cell lung carcinoma tissues. This was first shown by RT-PCR, and real-time quantitative PCR further indicated that the expression levels of ADAM28m and ADAM28s are significantly at least 9-fold higher in the carcinoma tissues than in the control non-carcinoma lung tissues. Predominant expression of these ADAM28 species in the carcinoma tissues was also supported by the findings of *in situ* hybridization, immunohistochemistry and immunoblotting. Previous studies have described the expression of ADAM species in human malignant tumors: ADAM12 in breast, colon and stomach carcinomas[3] and glioblastomas,[13] ADAM10 and ADAM15 in hematological malignancies,[23] and ADAM10, ADAM17 and ADAM20 in gastric carcinomas.[24] Among these ADAM species, only the ADAM12 expression seems to be more selective in carcinoma tissues, because it is localized to carcinoma cells in the breast, colon and stomach carcinomas without definite expression in the non-neoplastic tissues.[3] In addition, our recent study on the expression of ADAM species in human gliomas demonstrated that ADAM12 is selectively overexpressed in glioblastomas with correlation to proliferative activity of glioblastoma cells. In our present study, ADAM12 was expressed in 83% of the lung carcinomas, whereas it was also detected in 17% of the control lung tissues. These data suggest that ADAM12 may be a common ADAM species overexpressed

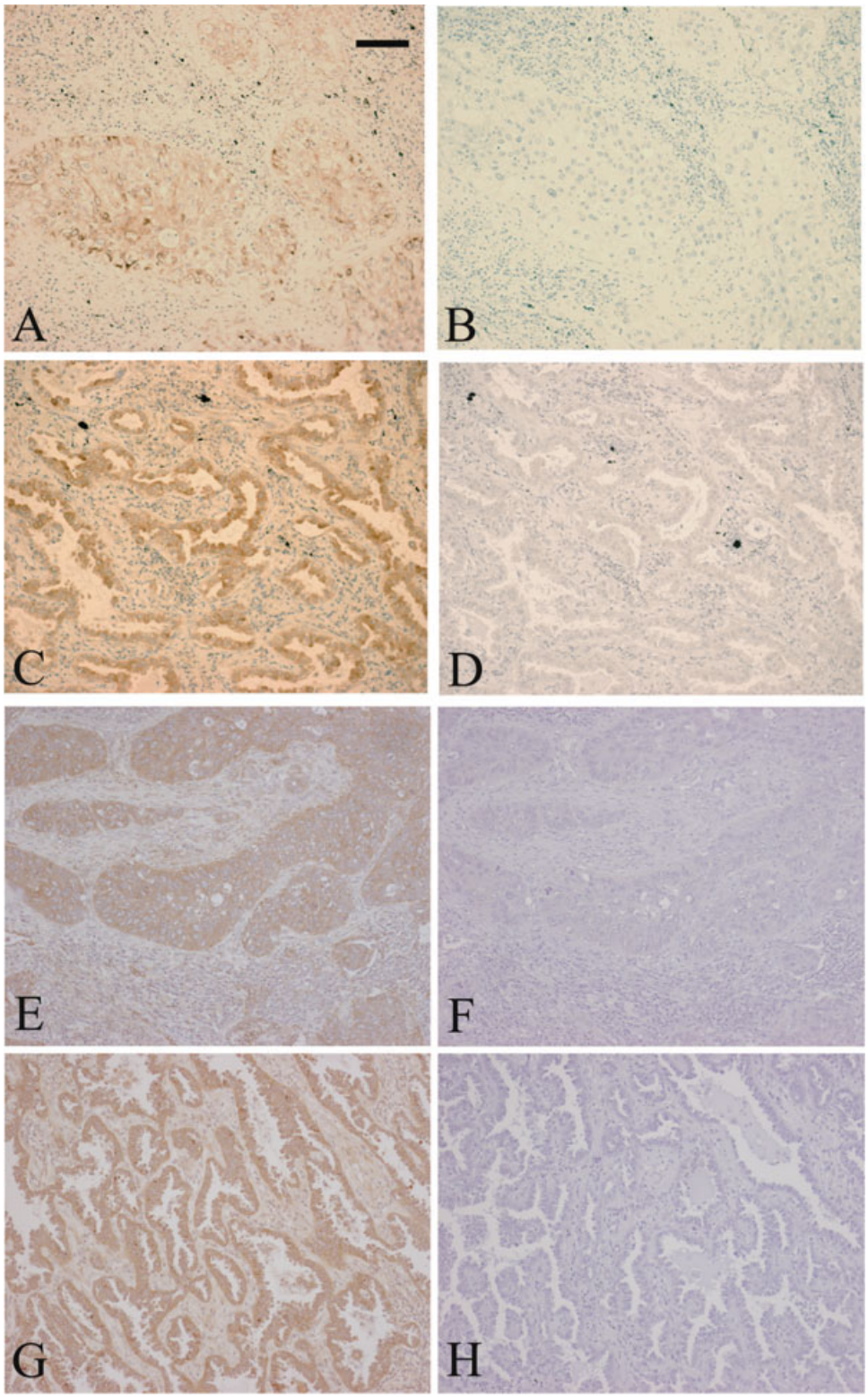

Figure 8 – Immunolocalization of ADAM28 in the lung carcinoma tissues. Paraffin sections of squamous cell carcinoma and adenocarcinoma tissues were immunostained with monoclonal anti-ADAM28 antibody (*a*,*c*) or polyclonal anti-ADAM28 antibodies (*e*,*g*) as described in Material and Methods. Note that ADAM28 is immunostained mainly in the carcinoma cells (*a*,*c*,*e*,*g*), whereas only a background signal is observed with non-immune IgG (*b*,*d*,*f*,*h*). (*a*,*b*,*e*,*f*) Squamous cell carcinoma. (*c*,*d*,*g*,*h*) Adenocarcinoma. Scale bar = 50 μm.

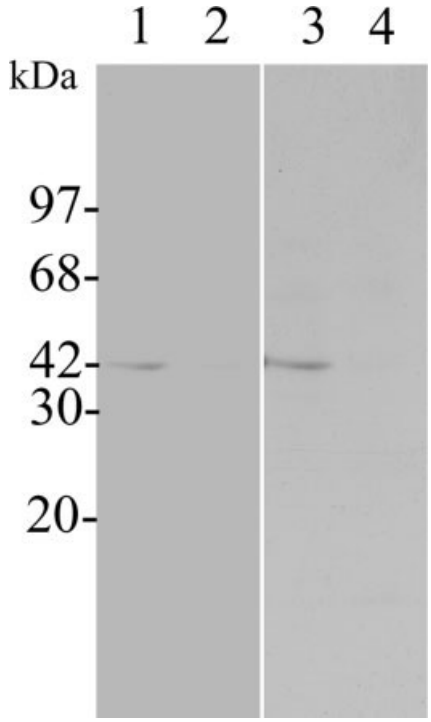

FIGURE 9 – Immunoblotting of ADAM28 in the lung carcinoma tissues. Homogenates (20 μg/lane) from squamous cell carcinoma and control non-carcinoma lung tissues (*lanes 1,2*) and adenocarcinoma and control lung tissues (*lanes 3,4*) were subjected to immunoblotting using monoclonal anti-ADAM28 antibody (297-2F3) as described in Material and Methods. Note that a positive band of 42 kDa in the lung carcinoma tissues (*lanes 1,3*).

in human malignant tumors. Our *in situ* hybridization and immunohistochemistry have indicated that ADAM28 is expressed predominantly by the carcinoma cells in squamous cell carcinomas and adenocarcinomas. Some lymphoid cells infiltrated in the lung carcinoma tissues were also labeled positively by these methods, confirming the previous study showing the ADAM28 expression in peripheral lymphocytes.[25,26] Because the staining in the lymphoid cells was weak and sporadic, however, it is very likely that carcinoma cells are mainly responsible for the ADAM28 expression in the human non-small cell lung carcinoma tissues.

Immunoblotting of the tissue homogenates using the monoclonal anti-ADAM28 antibody demonstrated an immunoreactive band of 42 kDa only in the carcinoma tissues. Both ADAM28m and ADAM28s are synthesized in proenzymes and processed to active forms without propeptides. Although some members of the ADAM family possess the furin-recognition site and are activated by furin,[27,28] ADAM28 lacks the furin-cleavage site. Our previous biochemical studies demonstrated that proADAM28s can be processed to a 42-kDa active form and some degradation fragments by the action of MMP-7, although it is not known whether MMP-7 is a physiological activator for proADAM28. In our present study, as shown in Figure 1*a*, our monoclonal antibody specifically recognizes the metalloproteinase domain of ADAM28. According to the specificity of the antibody and the molecular weight, the 42-kDa fragments detected in the non-small cell lung carcinoma tissues are considered the active forms of ADAM28s or ADAM28m shed from the cell membranes. The immunoblotting data suggest that ADAM28 species seem to be expressed in active form in the carcinoma tissues, although the activation mechanism remains to be unknown.

One of the interesting findings in our present study is that the mRNA expression levels of ADAM28m and ADAM28s are significantly higher in the carcinomas with a tumor size of >30 mm in diameter than in those of ≦30 mm. In support of the finding, the expression levels of ADAM28m and ADAM28s directly correlated with MIB1-positive cell index of non-small cell lung carcinomas, suggesting the involvement of ADAM28 in carcinoma cell proliferation. We have reported recently that ADAM28 releases intact IGF-I from the IGF-I/IGFBP-3 complex by selective cleavage of IGFBP-3.[8] IGFBP-3, a main species of the IGFBP family, binds with IGF to make the complexes and inhibits their mitogenic and anti-apoptotic activities by sequestering them away from its receptor in the extracellular environment.[29,30] Serum IGFBP-3 levels are known to negatively correlate with human lung carcinoma risk[30] and IGFBP-3 inhibits the cell growth of non-small lung cancer cells through suppression of mitogen-activated protein kinase.[31] These data suggest the possibility that ADAM28 plays a role in proliferation of non-small cell lung carcinomas by facilitating availability of IGF through digestion of IGFBP-3. Further work is, however, needed to demonstrate the hypothesis at the cellular and tissue levels.

Our present study has demonstrated that the expression levels of ADAM28m and ADAM28s are significantly higher in the lung carcinomas with lymph node metastasis than in those without metastasis. This is the first finding suggesting the involvement of an ADAM member in lymph node metastasis of human malignant tumors. At the present time, however, we do not have sufficient data to explain the roles of ADAM28 in the metastasis. Several ADAM including ADAM28 interact with integrins on the cell membranes, and the disintegrin domain potentially plays a role in integrin binding and dissociation, which are key steps in cancer cell migration.[32,33] The disintegrin domain of ADAM28 is known to interact with integrin α4β1,[34] and lung carcinoma cell lines express β1 integrins, which carry out an important role in cancer cell migration toward fibronectin, laminin and type IV collagen.[35] Among the ADAM members, the metalloproteinase domain of ADAM28 is most homologous to that of the hemorrhagic metalloproteinase Jararhagin found in the venom of *Bothrops jararaca*.[36,37] Because this snake venom has proteolytic activities to various substrates such as fibrinogen, von Willebrand factor and type IV collagen,[37,38] ADAM28 has the possibility to degrade substrates other than myelin basic protein[7] and IGFBP-3.[8] In addition, some members of the ADAM family such as ADAM9, ADAM12 and ADAM17 are involved in shedding of growth factors and cytokines including HB-EGF, transforming growth factor-α and TNF-α,[11,12] all of which may be implicated in cancer growth and progression.[39,40] Although information about sheddase activity of ADAM28 is limited to CD23,[10] it may be possible to speculate that activated forms of ADAM28 exhibit sheddase activity to growth factors and cytokines related to invasion and metastasis. Further experimental work is definitely needed to elucidate the mechanisms of how ADAM28 is involved in the metastasis.

In summary, we have provided the first evidence that among 11 different ADAM species with putative metalloproteinase activity, ADAM28m and ADAM28s are overexpressed selectively in the human non-small cell lung carcinomas with correlations to carcinoma cell proliferation and lymph node metastasis. Our data support the hypothesis that ADAM28 plays a role in lung cancer growth and progression, and suggest that this metalloproteinase could be a new target of cancer therapy, although further investigation is necessary.

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