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# Highly sensitive detection of invasive lung cancer cells by novel antibody against amino-terminal domain of laminin γ2 chain

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**Key words**

Biomarker, laminin γ2, lung cancer, monoclonal antibody, tumor invasion

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**The laminin γ2 chain, a subunit of laminin-332 (α3β3γ2), is a molecular marker for invasive cancer cells, but its pathological roles in tumor progression remain to be clarified. It was recently found that the most N-terminal, domain V (dV) of γ2 chain has activities to bind CD44 and stimulate tumor cell migration and vascular permeability. In the present study, we prepared a mAb recognizing γ2 dV. Immunoblotting with this antibody, for the first time, showed that proteolytic fragments containing dV in a range of 15–80 kDa were highly produced in various human cancer cell lines and lung cancer tissues. In immunohistochemistry of adenocarcinomas and squamous cell carcinomas of the lung, this antibody immunostained the cytoplasm of invasive tumor cells and adjacent stroma much more strongly than a widely used antibody recognizing the C-terminal core part of the processed γ2 chain. This suggests that the dV fragments are highly accumulated in tumor cells and stroma compared to the processed γ2 protein. The strong tumor cell staining with the dV antibody correlated with the tumor malignancy grade. We also found that the laminin β3 and α3 chains were frequently overexpressed in tumor cells and tumor stroma, respectively. The cytoplasmic dV detection was especially prominent in tumor cells infiltrating stroma, but low in the cells surrounded by basement membranes, suggesting that the active tumor–stroma interaction is critical for the aberrant γ2 expression. The present study suggests important roles of laminin γ2 N-terminal fragments in tumor progression.**

The basement membrane protein Lm332, a heterotrimer of laminin α3, β3, and γ2 chains, plays critical roles in the structure and function of epithelial tissues.[1–3] This laminin was originally identified as an ECM protein secreted by keratinocytes,[4,5] and as a cell-scattering factor secreted by human cancer cell lines.[6] The strong cell motility activity of Lm332 suggested its possible roles in tumor invasion and metastasis.[7–9] As Lm332 is the only laminin containing the Lm-γ2 chain, a number of immunohistochemical studies of various human cancers have been undertaken using anti-γ2 antibodies.[9,10] These studies reported that Lm-γ2 is frequently overexpressed at invasion fronts of many types of human cancers of various organs such as the colorectum,[11,12] pancreas,[13] stomach,[14] lung,[15–17] esophagus,[18] tongue,[19] and others.[9,10] Many of them showed that the characteristic expression of the Lm-γ2 chain is associated with poor prognosis and metastasis.[13,18,19] Moreover, it was found that Lm332 promotes tumor growth *in vivo*.[20–22] Several studies, including ours, have shown that Lm-γ2 is overexpressed as a monomer form in invasive cancer tissues[14,16,23] and in cultured cancer cells activated by growth factors.[24,25] These findings give rise to the hypothesis that the tumor invasion marker Lm-γ2 may play some active roles in the invasive growth and metastasis of human cancer cells. However, the exact functions of Lm-γ2 in tumor progression are still unclear.

Laminin-γ2 (150 kDa) is cleaved at its short arm by bone morphogenetic protein-1/mammalian tolloid and MMPs, releasing an N-terminal fragment (γ2pf) with 45 kDa as a major proteolytic fragment.[7,26,27] This proteolytic processing increases the cell motility activity of Lm332,[27,28] but inhibits its matrix assembly.[29] It is also known that additional proteolytic cleavages of Lm-γ2 at the C-terminal domain of the short arm (i.e., domain III) by MMPs and elastase generate cell motility fragments.[30,31] Laminin γ2pf-like fragments have been found in conditioned media of cultured cancer cells and sera from human cancer patients.[32] It was recently found that recombinant proteins of γ2pf and its most N-terminal fragments promote tumor cell invasion and vascular permeability *in vivo* and *in vitro*.[24,33,34] CD44 and syndecan-1 are thought to be receptors for the fragments on cancer cells.[35,36] These findings support active roles of the N-terminal fragments of Lm-γ2 in cancer progression.

Lung cancer is one of the most common cancers and the leading cause of cancer-related death in many countries.[37] The prognosis of patients with lung cancers is generally very

poor. It is critically important for cancer treatments to discriminate invasive carcinomas from non-invasive ones. Laminin-332 is a major component of the bronchioloalveolar basement membrane,[38,39] and Lm-γ2 is often overexpressed in invasive lung cancer tissues.[15–17] Although past immunohistochemical studies of lung cancers showed the relationship between Lm-γ2 expression and tumor invasiveness, they were unable to show the distribution of Lm-γ2 N-terminal fragments because of the lack of suitable antibodies. In the present study, we prepared mouse mAbs recognizing different regions of γ2pf and investigated the production of γ2 N-terminal fragments by various cancer cell lines and its distribution in non-small-cell lung cancers. To show whether the γ2 chain is associated with the α3 and β3 chains in the cancer tissues, localization of the three Lm332 chains was also investigated in detail.

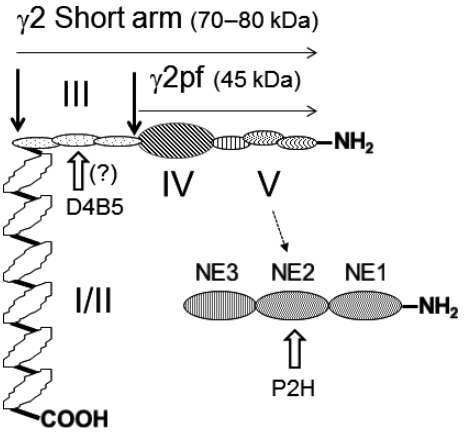


**Fig. 1.** Domain structure of laminin γ2 (Lm-γ2) chain. Lm-γ2 consists of domains I/II (or coiled-coil domain), III (or LEb), IV (or L4), and V (or LEa) from the C-terminus.[2] Domain V (dV) consists of three N-terminal EGF-like repeats (NE1–3). Domain I/II is associated with the laminin β3 and α3 chains to form the Lm332 heterotrimer. P2H recognizes NE2 of dV, and D4B5 recognizes an unidentified EGF-like repeat of domain III (dIII). Solid arrows, cleavage sites by endogenous proteinases; open arrows, epitopes for mAbs (D4B5 and P2H); γ2pf, a 45-kDa fragment released by a major proteolytic cleavage.

## Materials and Methods

**Tumor tissues.** Human tissue specimens of 29 lung cancers (15 ADCs and 14 SCCs) were obtained from patients who received surgery for lung cancers at the KCC Hospital (Kanagawa, Japan) between 2006 and 2008 for the ADCs and between 2006 and 2009 for the SCCs, and provided by Human Cancer Tissue Bank of KCC. Disease stage was determined according to the TNM classification of the International Union Against Cancer.[40] Histological typing of the tumors was determined according to the 2015 WHO Classification of Tumors.[41] The study protocol was approved by the Ethical Committees of both KCC and Kihara Institute for Biological Research, Yokohama City University (Yokohama, Japan), and carried out according to the guidelines of the 1995 Declaration of Helsinki. Written informed consent was obtained from each patient in the KCC Hospital.

**Cell culture.** Three human lung carcinoma cell lines (SCC VMRC-LCP, giant cell carcinoma Lu65, and ADC PC3), cervix epidermoid carcinoma line CaSki, three gastric carcinoma lines (MKN-74, MKN-45, and STKM-1), two mammary carcinoma lines (MMK-29 and MDA-MB-231), ovarian mucinous cystadenocarcinoma line MCAS, and fibrosarcoma line HT1080. MDA-MB-231 (ATCC HTB-26) was obtained from ATCC (Manassas, VA, USA), and MCAS (JCRB0240) from the JCRB Cell Bank (Osaka, Japan). The other cell lines were described in our previous studies.[6,42] These cells were maintained in DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS (Nichirei Biosciences, Tokyo, Japan) and antibiotics.

**Preparation of mouse mAbs against N-terminal domains of Lm-γ2 chain.** Recombinant proteins of the following Lm-γ2 fragments, all of which contained the His-tag sequence in their C-termini, were prepared as previously reported (see Fig. 1): γ2pf, dV, and three deletion mutants of dV (NE1/2, NE1/3, and NE2/3).[34,36] To prepare mouse mAbs, γ2pf was immunized into BALB/c mice. Hybridoma cell lines that secreted mAbs against γ2pf were prepared by the fusion of spleen cells from the immunized mice and the mouse myeloma cell line P3U1. Epitopes for the antibodies were determined by ELISA using γ2pf, dV, and the three dV deletion mutants. In the present study, clone P2H, an antibody recognizing NE2, was used in IHC and immunoblotting.

**Analysis of Lm-γ2 fragments by immunoblotting.** To analyze Lm-γ2 fragments produced by cultured human cancer cell lines, conditioned medium was collected from confluent culture of each cell line after 2 days of incubation in serum-free medium, and treated with 10% (w/v) trichloroacetic acid to precipitate protein. The protein obtained from 1 mL conditioned medium was applied to SDS-PAGE. To analyze γ2 fragments produced in cancer tissues, two serial frozen sections with an approximate size of 5 × 5 × 0.005 mm were combined and extracted with 100 μL lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% (v/v) Triton X-100, 1 mM EDTA, and protease inhibitors and centrifuged at 21 500 × g for 10 min. Protein in the resultant supernatant was precipitated with 4 volumes of cold acetone and dissolved in 50 μL SDS sample buffer (soluble fraction), while the precipitate was again extracted with 50 μL SDS sample buffer (insoluble fraction). Twenty microliters of each fraction was applied to SDS-PAGE. The electrophoresis was carried out on Any kD TGX gels (Bio-Rad, Hercules, CA, USA) under reducing conditions. For immunoblotting, proteins separated by SDS-PAGE were transferred onto PVDF membranes, probed with indicated antibodies, and visualized by the ECL method (GE Healthcare, Little Chalfont, UK).

**Immunohistochemistry of human cancer tissues.** Five-micrometer serial sections of each tumor tissue were cut on a cryostat and stored at −80°C until use. For IHC, the sections were dried at room temperature for 30–60 min and fixed in ice-cooled acetone for 10 min. After subsequent drying, they were again briefly fixed in 10% (v/v) formalin, incubated in 0.3% hydrogen peroxide, and blocked with 10% (v/v) rabbit serum in PBS. These sections were processed according to the standard method and visualized with 3,3-diaminobenzidine using the Histofine kit (Nichirei Biosciences). In routine experiments, four kinds of mouse mAbs against the three Lm332 chains, which had been prepared in this or out past studies, were used as the first antibodies at the following concentrations: a new antibody against Lm-γ2 dV (P2H; 1 μg/mL), an antibody against Lm-γ2 dIII (D4B5; 2 μg/mL),[38] one against the α3 chain (BG5; 2 μg/mL),[43] and one against the β3 chain (12C; 3 μg/mL).[43]

**Semiquantitative evaluation of IHC staining.** In IHC, positive signals were found in tumor cells, stroma, and normal and neoplastic BMs. The immunostaining intensities obtained with the four antibodies were individually evaluated for tumor cells, stroma, and neoplastic BMs (tumor BMs), in which each

microscopic field (830 × 580 μm) to be scored commonly for the four antibodies was determined as the area showing the highest positive signals with any of the four antibodies. The immunostaining intensity was scored by two observers (K.M. and A.S.) as follows: 0, no or little signal; +1, weak signals; +2, moderate signals; and +3, strong signals. In the tumor cell staining, >50% tumor cells were immunopositive in most cases of the scores of +2 and +3.

**Statistical analysis.** Statistical significance was evaluated with a paired Student's *t*-test. A $P$-value < 0.05 was considered significant.

## Results

**Analysis of Lm-γ2 N-terminal fragments produced by human cancer cells with a new mAb.** The domain structure of the Lm-γ2 chain is shown in Figure 1.[2] In culture, Lm-γ2 is cleaved predominantly at an N-terminal edge of dIII, releasing a 45-kDa N-terminal fragment (γ2pf) consisting of dV and dIV. Mouse mAbs were prepared using recombinant γ2pf protein as an antigen. Of many antibody clones obtained, one clone that recognizes the second N-terminal EGF-like repeat (NE2) of dV, named clone P2H, was chosen in this study.

To show the metabolism of γ2pf, serum-free conditioned media of human cancer cell lines were analyzed by immunoblotting with the anti-dV antibody P2H. The conditioned medium of lung SCC line VMRC-LCP showed many weak bands in a wide molecular weight range of 15–80 kDa as well as the 45-kDa major band of γ2pf and a 150-kDa minor band of the uncleaved γ2 chain (Fig. 2a). Of these additional bands, the 80-, 22-, and 15-kDa bands appeared to correspond to the whole short arm, the full-length dV, and NE-1/2 or NE2/3, respectively. When analyzed with the anti-dIII antibody D4B5, the immunoblot showed the 105-kDa mature, or processed, γ2 chain (or γ2') consisting of dI/II and dIII and several minor bands including the 150-kDa and 80-kDa bands. No immunopositive band was detected by D4B5 in a low molecular weight region under 50 kDa. The lung giant cell carcinoma cell line Lu65 showed 22- and 15-kDa dV fragments as analyzed by the anti-dV antibody but only faint bands around 70 kDa by the anti-dIII antibody. We further analyzed the conditioned media of nine cancer cell lines.[39] All these cell lines released significant amounts of small dV fragments in addition to the γ2pf major band (Fig. 2b). In addition, the fragments of 70 and/or 80 kDa were highly detected in MCAS ovarian carcinoma, PC3 lung carcinoma, and HT1080 fibrosarcoma cells.

To identify enzymes responsible for the generation of small γ2 dV fragments, proteinases secreted by three cancer cell lines were analyzed by gelatin zymography (Fig. S1). Based on our past studies,[44,45] MMP-2, MMP-9, and a low activity of MMP-1 were identified in VMRC-LCP, MCAS, and HT-1080. The presence of the active form of MMP-2 also suggested the expression of MT1-MMP. In addition, soluble forms of matriptase (MT-SP1) were detected as an EDTA-resistant activity in VMRC-LCP and MCAS.[45] When the effects of proteinase inhibitors on the production of dV fragments by VMRC-LCP were examined, a weak but significant inhibition was obtained by the MMP inhibitor TAPI-1 (Fig. S2). The serine proteinase inhibitor aprotinin appeared to slightly inhibit the degradation of the 150-kDa γ2 chain. However, the majority of the dV fragments were not reduced by treatment with these inhibitors, suggesting that they might be intracellularly produced.

We also analyzed whether the dV fragments are produced in lung cancer tissues. When analyzed with the anti-dV antibody P2H, a Triton X-100-soluble fraction from an ADC showed an 80-kDa major band and relatively minor bands of 70, 45, and

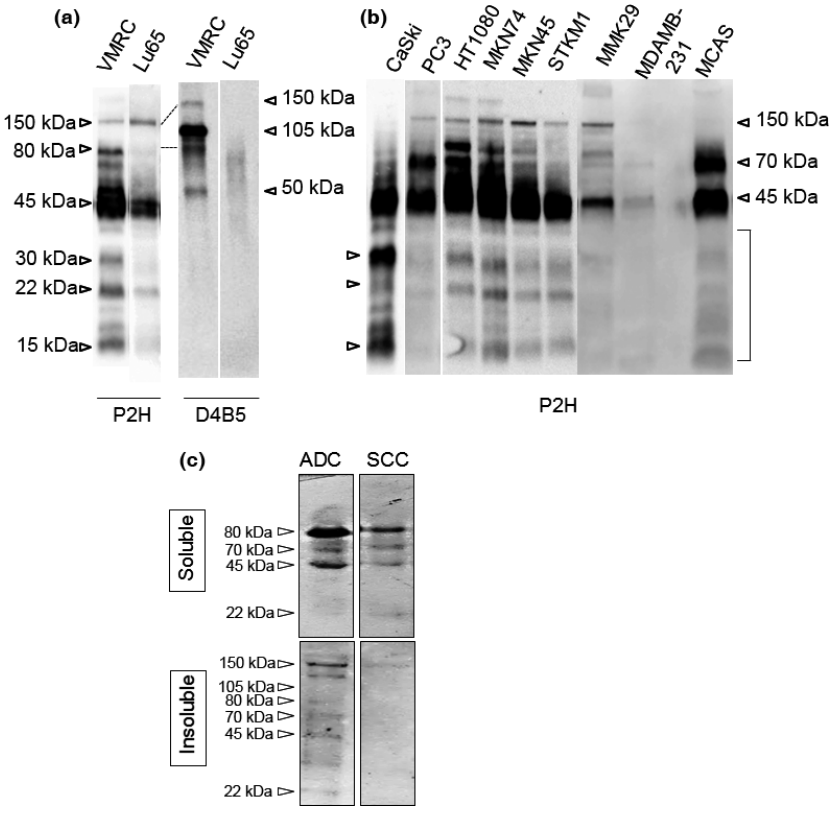


**Fig. 2.** Production of laminin γ2 (Lm-γ2) fragments in cultures of cancer cell lines and lung cancer tissues. (a) Immunoblotting with the anti-domain V (dV) antibody P2H and the anti-dIII antibody D4B5 of γ2 fragments released into conditioned media by the two lung carcinoma lines VMRC-LCP (VMRC) and Lu65. Arrowheads indicate γ2 or its fragments with their approximate molecular sizes in kDa. Note that P2H specifically detect γ2pf as a major band and small dV fragments of 15–30 kDa. The faint 150-kDa band corresponds to the uncleaved γ2 chain, while the 105-kDa major band detected by D4B5 corresponds to the cleaved, or processed, γ2 chain. (b) Immunoblotting of conditioned media from nine cancer cell lines with the P2H antibody. (c) Immunoblotting of soluble and insoluble fractions from an adenocarcinoma (ADC) and a squamous cell carcinoma (SCC) tissues with the anti-dV (P2H) antibody. Note that both soluble fractions contain an 80-kDa fragment as a major band, and γ2pf and small dV fragments can be faintly detected.

22 kDa, while a Triton X-100-insoluble fraction, which seemed to contain the Lm332 matrix, showed the unprocessed 150-kDa γ2 chain and many dV-containing fragments (Fig. 2c). Similar results were obtained with a lung SCC but at lower band intensity. These results verified that the 22-kDa fragment, presumably other small dV fragments too, are produced in human lung cancer tissues. The data also indicated that proteolytic cleavage of the Lm-γ2 chain occurs at C-terminal sites of dIII more frequently *in vivo* than *in vitro* (Fig. 1).

**Distribution of Lm-γ2 N-terminal fragments in lung ADC tissues.** To show the tissue distribution of Lm-γ2 N-terminal fragments in non-small-cell lung cancers, serial frozen sections of 15 ADCs and 14 SCCs were subjected to IHC staining with the anti-dV antibody P2H and with the anti-dIII antibody D4B5. These sections were also immunostained with the anti-α3 antibody BG5 and the anti-β3 antibody 12C. Immunopositive signals, which were detected in the cytoplasm of tumor cells, adjacent stroma, and tumor BMs, were scored into four grades (0, +1, +2, and +3).

In a case of a lepidic component of invasive, acinar ADC, non-neoplastic epithelial BMs close to tumor cells were strongly stained with all of the four antibodies, indicating the presence of the Lm332 heterotrimer assembled into the membrane structures (Fig. 3a–d). Although irregular BM-like structures surrounding tumor cells were poorly detected by the anti-dV antibody (Fig. 3a), they were clearly detected by the other three antibodies including the anti-dIII antibody (Fig. 3b–d). In contrast, only the anti-dV antibody clearly stained the cytoplasm of tumor cells. The differential staining patterns between the anti-dV and anti-dIII antibodies suggest that at least a part of the Lm-γ2 chain is cleaved intracellularly, and γ2pf and other dV fragments remain more stably than the processed, 105-kDa γ2 chain. At an invasion front of the same tumor specimen, where tumor cells had infiltrated stroma, positive signals were found in tumor cells and adjacent stroma intensely with the dV antibody but less markedly with the dIII antibody (Fig. 3e,f). The α3 and β3 chains were faintly detected only on BM-like structures, suggesting that Lm-γ2 was expressed mostly as a monomer form in this area (Fig. 3g,h). However, in many cases of invasive carcinoma tissues with tumor cells having infiltrated in a scattering manner or collectively into the stroma, these invading tumor cells showed strong cytoplasmic staining for not only γ2 dV but also the β3 chain (Fig. 4a–d).

In contrast to the above examples, the cytoplasmic staining of tumor cells for γ2 dV was only focally obtained in a case of lepidic ADC, where tumor cells were separated by continuous BM structures from the adjacent thick stroma (Fig. 4e–h). In this case, positive signals were found in the stroma for both γ2 dV and the α3 chain, and weakly for γ2 dIII but scarcely

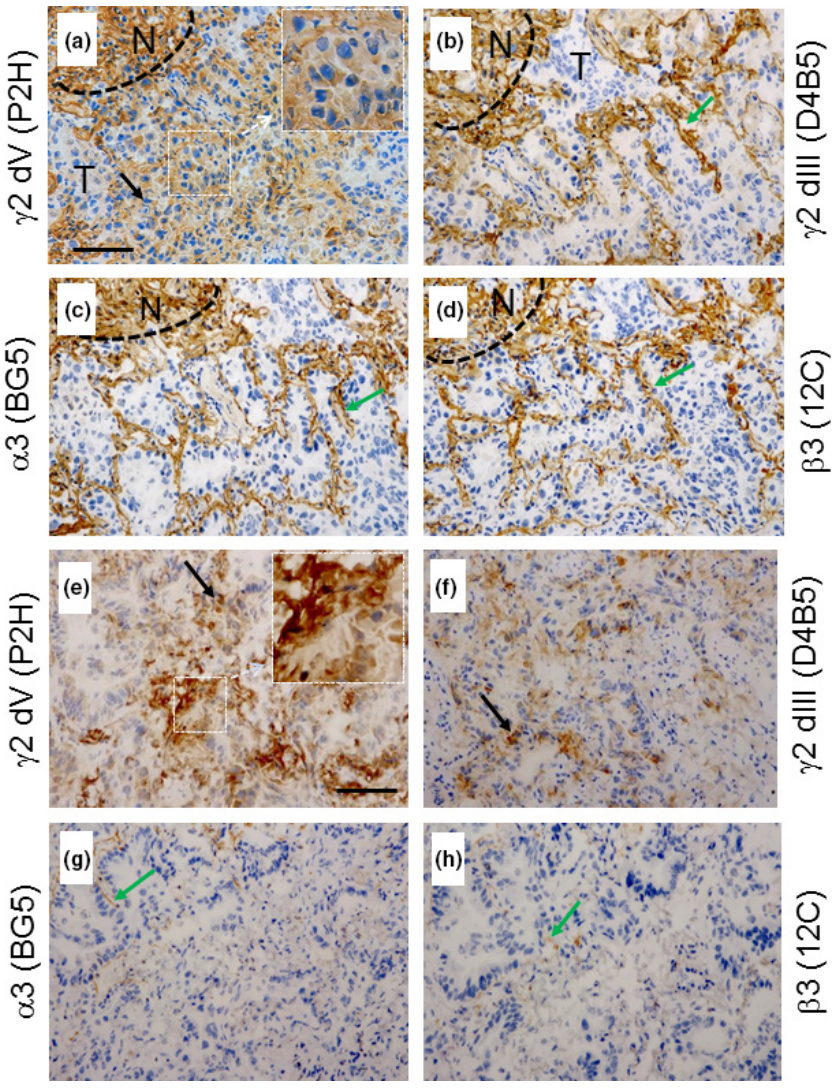


**Fig. 3.** Immunohistochemical staining of acinar adenocarcinoma (#15) for two domains of γ2 (dV and dIII) and α3 and β3 chains of laminin-332 (Lm332). Serial or close sections from the same tissue specimen were immunostained with antibodies against γ2 dV (P2H) (a,e), γ2 dIII (D4B5) (b, f), α3 (BG5) (c, g), and β3 (12C) (d, h). Microscopic fields showing lepidic pattern (a–d) and papillary pattern (e–h) were photographed. Insets show enlarged views of the areas shown by small dashed squares (a, e). Note that the cytoplasm of tumor cells is significantly stained only for γ2 dV in (a–d). N, non-neoplastic basement membranes (BMs); T, tumor cells. Black arrows, positive cytoplasmic signals; green arrows, BM-like structures surrounding tumor cells (tumor BMs). Scale bar = 100 μm.

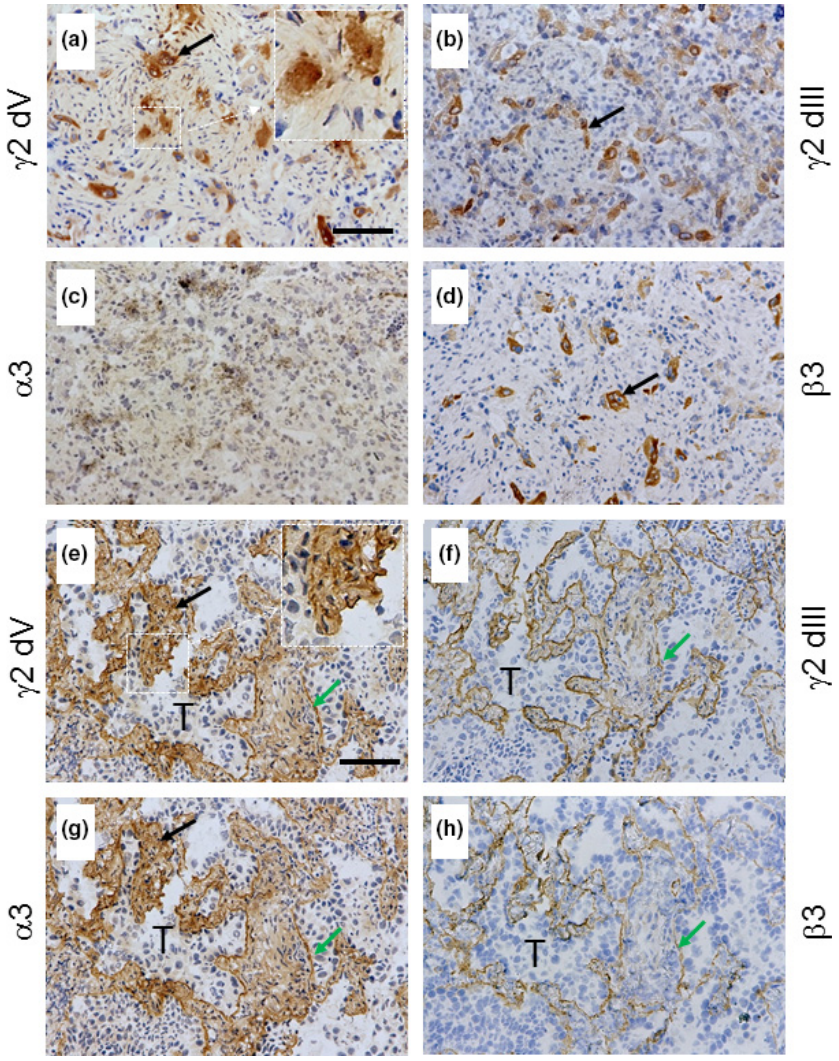


**Fig. 4.** Immunohistochemical staining of solid adenocarcinoma #5 (a–d) and lepidic adenocarcinoma #10 (e–h). In (a–d), tumor cells singly invading stroma show strong immunoreactivity for both domain V (dV) (a), dIII (b), and β3 (d), but not α3 (c). Stromal staining is seen for dV (a) and α3 (c). In (e–h), stroma adjacent to tumor cells was strongly stained for γ2 dV (a) and α3 (c), weakly for γ2 dIII (b), but not at all for β3 (d). Insets show enlarged views of the areas shown by small dashed squares (a,e). Black arrows, positive cytoplasmic or stromal signals; green arrows, tumor basement membranes. T, tumor cells. Scale bar = 100 μm.

for the β3 chain. The stromal overexpression of the γ2 chain was frequently accompanied with that of the α3 chain (Table 1).

**Distribution of Lm-γ2 N-terminal fragments in SCC tissues.** Although SCCs are clearly different from ADCs in their histological characteristics, immunostaining patterns were rather similar to each other. As shown in Figure 5, tumor cells collectively infiltrating stroma with abundant inflammatory cells were stained at the highest level for γ2 dV and moderately for dIII, but weakly or negligibly for β3 and α3, respectively. Tumor BMs were positive for both α3 and β3 but unclear for γ2 dV and dIII. In poorly differentiated carcinomas, the anti-dV antibody well stained both diffusely infiltrating tumor cells and the stroma (Fig. 6a) or tumor cells surrounded by irregular BM-like structures (Fig. 6b). In a moderately differentiated carcinoma, strong signals for γ2 dV were localized on tumor cells at the tumor–stroma interface (Fig. 6c), whereas the tumor cell staining was faint in a well differentiated carcinoma with continuous BM structures (Fig. 6d). In the last case, marked deposition of dV-positive fibrous structures, which were also positive for α3 and β3 (see Table 1), were seen in the stroma adjacent to tumor cells.

All quantitative data shown in Table 1 are summarized in Figure 7. The strong cytoplasmic staining of tumor cells was frequently observed for the γ2 and β3 chains, whereas strong stromal staining was observed for the γ2 and α3 chains. The immunopositive signals in tumor cells and stroma were much higher for γ2 dV than dIII in both ADCs ($P = 1 \times 10^{-4}$ and $3 \times 10^{-5}$, respectively) and SCCs ($P = 1 \times 10^{-3}$ and $2 \times 10^{-3}$, respectively). Although immunoreactivities of tumor BMs were comparable among the four antibodies, those for γ2 dV were significantly lower than those for γ2 dIII ($P = 0.01$) in ADCs, suggesting that γ2 dV had partially been lost in the Lm332 matrix.

As the present study analyzed only a limited number of tumor samples, we were unable to investigate the relationship between the expression data and the prognosis of cancer patients. However, the strong staining of tumor cells for γ2 dV seemed to correlate with cancer malignancy. The rating of score 3 positivity was 75% in poorly differentiated ADCs, which corresponded to malignancy grade G3, but only 27% in the other tumors (G1 + G2). This was also true in SCCs: 78% for G3 *versus* 40% for G1 + G2.

## Discussion

In this study, we prepared a new mAb (clone P2H) against the most N-terminal domain of the γ2 chain (dV). Using this and three other antibodies, we showed the distribution of γ2 short arm fragments and the three Lm332 chains in ADCs and SCCs

**Table 1. Relative expression of three Lm332 chains in two types of lung cancer tissues**

| ADC No. | Stage | Diff. grade† | Tumor cells | | | | Stroma | | | | Tumor BMs | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | γ2dV | γ2dIII | α3 | β3 | γ2dV | γ2dIII | α3 | β3 | γ2dV | γ2dIII | α3 | β3 |
| 1 | 1b | p/d | 3 | 3 | 0 | 3 | 2 | 2 | 2 | 1 | 0 | 0 | 0 | 0 |
| 2 | 1a | w/d | 1 | 0 | 0 | 1 | 2 | 1 | 2 | 1 | 0 | 1 | 2 | 1 |
| 3‡ | 1b | m/d | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| 4‡ | 2b | p/d | 3 | 2 | 0 | 2 | 2 | 1 | 2 | 0 | 0 | 0 | 1 | 0 |
| 5‡ | 1b | p/d | 3 | 2 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | 1a | m/d | 3 | 1 | 0 | 1 | 2 | 1 | 1 | 0 | 0 | 1 | 3 | 2 |
| 7 | 1a | w/d | 2 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 2 | 2 | 3 | 1 |
| 8 | 3a | m/d | 3 | 2 | 1 | 1 | 2 | 1 | 1 | 0 | 0 | 1 | 2 | 1 |
| 9 | 1a | w/d | 2 | 0 | 0 | 2 | 1 | 0 | 3 | 0 | 1 | 1 | 2 | 2 |
| 10 | 1b | m/d | 0 | 0 | 0 | 0 | 3 | 1 | 2 | 0 | 3 | 3 | 3 | 2 |
| 11 | 1a | p/d | 1 | 1 | 1 | 1 | 2 | 0 | 2 | 0 | 0 | 2 | 2 | 1 |
| 12‡ | 2b | m/d | 2 | 1 | 0 | 1 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| 13 | 1b | w/d | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| 14 | 2a | m/d | 1 | 0 | 0 | 0 | 2 | 1 | 1 | 0 | 1 | 3 | 2 | 3 |
| 15 | 2a | m/d | 3 | 1 | 0 | 0 | 3 | 1 | 1 | 0 | 1 | 3 | 3 | 3 |
| Mean | | | 1.87 | 0.87 | 0.2 | 0.93 | 1.73 | 0.73 | 1.4 | 0.13 | 0.53 | 1.13 | 1.53 | 1.07 |
| (SD) | | | 1.13 | 0.99 | 0.41 | 0.96 | 0.8 | 0.59 | 0.83 | 0.35 | 0.92 | 1.19 | 1.24 | 1.1 |

| SCC No. | Stage | Diff. grade† | Tumor cells | | | | Stroma | | | | Tumor BMs | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | γ2dV | γ2dIII | α3 | β3 | γ2dV | γ2dIII | α3 | β3 | γ2dV | γ2dIII | α3 | β3 |
| 1‡ | 1a | p/d | 3 | 3 | 0 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | 1b | m/d | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 2 | 3 | 1 | 2 |
| 3 | 1b | p/d | 3 | 3 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| 4 | 1b | w/d | 1 | 0 | 0 | 1 | 3 | 3 | 2 | 1 | 2 | 1 | 1 | 2 |
| 5 | 1b | p/d | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 3 | 3 | 3 | 3 |
| 6 | 1b | p/d | 3 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 |
| 7 | 1b | p/d | 3 | 1 | 0 | 2 | 2 | 0 | 1 | 0 | 3 | 2 | 3 | 3 |
| 8 | 2b | m/d | 3 | 1 | 1 | 2 | 1 | 0 | 1 | 0 | 3 | 3 | 3 | 3 |
| 9‡ | 2b | p/d | 3 | 2 | 2 | 3 | 3 | 2 | 3 | 3 | 1 | 0 | 1 | 1 |
| 10 | 1a | m/d | 3 | 2 | 0 | 3 | 2 | 1 | 0 | 0 | 0 | 2 | 2 | 2 |
| 11 | 1b | p/d | 3 | 2 | 0 | 3 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 1 |
| 12 | 2b | p/d | 1 | 0 | 0 | 0 | 2 | 1 | 1 | 0 | 2 | 2 | 3 | 3 |
| 13 | 1b | m/d | 3 | 3 | 2 | 3 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 |
| 14 | 1b | p/d | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 3 | 1 | 2 |
| Mean | | | 2.43 | 1.5 | 0.5 | 1.64 | 1.36 | 0.71 | 0.79 | 0.29 | 1.21 | 1.43 | 1.5 | 1.79 |
| (SD) | | | 0.94 | 1.02 | 0.76 | 1.08 | 1.01 | 0.99 | 0.89 | 0.83 | 1.25 | 1.28 | 1.09 | 0.98 |

†w/d (well differentiated), m/d (moderately differentiated), and p/d (poorly differentiated) correspond to the malignancy grades G1, G2 and G3, respectively. ‡Patients who had recurrence or metastasis within 5 years after surgery.

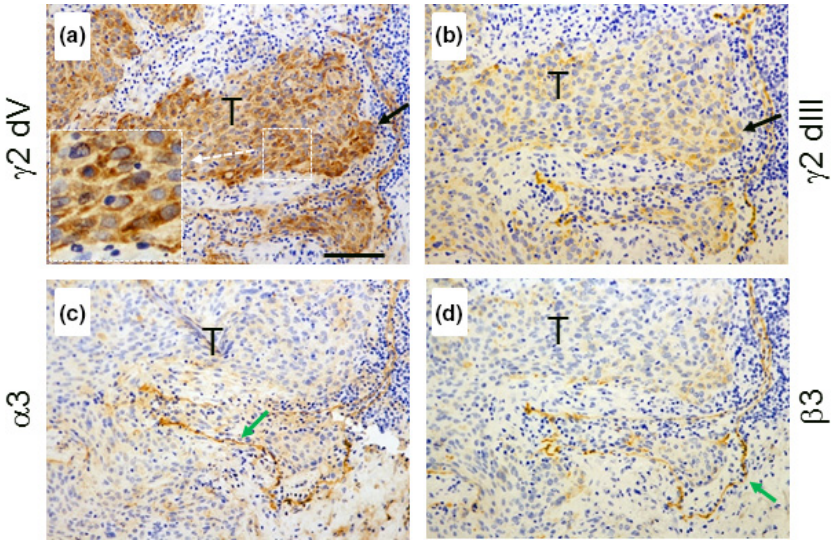


**Fig. 5.** Immunohistochemical staining of poorly differentiated squamous cell carcinoma #6 for γ2 domain V (dV) (a), γ2 dIII (b), α3 (c), and β3 (d). Tumor cells (black arrows) collectively invading stroma show immunoreactivity strongly for dV (a) and moderately for γ2 dIII (b) but poorly for α3 (c) and β3 (d). Basement membrane-like structures (green arrows) are seen for α3 (c) and β3 (d). Insets show an enlarged view of the area shown by a small dashed square (a). Scale bar = 100 μm.

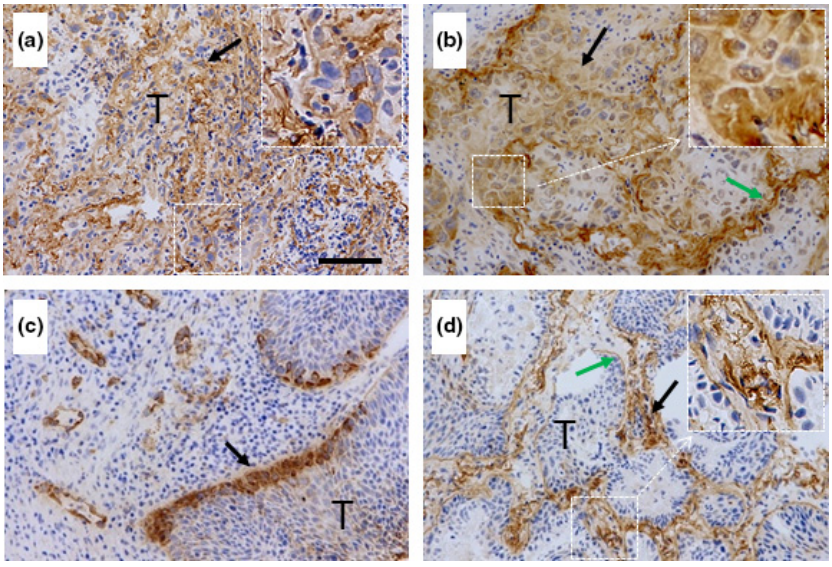


**Fig. 6.** Immunohistochemical staining of four squamous cell carcinomas with different grades for γ2 domain V (dV). (a) Squamous cell carcinoma (SCC) #9, poorly differentiated; (b) SCC #5, poorly differentiated; (c) SCC #10, moderately differentiated; (d) SCC #4, well differentiated. Strong immunoreactivity (black arrows) is seen in tumor cells infiltrating stroma (a), surrounded by basement membrane-like structures (b) and at the tumor–stroma interface (c), but scarcely in the cells surrounded by continuous basement membrane structures (green arrow) (d). In (d), marked deposition of dV-containing fibrils (black arrow) is seen in the stroma. Insets show enlarged views of the areas shown by small broken squares (a, b, d). Scale bar = 100 μm.

of the lung by IHC. We also showed the metabolism of the γ2 short arm *in vitro* and *in viv*o by immunoblotting with these antibodies.

Our immunoblotting data with the anti-dV antibody for the first time revealed that small domain V fragments, as well as the 45-kDa fragment (γ2pf), were produced at high levels by various cultured cancer cell lines. Additionally, the lung cancer tissues contained an 80-kDa fragment as a major component, in addition to γ2pf and small dV fragments. The differential proteolysis between *in vitro* and *in viv*o suggests that proteinases expressed by stromal cells, such as activated fibroblasts and inflammatory cells, are also involved in the γ2 chain cleavage. Although further analysis is required, the present study suggested that small dV fragments were at least partly produced intracellularly.

In spite of a number of past IHC studies on Lm-γ2 expression, to our knowledge, none of them investigated the distribution of its N-terminal fragments. In the present IHC study, the dV antibody showed much stronger signals than the dIII antibody in both tumor cells and stroma, although the signal intensity was reversed in tumor BMs. Most of the ADCs and SCCs tested more or less showed immunoreactivity for γ2 dV in the cytoplasm of invasive cancer cells. This immunoreactivity correlated with the malignancy grade of tumors. It is not peculiar that two antibodies recognizing different epitopes of one antigen detect the antigen differently, depending on its molecular states or conformational changes. Indeed, there are reports that some anti-dIII antibodies show poor cytoplasmic reactivity in frozen sections compared to paraffin sections.[15] In the present study, however, the anti-dIII antibody D4B5 produced strong cytoplasmic positivity in some cases of cancer tissues (Table 1). Our results from both immunoblotting and IHC rather suggest that γ2 dV fragments remain in tumor cells and stroma more stably than dIII fragments. The experiments with proteinase inhibitors, which suggested the intracellular production of dV fragments, also supports this possibility (Fig. S2). This might allow the anti-dV antibody P2H to detect invasive cancer cells more effectively than the dIII antibody. To the contrary, the γ2 chain of the Lm332 heterotrimer assembled into tumor BMs appears to have mostly been processed releasing its N-terminal fragments. Although positive signals for γ2 dV were strongly detected in the cytoplasm of tumor cells, tumor cells were also shown to secrete dV fragments *in vitro*, as shown by immunoblotting data. The Lm-γ2 chain or its short

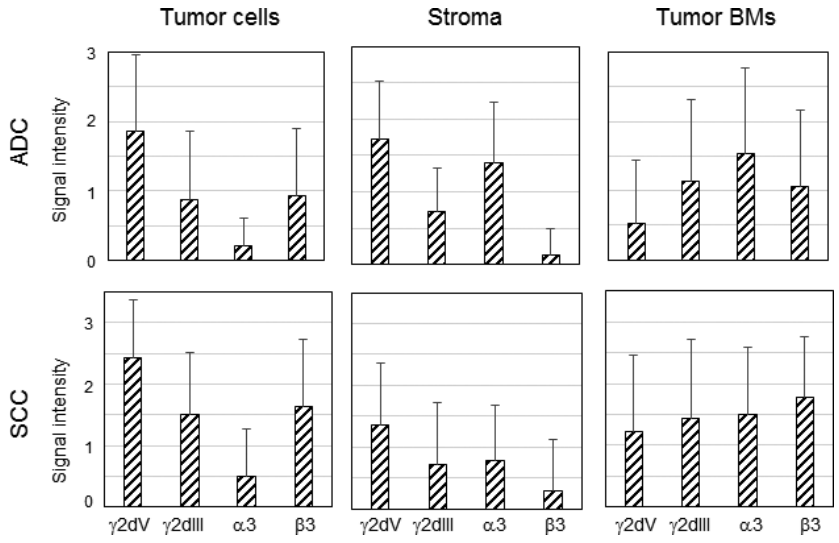


**Fig. 7.** Summary of immunohistochemistry with four antibodies against laminin-332 subunits. Each column indicates the mean score ± SD (bar) for the relative immunoreactivity with the indicated antibody in 15 adenocarcinoma (ADC; upper panels) and 14 squamous cell carcinoma (SCC; lower panels) tissue samples. Antibodies used are P2H (γ2 dV), D4B5 (γ2 dIII), BG5 (α3), and 12C (β3).

arm stimulates tumor cell invasion *in vivo* and *in vitro*,[24,33] and recombinant dV fragments promote transendothelial migration of tumor cells *in vitro* and vascular permeability *in vivo*.[34] We recently found that domain V fragments promote migration of cancer cells by interacting with CD44 on the cell surface.[35] Therefore, the accumulation of dV fragments in the tumor microenvironment seems to favor tumor cell invasion and metastasis. The dV fragments are thought to be possible targets to develop new anticancer drugs.

There are some IHC studies that investigated Lm-γ2 expression in lung cancer tissues.[15–17,33] Moriya *et al.* found, using a γ2 dIII antibody, that the cytoplasmic positivity of lung ADC cells for Lm-γ2 is well correlated with poor patient prognosis, although they did not detect the γ2 expression in tumor BMs or stroma.[17] This correlation has been confirmed by a recent study.[33] The immunostaining patterns of the γ2 expression in the present study are essentially consistent with those reported in past studies with anti-dIII antibodies, but our anti-dV antibody P2H stained invasive tumor cells much more intensely than the anti-dIII antibody D4B5. Our previous study showed frequent overexpression of the Lm-γ2 chain monomer in sclerosing bronchioloalveolar ADC of the lung.[16] In agreement with this report, immunostaining with the dV antibody was especially prominent in tumor cells collectively or diffusely infiltrating into desmoplastic fibrous stroma in both ADCs and SCCs. In contrast, the cytoplasmic staining was absent or very poor in tumor cells separated by thick continuous BM structures, although stromal staining was often found in these cases. It seems evident that active interaction between tumor cells and stromal cells induces γ2 expression in tumor cells. In this regard, it should be noted that γ2 expression is associated with epithelial–mesenchymal transition of carcinoma cells, which is induced by transforming growth factor-β, tumor necrosis factor-α, epidermal growth factor, and other soluble factors *in vitro*.[24]

Of a number of IHC studies of Lm-γ2 expression, only a few have analyzed the expression of the three Lm332 chains.[14,16] In the present study, γ2 signals in tumor cells were often associated with β3 signals, whereas those in tumor stroma were with α3 signals. Franz *et al.*[46] reported that the expression of the Lm-γ2, α3, α4, and α5 chains increases in the stroma of oral SCCs with rising cancer grade. It has been shown that mesenchymal cells contribute to the stromal γ2 expression.[47] Our results suggest that the γ2 chain is mainly expressed as its monomer or the β3γ2 heterodimer in lung cancer cells at an invasion front. As no laminin heterotrimers containing the α3 and γ2 chains without the β3 chain have been identified,[2] the stromal γ2 chain seems to be mainly expressed as the monomer form. It is possible that the stromal α3 chain exists as Lm321 and/or Lm311 heterotrimers. In any form of Lm-γ2 complexes, N-terminal parts of the γ2 chain seem to be mostly released from the C-terminal core structure.

Immunohistochemical detection of Lm-γ2 facilitates the assessment of invasiveness and improves diagnostic reproducibility in some types of cancers.[48,49] In the present study, we developed for the first time antibodies recognizing the most N-terminal γ2 domain, and one of the anti-dV antibodies detected invasive lung carcinoma cells at a high sensitivity. Such anti-dV antibodies seem useful for the pathological assessment of invasiveness and malignancy in various types of human cancers.

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## Disclosure Statement

The authors have no conflict of interest.

## Abbreviations

| | |
|---|---|
| γ2pf | 45-kDa fragment produced by proteolytic cleavage of laminin γ2 chain |
| ADC | adenocarcinoma |
| BM | basement membrane |
| dIII/dIV/dV | domains III/IV/V of laminin γ2 chain |
| IHC | immunohistochemistry |
| KCC | Kanagawa Cancer Center |
| Lm332 | laminin-332 |
| Lm-γ2 | laminin γ2 |
| NE | N-terminal EGF-like repeat |
| SCC | squamous cell carcinoma |

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## Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Fig. S1.** Analysis of proteinases secreted by VMRC-LCP, MCAS, and HT1080 cells by gelatin zymography.

**Fig. S2.** Effects of serine proteinase and metalloproteinase inhibitors on production of γ2 domain V fragments by VMRC-LCP human lung carcinoma cells.