

## Enhanced Expression of Aggrus (T1alpha/Podoplanin), a Platelet-Aggregation-Inducing Factor in Lung Squamous Cell Carcinoma

Yukinari Kato<sup>a, c</sup> Mika Kaneko<sup>a</sup> Makoto Sata<sup>b</sup> Naoya Fujita<sup>c</sup>  
Takashi Tsuruo<sup>c, d</sup> Motoki Osawa<sup>a</sup>

<sup>a</sup>Department of Experimental and Forensic Pathology and <sup>b</sup>First Department of Internal Medicine, Yamagata University School of Medicine, Yamagata, and <sup>c</sup>Institute of Molecular and Cellular Biosciences, University of Tokyo, and <sup>d</sup>Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan

### Key Words

Aggrus · Lung cancer · Platelet aggregation · Squamous cell carcinoma · Tumor marker

### Abstract

Aggrus (T1 $\alpha$ /podoplanin, known as a specific marker for type I alveolar cells or lymphatic endothelial cells) is a transmembrane sialoglycoprotein that aggregates platelets. Previously, we showed that upregulated expression of Aggrus occurs in colorectal tumors or testicular tumors and could be associated with platelet-aggregating activity and metastatic ability. In testicular tumors, Aggrus is specifically expressed in seminoma. The present study investigates Aggrus expression in human primary lung cancer tissues of different types. Microarray analysis demonstrated that *aggrus* was significantly expressed in squamous cell carcinoma (10/15; 66.7%). Immunohistochemical analysis also showed that the incidence of positive staining in sections of squamous cell carcinoma (7/8; 87.5%) was higher than that in adenocarcinoma (2/13; 15.4%). Furthermore, Aggrus expression was detected in a squamous cell carcinoma cell line, NCI-H226, by real-time PCR. These findings indicated that overexpression of Aggrus occurred in squamous cell carcinoma of the lung. Therefore, Aggrus could be a useful diagnostic marker for squamous cell carcinoma of the lung.

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### Introduction

Lung cancer is the leading cause of cancer death worldwide, accompanied by a continuing increase of patients [1]. The major classification of carcinomas consists of small cell lung carcinoma (SCLC) or non-SCLC (NSCLCs). Furthermore, NSCLCs are divided histologically into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. The NSCLCs must be diagnosed precisely at the time of surgical resection as distinct from SCLC because prognosis depends on the histopathological types. Therefore, specific diagnostic markers have been sought to evaluate types and malignancy.

We have previously established several clones possessing different platelet-aggregation-inducing capabilities and metastatic abilities from a mouse colon adenocarcinoma 26 cell line [2]. The expression level of Aggrus, a platelet-aggregating factor, was correlated with the platelet-aggregating activity and metastatic ability in a mouse colon adenocarcinoma 26 cell line [3, 4]. Cloning of cDNA revealed that Aggrus was identical to a separately isolated protein of mouse T1 $\alpha$  antigen (also known as gp38P/OTS-8) [5–7]. In experiments using the 8F11 antibody that neutralized platelet aggregation, the platelet-aggregation-stimulating (PLAG) domain was identified to a portion spanning amino acid sequence 28–39 of mouse Aggrus molecules [5]. Amino acid sequences of PLAG domains are highly conserved in orthologues of

mouse Aggrus and human Aggrus (also known as hT1 $\alpha$ -2/gp36/podoplanin) despite a relatively low overall amino acid identity (39%) [5, 8]. Residual mutagenesis to threonine in the domain abolished PLAG activities of both human and mouse Aggrus. It was also evident that overexpression of the *aggrus* gene occurred in human intestinal cancer tissues or testicular tissues, which was displayed on cancer profiling arrays [5, 9].

Aggrus expression was also found in lymphatic endothelium [10], alveolar type I cells in rat lung [11], choroid plexus, ciliary epithelium of the eye, intestine, kidney, thyroid and esophagus of the fetal rat [12] and in MC3T3-E1 mouse osteoblast cells [7]. Scholl et al. [13] reported that PA2.26/Aggrus is concentrated in actin-rich microvilli and plasma membrane projections, such as filopodia, lamellipodia and ruffles, where it colocalizes with members of the ERM (ezrin, radixin, moesin) protein family, indicating that PA2.26/Aggrus is involved in cell migration. Ramirez et al. [14] generated *T1 $\alpha$ /aggrus* null mice containing a targeted mutation in the *T1 $\alpha$ /aggrus* gene locus. Homozygous null mutant mice die at birth of respiratory failure. Recently, we indicated that sialylated core 1 structures were critical for the platelet aggregation activity [15, 16].

This study uses real-time PCR and immunohistochemical analysis to investigate expression of Aggrus in clinical specimens of NSCLCs and cells of four lung cancer cell lines.

## Materials and Methods

### Cell Culture Conditions

Human lung cancer cell lines, A549, NCI-H23, NCI-H226 and NCI-H522 [17–20] were cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in humidified air containing 5% CO<sub>2</sub>.

### Cancer Profiling Array Analysis

Cancer Profiling Arrays I and II were purchased from BD Biosciences (Brentford, Mass., USA). Cancer Profiling Array I consists of 241 cDNA pairs and Array II consists of 160 cDNA pairs, synthesized from human tumorigenic and corresponding normal tissues. Each pair was independently normalized based on the expression of four housekeeping genes and immobilized in separate dots. The full-length <sup>32</sup>P-labeled human *aggrus* cDNA was used for hybridization; the radioactivity of each dot was visualized and quantified with a BAS1000 Bio-Imaging analyzer (Fuji Film, Tokyo, Japan). The expression level of human *aggrus* mRNA in individual patients was normalized by measuring the radioactivity of each dot after hybridization with <sup>32</sup>P-labeled *ubiquitin* cDNA [5]. Statistical significance of *aggrus* mRNA expression in squamous cell carcinoma and adenocarcinoma tissues was performed using paired t test.

### Quantitative Real-Time PCR

The first-strand cDNA samples of patients 3, 20 and 21 were purchased from BD Biosciences. Total RNAs were prepared from four lung cell lines employing an RNeasy mini prep kit (Qiagen, Hilden, Germany). First-strand cDNAs were synthesized by M-MLV reverse transcriptase (USB, Cleveland, Ohio, USA) via priming nine random oligomers according to the manufacturer's instructions. The primer set for *aggrus* was as follows: the forward primer was 5'-GGAAGGTGTCAGCTCTGCTC-3' and the reverse primer was 5'-CGCCTTCCAAACCTGTAGTC-3'. The primer set for  $\beta$ -actin was: 5'-ACTCTTCCAGCCTTCCTCCTG-3' and 5'-ATCTCCTTCTGCATCCTGTCCGG-3'. Real-time PCR was performed using the QuantiTect SYBR Green PCR (Qiagen). The PCR conditions were 95°C for 15 min for 1 cycle, followed by 40 cycles of 94°C for 15 s, 55°C for 25 s, 72°C for 20 s, and 0.2°C s<sup>-1</sup> to 40°C to perform a melting curve analysis. Standard curves for templates of *aggrus*, and an endogenous control,  $\beta$ -actin, were generated by serial dilution of the PCR products (1  $\times$  10<sup>8</sup> copies/ $\mu$ l to 1  $\times$  10<sup>3</sup> copies/ $\mu$ l).

### Immunohistochemistry

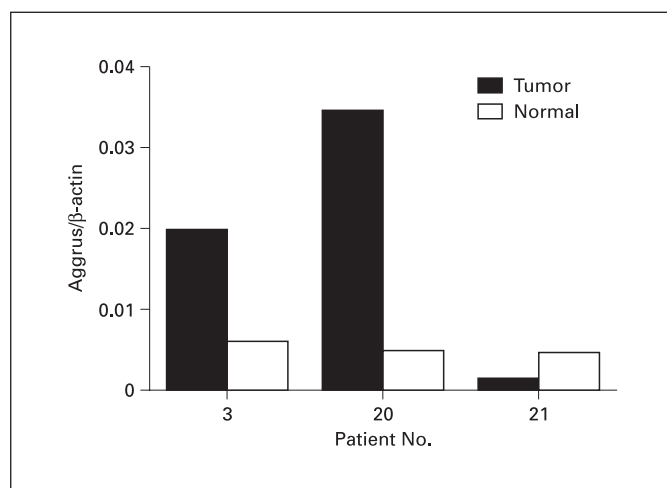
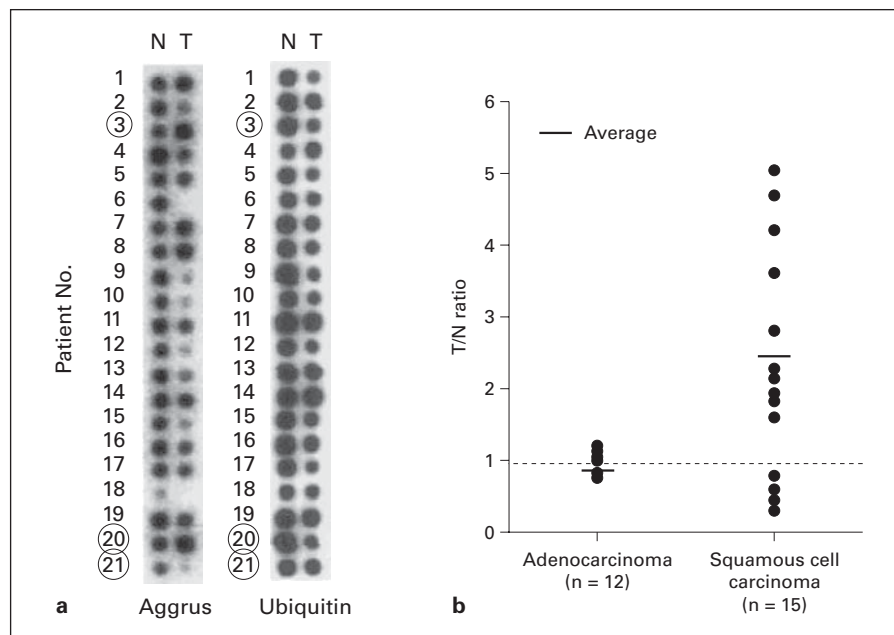
A lung tissue array was purchased from Ambion (Austin, Tex., USA). Anti-human Aggrus polyclonal antibody (TT679) was prepared in rabbits by immunization of a synthetic oligopeptide, CEGGVAMPGAEDDVV, corresponding to amino acids 38–51 of the human Aggrus sequence plus N-terminal cysteine. Affinity purification was performed using a gel column, which contained the chemically linked peptide [5]. The monoclonal antibody to human cytokeratin 19 (BA17) was obtained from Research Diagnostics (Flanders, N.J., USA). The antibodies were incubated with deparaffinized and rehydrated sections at a concentration of 1  $\mu$ g/ml at 23°C for 4 h. The DAKO CSA system (DakoCytomation, Glostrup, Denmark) was employed for subsequent development, in which procedures were performed according to the manufacturer's instructions.

## Results

### Expression of *aggrus* in Lung Squamous Cell Carcinoma Tissues

Expression of the *aggrus* gene was estimated for human lung cancer tissues displayed in the Cancer Profiling Arrays I and II. In these arrays, cDNA pairs from primary tumor tissues and corresponding normal tissues from individual patients were immobilized on a nylon membrane. We revealed here the increase in *aggrus* mRNA expression in squamous cell carcinoma of the lung compared with adenocarcinoma in Cancer Profiling Array I (fig. 1a). Results of Cancer Profiling Array II have been described previously [5]. Signal intensities were detected semiquantitatively after hybridization with <sup>32</sup>P-labeled cDNA probes of human *aggrus* and *ubiquitin* (fig. 1a). The level of *aggrus* expression was represented as a relative value to that of *ubiquitin* expression. The T/N ratio was more than 1.5 in 10 of 15 squamous cell carcinoma

**Fig. 1.** Increased *aggrus* mRNA expression in squamous cell carcinoma of lung. Human *aggrus* mRNA expression in tumor (T) and normal (N) tissues in the same patient was assessed by hybridizing  $^{32}\text{P}$ -labeled human *aggrus* cDNA to Cancer Profiling Arrays I and II. Radioactivity of each dot was visualized in Profiling Array I (a) and quantified in Profiling Arrays I and II (b). b The relative *aggrus* mRNA expression level in the each dot was normalized by measuring radioactivity of each dot after hybridization with  $^{32}\text{P}$ -labeled *ubiquitin* cDNA. The T/N ratio was calculated by dividing the normalized radioactivity of tumor samples with that of normal tissues in individual patients. Horizontal columns = Average T/N ratio in either adenocarcinoma or squamous cell carcinoma.



**Fig. 2.** Quantitative analysis of *aggrus* transcripts in lung tumors and corresponding normal tissues by real-time PCR. The first-strand cDNA samples derived from tumorigenic and corresponding normal tissues of patients 3, 20 and 21 were used as templates for real-time PCR. The respective expression levels of *aggrus* were normalized to that of the  $\beta$ -actin transcript.  $Aggrus/\beta$ -actin = *Aggrus* mRNA/ $\beta$ -actin mRNA ratio.

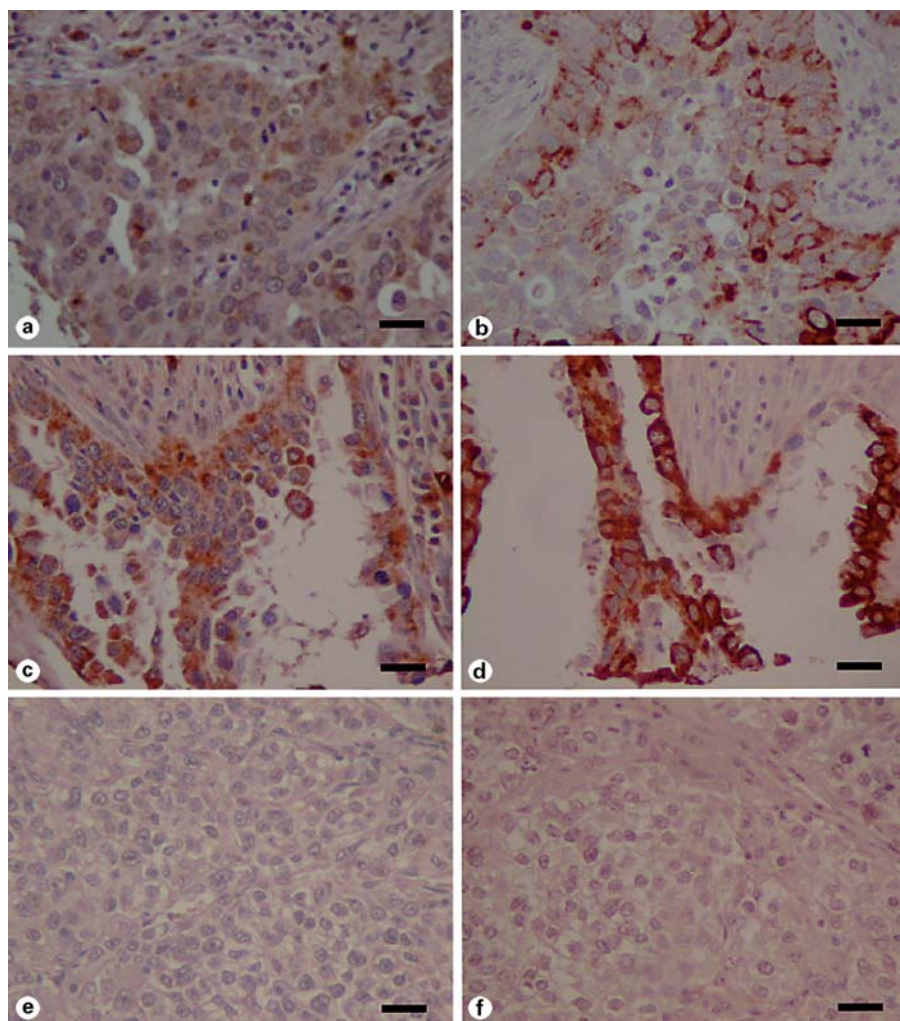
specimens (67%), in contrast to lower ratios exhibited by adenocarcinoma specimens. As shown in figure 1b, the mean value in squamous cell carcinoma was significantly higher than that in adenocarcinoma ( $p < 0.01$ ).

We could obtain cDNA prepared from only 3 squamous cell carcinoma patients (patients 3, 20 and 21 cir-

cled in fig. 1a) and performed quantitative real-time PCR to confirm array analysis results (fig. 2). Consistent with the array analysis, quantitative PCR analysis revealed that human *aggrus* mRNA expression was markedly enhanced in squamous cell carcinoma derived from patients 3 and 20, but not from patient 21.

#### *Expression of Aggrus Protein in Lung Squamous Cell Carcinoma Tissues*

Previously, we generated polyclonal antibody to human Aggrus (TT679) since the 8F11 antibody, which can bind to mouse Aggrus protein and neutralize mouse Aggrus-induced platelet aggregation, could not recognize human Aggrus [5]. The TT679 antibody specifically recognized the 36-kDa human Aggrus protein and immunohistochemically reacted with colorectal tumors or seminoma [5, 9]. TT679 also stained lymphatic vessels in colon tissues like several anti-podoplanin antibodies [9]. In this study, we stained 21 lung cancer specimens (8 squamous cell carcinomas and 13 adenocarcinomas). Strong staining was observed in almost all squamous cell carcinoma specimens (fig. 3a, c), but specimens from adenocarcinoma patients did not stain (fig. 3e). Furthermore, no staining was observed in normal lung (data not shown). Seven of 8 squamous cell carcinoma patients (87.5%) showed positive staining of Aggrus, whereas only 2 of 13 adenocarcinoma patients (15.4%) showed positive staining ( $p < 0.01$ ). Subsequently, we compared the TT679 reactivity with anti-human cytokeratin 19 monoclonal



**Fig. 3.** Aggrus protein was strongly expressed in squamous cell carcinoma of the lung. Immunostaining was done by TT679 (**a, c, e**) or BA17 (**b, d, f**) with a DAKO CSA System. Squamous cell carcinoma from patient 31 (**a, b**), squamous cell carcinoma from patient 42 (**c, d**) and adenocarcinoma from patient 35 (**e, f**) are shown. Bars = 10  $\mu\text{m}$ .

antibody (BA17), which is used as a tumor marker of squamous cell carcinoma (fig. 3b, d, f) [21]. Five of 8 squamous cell carcinoma patients (62.5%) showed positive staining of cytokeratin 19 by BA17. Interestingly, squamous cell carcinoma specimens from patients 42 and 51 were stained only by TT679, not by BA17, indicating that TT679 is highly reactive with squamous cell carcinoma compared with BA17. Of 13 adenocarcinoma specimens, 2 adenocarcinoma specimens (patients 49 and 52) were stained by both TT679 and BA17, while the others neither stained by TT679 nor by BA17.

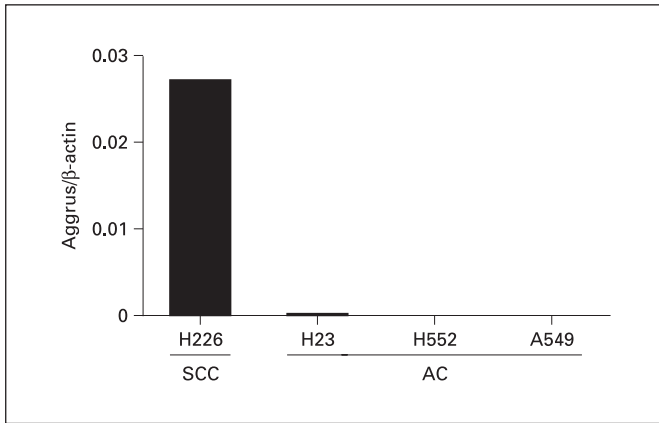
#### *Expression of aggrus in Culture Cells Derived from Lung Cancer*

Expression of *aggrus* was examined further in four established cell lines of human lung cancer. Total mRNA

was prepared from cultured cells of one squamous cell carcinoma cell line (NCI-H226) and three adenocarcinoma cell lines (NCI-H23, NCI-H522 and A549). In real-time RT-PCR analysis, a high level of *aggrus* expression was evident only in NCI-H226 cells, but not in the others (fig. 4).

#### **Discussion**

Human Aggrus was expressed in colorectal tumors [5] and testicular tumors [9]. In testicular tumors, Aggrus expression was high in seminomas, but its expression was not observed in embryonal carcinomas [9]. On the other hand, *aggrus* expression in lung tumors varied from patient to patient in Cancer Profiling Array II [5]. Therefore,



**Fig. 4.** Expression of *aggrus* in the squamous cell carcinoma cell line (SCC). Total RNAs were prepared from the indicated human lung cancer cell lines. The respective expression levels of *aggrus* were normalized to that of the  $\beta$ -actin transcript. AC = Adenocarcinoma; *aggrus*/ $\beta$ -actin = *aggrus* mRNA/ $\beta$ -actin mRNA ratio.

this study estimated the human *aggrus* expression in human lung cancer of different histological types. As shown in figure 1, we revealed the increase in *aggrus* mRNA level in squamous cell carcinoma of the lung compared with corresponding normal lung tissues in Cancer Profiling Array. Furthermore, the increase was not seen in adenocarcinoma of the lung, indicating that overexpression of *aggrus* is specific for squamous cell carcinoma. In immunohistochemical analysis, human Aggrus was also specifically detected in squamous cell carcinoma tissues (fig. 3). Human Aggrus is also known as hT1 $\alpha$ -2, a specific marker of type I alveolar cells [8]; furthermore, it was also expressed in bronchial epithelium (data not shown). Squamous cell carcinoma arises in the larger, more central bronchi, while adenocarcinoma are usually more peripherally located. From now on, the reason why Aggrus was specifically overexpressed in squamous cell carcinoma should be clarified, although both squamous cell carcinomas and adenocarcinomas were known to be derived from the bronchial epithelium.

We investigated the expression of *aggrus* in cell lines derived from squamous cell carcinoma or adenocarcinoma. As shown in figure 4, *aggrus* was specifically detected in NCI-H226, a squamous cell carcinoma cell line, by real-time PCR analysis. Previously, we clarified the expression of Aggrus on NCI-H226 by flow cytometry and showed the platelet-aggregation-inducing ability of NCI-H226 [15]. In our previous studies, we observed the expression of mouse *aggrus* in a mouse colon adenocarcinoma cell line (NL-17) and a melanoma cell line (B16-

F10) that have platelet-aggregation-inducing capability. Furthermore, their pulmonary metastatic abilities were highly associated with their platelet-aggregation-inducing capabilities [5]. Therefore, NCI-H226 cells might also have metastatic ability in vivo. Since we could not obtain sufficient clinical information about patients examined in this study, we could not discuss the relationship of the expression level of Aggrus with the clinical prognosis of squamous cell carcinoma patients. Further studies, including careful clinical follow-ups of squamous cell carcinoma patients, are needed to evaluate the significance of Aggrus with respect to prognosis. However, it is intriguing to speculate that overexpression of Aggrus in lung squamous cell carcinoma might be associated with its migrating activity [13].

NSCLCs are a heterogeneous group of tumors with variable clinical courses [1]. However, most studies that have investigated treatment efficacy for NSCLCs have grouped squamous cell carcinoma and adenocarcinoma of the lung together as NSCLCs, despite likely biological differences between the two histological subtypes. Indeed, clinical prognosis is better for squamous cell carcinoma than for adenocarcinoma. Therefore, the availability of a reliable tumor marker that would reflect the change in tumor burden would be helpful to: specify treatment protocols; determine which patients need more aggressive surgery, adjuvant chemotherapy, or radiotherapy; monitor treatment efficacy, and to assist in decision-making for further therapy in NSCLCs. For the detection of changes in tumor behavior of NSCLCs, several tumor markers have been evaluated, including carcinoembryonic antigen, squamous cell carcinoma antigen and soluble cytokeratin 19 fragment (CYFRA21-1) [22–24]. Some study results have revealed that these markers may contribute to diagnosis, staging, monitoring, and prognosis of NSCLCs to some extent, but that they are not yet ideal, especially in squamous cell carcinoma [22–24]. Sensitivity of CYFRA21-1, which seems to have the highest sensitivity in squamous cell carcinoma of lung thus far, is 57% for squamous cell carcinoma and 27% for adenocarcinoma of the lung [24], whereas those of Aggrus are 67 and 0%, respectively, when the cutoff values of the T/N ratio were defined as 1.5 in Cancer Profiling Array. Regarding immunostaining of Aggrus by TT679, 87.5% of squamous cell carcinomas and 15.4% of adenocarcinomas showed positive staining. These data indicate a higher sensitivity and specificity of Aggrus for lung squamous cell carcinoma than CYFRA21-1. The detection of Aggrus protein in this study was limited to the tissues from lung cancer patients. Therefore, we should produce the

ELISA system that can detect the serum Aggrus and compare its specificity with other tumor markers of squamous cell carcinoma.

In conclusion, Aggrus is overexpressed in squamous cell carcinoma of the lung, and NCI-H226 cells, which expressed Aggrus on the cell membrane, could possess platelet-aggregation-inducing ability. Therefore, Aggrus may be associated with platelet-aggregating activity and metastatic abilities of squamous cell carcinoma of the lung. Furthermore, Aggrus could be a novel sensitive and specific tumor marker for patients with lung squamous

cell carcinoma. In the future, Aggrus will be useful as a tumor marker if it can be detected in the serum of patients.

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