Table 2. *Continued*

Patient characteristics	Patients' willingness to continue living at home: Present (n = 64) No. of patients (%)	Absent (n = 9) No. of patients (%)	p value
E. Desire for home care			
Present	42 (89)	5 (11)	0.34[2]
Absent	22 (85)	4 (15)	
F. PFC discharge-related information			
Relationship with patient			
Spouse	49 (91)	5 (9)	0.33[1]
Child	8 (73)	3 (27)	
Parent	3 (100)	0 (0)	
Sibling	2 (67)	1 (33)	
Friend	1 (100)	0 (0)	
Other	1 (100)	0 (0)	
Secondary caregiver(s)			
Present	58 (87)	9 (13)	0.49[2]
Absent	6 (100)	0 (0)	
PFC satisfaction with discharge care[3] (range 0–32)			
<21 points	11 (92)	1 (8)	0.43[1]
21–25 points	21 (91)	2 (9)	
26–27 points	10 (83)	2 (17)	
>≠28 points	22 (85)	4 (15)	
G. Patient care status			
Support and information are available when there are changes in care status			
Not true	15 (100)	0 (0)	0.28[1]
Marginally true	11 (79)	3 (21)	
Somewhat true	12 (92)	1 (8)	
Quite true	12 (86)	2 (14)	
Very true	14 (82)	3 (18)	
You feel healthy			
Not true	5 (71)	2 (29)	0.79[1]
Marginally true	11 (92)	1 8)	
Somewhat true	17 (100)	0 (0)	
Quite true	15 (88)	2 (12)	
Very true	16 (80)	4 (20)	
Respite from care			
Not true	2 (100)	0 (0)	1.00[1]
Marginally true	2 (67)	1 (33)	
Somewhat true	17 (85)	3 (15)	
Quite true	24 (96)	1 (4)	
Very true	19 (83)	4 (17)	
Additional support etc			
Not true	31 (97)	1 (3)	0.01**[1]
Marginally true	11 (85)	2 (15)	
Somewhat true	14 (93)	1 (7)	
Quite true	5 (83)	1 (17)	
Very true	3 (43)	4 (57)	
Satisfied with life (satisfied with present QOL)			
Not true	4 (100)	0 (0)	0.19††[1]
Marginally true	7 (100)	0 (0)	
Somewhat true	10 (83)	2 (17)	
Quite true	18 (95)	1 (5)	
Very true	2 (25)	6 (75)	

[1]Cochran-Armitage's trend test, [2]Fisher's exact test, [3]Percentile point
††$P < 0.2$, †$P < 0.1$,*$P < 0.05$, **$P < 0.01$

Table 3. *Result of hierarchical regression analysis on patients' willingness to continue living at home (n = 73)*

	Model 1		Model 2		Model 3		Model 4	
	OR[a]	(95% CI[b])	OR[a]	(95% CI[b])	OR[a]	(95% CI[b])	OR[a]	(95% CI[b])
Patient characteristics								
Age (years)		—		—		—		—
Sex (1. Male/0. Female)		—		—		—		—
Performance Status (0–4)		—		—		—		—
No. of medical treatments (0–4)	0.49*	(0.23–0.97)	0.44*	(0.19–0.90)	0.39*	(0.13–0.94)	0.20*	(0.05–0.72)
Desire for home care (1. Present/0. Absent)	3.32	(0.74–17.34)	3.26	(0.64–20.71)		—		—
Patient discharge-related information								
Consistency with care envisioned by patient			2.70*	(1.34–6.41)	2.39	(0.95–7.19)	2.77*	(1.08–8.62)
Patient QOL								
Physical well-being					0.86	(0.67–1.01)	0.83	(0.61–1.02)
Social well-being						—		—
Emotional well-being						—		—
Functional well-being					1.36*	(1.06–1.94)	1.45*	(1.08–2.17)
PFC status								
Age (years)								—
Gender (1. Male/0. Female)								—
Additional support								—
Satisfied with life (satisfied with current QOL)							2.37*	(1.15–5.77)
R^{2c}	0.09		0.16		0.		0.26	
MR^{2d}	0.17		0.30		0.39		0.50	

*$p < .05$
— denotes item was not selected by backward elimination ($p > .2$)
[a]Adjusted odds ratio
[b]95% confidence interval
[c]Coefficient of determination
[d]Max-rescaled coefficient of determination

was found to be associated with the desire to maintain the current home care.

The support of the caregiver is an essential part of home care, and it seems that patients are sensitive to the situation of the caregivers close to them and worry about their caregivers' well-being since it relates to their giving care at home. The caregivers' satisfaction with life appears to bolster the willingness of patients to continue home care.

In the present study, the model contribution ratios were increased by adding the variables of caregiver status to those of patient characteristics, indicating that the attitudes and well-being of caregivers are important factors in the willingness of patients to continue home care and should be taken into account.

Assistance during the Early Phase of Home Care of Terminally Ill Cancer Patients to Promote Its Continuance

Taking account of the caregiver's status is essential if appropriate assistance is to be given during the early phase of home care. Our results indicate that efforts to promote consistency between the care envisioned by the patient and its reality are important, as are measures to reduce patients' fears of difficulties resulting from medical treatments. Thus, there is a substantial need to improve discharge assistance and continuing care, for example, via outpatient counseling for both patients and caregivers (Naylor et al., 1999, 2000; Naylor, 2000).

The importance of the role of caregivers, who are in closest contact with patients, was confirmed by the finding that the level of satisfaction with life of caregivers is associated with the willingness of patients to continue home care. Therefore assessment over time and finding a place to discuss such matters as the feelings about their current lives, not only of patients but also of caregivers, is desirable.

Our results suggest that the following aspects of care should be considered in the development of high quality transitional care from CCCs to the patient's own home in Japan: tailoring a support system for cancer pain relief and other physical suffering, coordinating care with other medical fa-

cilities, explaining the medical condition to the patient, coordinating the patient/family relationship with regard to telling the patient the diagnosis, and coordinating the provision of welfare services such as nursing, equipment rental, and provision of home helpers.

We recognize that this study has several limitations. The percentage of valid responses was low. However, virtually no studies exist in which an equal number of responses have been obtained from terminally ill cancer patients and their caregivers (Rinck et al., 1997). In addition, this study was cross-sectional. Future work should measure changes in the willingness of patients to continue home care over time and to further elucidate factors affecting this outcome, such as changes occurring in the living environment during home care.

This is the first quantitative study of the transition period from CCC to home care experienced by both terminally ill cancer patients and their caregivers in Japan. An understanding of the factors that determine the willingness of patients to continue living at home is necessary for planning the support required for a smooth transition to care at home and for providing solutions to problems encountered by health care professionals who provide home care.

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Prognostic Significance of Thin-Section CT Scan Findings in Small-Sized Lung Adenocarcinoma*

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Objectives: **The purpose of this study is to evaluate the prognostic importance of thin-section (TS) CT scan findings in small-sized lung adenocarcinomas.**

Patients and methods: **We reviewed TS-CT scan findings and pathologic specimens from 359 consecutive patients who underwent surgical resection for peripheral lung adenocarcinomas ≤ 20 mm in diameter during the period from July 1997 to May 2006. By using TS-CT scan images, tumors were defined as air-containing types if the maximum diameter of tumor opacity on mediastinal window images was less than or equal to half of that seen on lung window images, and as a solid-density type if the maximum diameter on the mediastinal window images was more than half of that on lung window images. We compared TS-CT scan findings to pathologic findings (*ie*, lymph node metastasis, pleural invasion, vessel invasion, and lymphatic invasion) and prognosis. The following prognostic factors were analyzed by χ^2 test and Cox proportional hazard model: age; gender; tumor size; pathologic stage; TS-CT scan findings; histologic subtypes defined by Noguchi et al (*ie*, Noguchi type); pleural involvement; lymphatic invasion; and vascular invasion.**

Results: **No pathologic invasive findings or recurrence were found in patients with air-containing-type tumors. Pathologic invasive findings and recurrence were found in 10 to 30% of patients with solid-density-type tumors. The air-containing type tumors seen on TS-CT scans and Noguchi type A or B tumors were demonstrated as prognostic factors for good outcome by χ^2 test ($p < 0.001$). Multivariate analyses revealed lymphatic permeation as a significant prognostic factor.**

Conclusion: **The TS-CT scan findings were important predictive factors for postsurgical outcome in patients with lung adenocarcinoma.** ***(CHEST 2008; 133:441–447)***

Key words: bronchioloalveolar cell carcinoma; ground-glass opacity; limited surgery; noninvasive cancer

Abbreviations: BAC = bronchioloalveolar cell carcinoma; GGO = ground-glass opacity; HU = Hounsfield units; TS = thin section

The number of patients with small-sized lung carcinoma has been increasing due to the routine clinical use of CT scanning and the increasing use of helical CT scan screening for lung cancer. Adenocarcinoma is the most common histologic type of lung cancer in those cases. The population of lung adenocarcinoma is heterogeneous, and many subtypes of adenocarcinoma have been advocated.[1,2] For example, Noguchi et al[1] classified small-sized lung adenocarcinoma into six subtypes based on tumor growth patterns. In this study, a type A or B tumor was localized bronchioloalveolar cell carcinoma (BAC), which showed no lymph node metastasis, rare vascular and pleural invasion, and excellent prognosis (5-year survival rate, 100%). A type C tumor was BAC with foci of active fibroblast proliferation, and showed pathologic invasive findings, and poor prognosis (5-year survival rate, 74.8%). A type D, E, or F tumor was adenocarcinoma without BAC and showed worst prognosis (5-year survival rate, 52.4%). Although these pathologic characteristics are useful as prognostic indicators, the results are defined only after surgery. If we have techniques by which we know the biological behavior of the tumor

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and prognosis before treatment, they may be useful for planning therapy.

Many investigators reported that preoperative CT scan findings were related to the pathologic features and prognosis after resection of the tumor. The ratio of ground-glass opacity (GGO), defined as a hazy increase in lung attenuation without obscuring the underlying vascular marking on the CT scan, was associated with the histologic type of the tumor and survival. One of the purposes of these studies was to determine noninvasive carcinoma, defined as a tumor without lymph node metastasis, pleural invasion, vascular invasion, and lymphatic invasion by using thin-section (TS) CT scan images. However, there are few articles accurately determining noninvasive carcinoma by TS-CT scan images. If we determine a diagnosis of noninvasive carcinoma using CT scan images, they are useful for deciding on the surgical procedure to be used, especially lesser resection. This study was carried out to determine whether TS-CT scan findings were good indicators of noninvasive carcinoma of the lung, and also to clarify whether TS-CT scan findings were related to the prognosis.

Materials and Methods

We reviewed TS-CT scan findings and pathologic specimens from 359 consecutive patients who underwent surgical resection for peripheral adenocarcinomas ≤ 20 mm in diameter during the period from July 1997 to May 2006. All patients underwent physical examination, chest roentgenography, CT scan of the chest and abdomen, bone scintigraphy, and MRI of the brain for the staging and evaluation of resectability before the operation. The patients with disease of clinical stage IIB or less underwent surgery. We also surgically treated the patients with clinical N2 disease without evidence of mediastinal lymph node metastasis proven by mediastinoscopy. This study was approved by our institutional review board after confirmation of informed consent by the patients for us to review their records and images. Chest CT scan images were obtained by a commercially available scanner (X-Vigor/Real or Aquilion M/16 CT scanner; Toshiba Medical Systems; Tokyo, Japan). Conventional CT scan images were obtained serially from the thoracic inlet to the lung bases at 120 kV peak spacing, 512 × 512 pixel resolution, and 1-s scanning time. TS images targeted to the tumor were obtained serially at 120 kVp and 200 mA, with 2-mm section thickness, pitch 1, section spacing of 1 to 2 mm, 512 × 512 pixel resolution, and 1-s scanning time, using a high-spatial-reconstruction algorithm with a 20-cm field of view. These images were printed as photographs on each sheet of films using a mediastinal window level setting (level, 40 Hounsfield units [HU]; width, 400 HU) and a pulmonary window level setting (level, −600 HU; width, 1,600 HU).

While contrast medium (60 mL) was infused IV during imaging, lesion sites were translocated in a helical scan mode with a CT scan table speed of 2 mm/s; TS-CT scan images were obtained at one breath hold (120 kVp; 200 mA). The time interval between CT scan examination and subsequent surgery was ≤ 2 weeks in all patients. All CT scan images were reviewed by four thoracic oncologists who were not informed of the pathologic findings. They obtained the maximum dimension of the tumor using a pulmonary window level setting and the maximum dimension of the tumor using a mediastinal window level setting from the TS-CT scan images.

Tumors were defined as air-containing types if the ratio of the maximum dimension of the tumor using a mediastinal window level setting to the maximum dimension of the tumor using a pulmonary window level setting was ≤ 50%, and were defined as solid-density types if it was > 50%. Examples of CT scan images of the two groups are shown in Figures 1 and 2.

Each pattern based on TS-CT scan images was evaluated in terms of pathologic findings and survival outcome. We evaluated pathologic stage (TNM system), pleural involvement, vascular invasion, and lymphatic invasion. In addition, pathologic subtypes defined by Noguchi et al[1] (called hereafter *Noguchi type*) were evaluated.

The statistical significance of the difference between the incidence of relapse and TS-CT scan findings or Noguchi type was assessed by χ^2 tests. Relapse-free survival was calculated by the Kaplan-Meier method. Log-rank tests were used to compare the Kaplan-Meier curves. The Cox proportional hazards model was applied for multivariate analysis. Significance was defined as $p < 0.05$.

Results

Patient and tumor characteristics are listed in Table 1. There were 60 cases in which the largest diameter of the lesion was ≤ 10 mm, 130 cases in which it was 11 to 15 mm, and 169 cases in which it was 16 to 20 mm. There were 152 patients with air-containing-type tumors, and 207 patients with solid-density-type tumors. Table 2 shows the relationship between TS-CT scan findings and pathologic findings. No patients with air-containing-type tumors had lymph node metastasis, pleural involvement, vascular invasion, or lymphatic permeation. Among patients with solid-density-type tumors, 23 (11%) had lymph node metastasis, 45 (22%) had pleural involvement, 69 (33%) had vascular invasion, and 41 (20%) had lymphatic permeation. Table 3 shows the relationship between TS-CT scan findings and

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FIGURE 1. TS-CT scan findings of an air-containing-type tumor (diameter, 13 mm) on lung window setting images (*left*, *a*) and on mediastinal window setting images (*center*, *b*). The histologic specimen (*right*, *c*) shows BAC (hematoxylin-eosin, original × 6).

pathologic stage. All patients with air-containing-type tumors had pathologic stage IA disease. In contrast, 39 patients (19%) with solid-density-type tumors had pathologic stage IB or greater disease. Table 5 shows the relationship between TS-CT scan findings and Noguchi type tumors. Among 152 patients with air-containing-type tumors, 79 patients received lobectomy, while 73 underwent limited resections (*ie*, segmentectomy or wedge resection) because of their small size (median tumor diameter, 11 mm). Among 207 patients with solid-density-type tumors, 3 patients underwent pneumonectomy and 155 underwent lobectomy, while 49 underwent limited resections because of their being elderly or having pulmonary hypofunction.

Table 2 shows the relationship between TS-CT scan findings and cancer relapse after surgery. No postoperative cancer relapse was seen in patients with air-containing-type tumors; in contrast, relapse was found in 31 patients (15%) with solid-density-type tumors. The relapse-free survival of 207 patients for whom ≥ 3 years have passed since surgery is shown in Figure 3. Patients with air-containing-type tumors had a 100% 5-year relapse-free survival rate, which was significantly better than that for patients with solid-density-type tumors ($p < 0.001$).

We assessed prognostic factors in 207 patients for whom ≥ 3 years had passed since undergoing surgery. Table 5 shows the relationship between cancer relapse and TS-CT scan findings or Noguchi type. No cancer relapse was seen patients with air-containing-type tumors or patients with Noguchi type A or B tumors. The presence of both air-containing-type and Noguchi type A or B tumors were demonstrated as significant prognostic factors for good outcome by χ^2 tests ($p < 0.001$). The reason for using χ^2 tests but not Cox proportional hazards models to analyze the prognostic factors for TS-CT scan findings and Noguchi type tumors was due to the difficulty in conducting a statistical analysis at the time of no relapse event in the patient group with air-containing-type tumors or Noguchi type A or B tumors. Then, a multivariate analysis with a Cox pro-

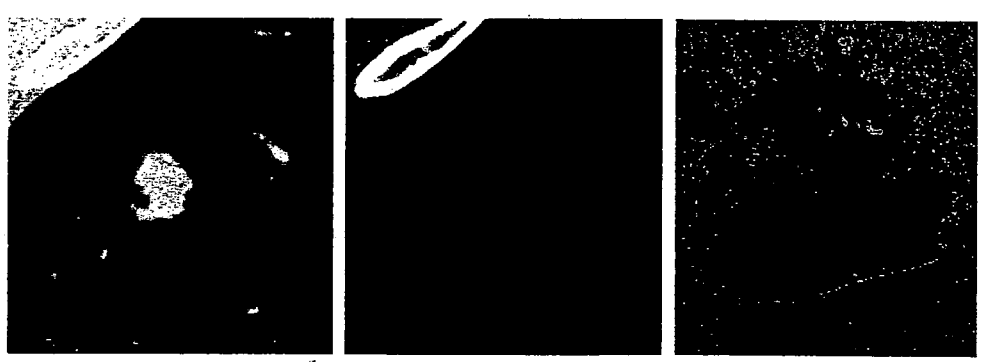

FIGURE 2. TS-CT scan findings for a solid-density-type tumor (diameter, 14 mm) on lung window setting images (*left*, *a*) and on mediastinal window setting images (*center*, *b*). The histologic specimen (*right*, *c*) shows poorly differentiated adenocarcinoma (hematoxylin-eosin, original × 6).

Table 1—*Patient and Tumor Characteristics**

Variables	Values
Patients, No.	359
Age, yr	29–86 (65)
Gender, No.	
Male	159
Female	200
Tumor size, mm	5–20 (15)
Noguchi type tumor, No.	
Type A	52
Type B	75
Type C	162
Type D	39
Type E	5
Type F	25
TS-CT scan findings, No.	
Air-containing-type tumor	152
Solid-density-type tumor	207

*Values are given as range (median) or No.

portional hazard model was performed in 116 patients without air-containing type tumors or Noguchi type A or B tumors. The results showed that lymphatic permeation was a significant prognostic factor (Table 6).

Discussion

In patients with small-sized lung adenocarcinomas, several authors[1,2] have shown that pathologic characteristics are correlated with prognosis. Noguchi et al[1] have used tumor growth patterns to classify small-sized adenocarcinomas into six subtypes (*ie*, types A to F). Small, localized BACs (*ie*, types A and B) have not yet metastasized to lymph nodes or invaded vessels or pleura, and are associated with an excellent prognosis (5-year survival rate, 100%). Localized BAC with central fibrosis formation (*ie*, type C) is thought to be advanced carcinoma, which progresses from type A or B and is associated with a poorer prognosis than before (5-year survival rate, 74.8%). The prognosis for patients with nonreplacement-type adenocarcinomas (*ie*, types D, E, or F) is worse than that for patients with replacement-type adenocarcinomas (*ie*, types A, B, and C) [5-year survival rate, 52.4%]. Suzuki et al[3] showed that the size of the central fibrosis was a prognostic factor among peripheral lung adenocarcinomas that were ≤ 3.0 cm in size. In this study, the patients with adenocarcinoma having central fibrosis ≤ 5 mm in the maximum dimension had a 5-year survival rate of 100%, whereas the other patients had a 5-year survival rate of 70%. Higashiyama et al[4] showed that the component area of BAC was correlated with postoperative survival in patients with small peripheral adenocarcinomas ≤ 2.0 cm in diameter. Patients with adenocarcinoma having a BAC component comprising < 50% of the tumor tissue showed a significantly poorer prognosis than those with ≥ 50%.

In TS-CT scan images, consolidation areas represent mostly the foci of fibrosis or tumors of a solid growth pattern, whereas GGO areas reflect areas of a growth pattern of tumor cells replacing alveolar lining cells such as BAC. Because the fibrotic foci increase with the progression of the tumor, and because these areas and advanced adenocarcinomas with a solid growth pattern demonstrate consolidation areas on CT scans, it is suggested that the percentage of the consolidation or GGO areas relative to the tumor is a prognostic indicator. Many investigators[5–22] have reported on the correlation among TS-CT scan findings, pathologic findings, and prognosis. These studies have shown that GGO ratios were very much associated with BAC ratios and had favorable prognostic factors. However, the methods used to calculate the percentage of GGO areas (*ie*, GGO ratio) differ in different articles. Besides, we have few articles that have accurately determined the presence of noninvasive carcinoma, which was defined as a tumor without lymph node

Table 2—*Relationship Between TS-CT Findings and Both Pathologic Findings and Recurrence*

	TS-CT Scan Findings	
Pathologic Findings	Air-Containing-Type Tumors (n = 152)	Solid-Density-Type Tumors (n = 207)
Lymph node metastasis	0	23
Pleural involvement	0	45
Lymphatic permeation	0	41
Vascular invasion	0	69
Recurrence	0	31

Table 3—*Relationship Between TS-CT Findings and Pathologic Stage*

	Pathologic Stage					
TS-CT Scan Findings	IA	IB	IIA	IIB	IIIA	IIIB
Air-containing-type tumor	152	0	0	0	0	0
Solid-density-type tumor	167	16	5	3	15	1

Table 4—*Relationship Between TS-CT Findings and Noguchi Type*

	Noguchi Type					
TS-CT Scan Findings	A	B	C	D	E	F
Air-containing-type tumor	49	53	49	0	0	1
Solid-density-type tumor	3	22	113	39	5	24

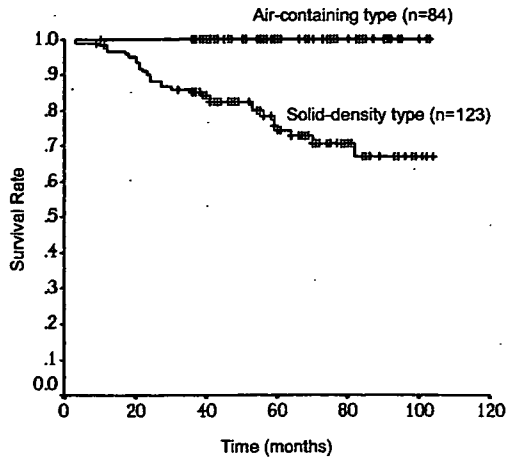

FIGURE 3. Relapse-free survival curves in patients with air-containing-type tumors and solid-density-type tumors.

metastasis, pleural invasion, vascular invasion, and lymphatic invasion, by TS-CT scan images. The parameters used to calculate the GGO ratio that have previously been reported are as follows: a GGO/tumor area ratio[5–10]; a consolidation/tumor dimension ratio[11–14]; a GGO/tumor volume ratio[15]; an area ratio of tumor on mediastinal window to that on the lung window[16,17]; a product of the dimension ratio of the tumor on the mediastinal window to that on lung window[18–20]; and a maximum dimension of tumor on the mediastinal window.[21] Matsuguma et al[8] reported on the relation between the proportion of the GGO and both clinicopathologic characteristics and tumor recurrence in patients with clinical T1N0M0 adenocarcinoma. In this study, the patients with a GGO ratio of ≥ 50% seen on high-resolution CT scans had neither lymph node metastasis nor lymphatic invasion and were alive without cancer recurrence. Ohde et al[12] reported the relation between the proportion of consolidation to GGO and pathologic invasive findings in patients with lung adenocarcinomas ≤ 3.0 cm. They showed that all tumors in which the ratio of the greatest diameter of consolidation to that of the tumor was ≤ 50% had neither lymph node metastasis nor vessel invasion and 5-year survival rate of 95.7%. Although only one cancer relapse was seen in tumors with a ratio of the greatest diameter of consolidation to that of the tumor of ≤ 50% in the study by Ohde et al[12]; the methods used to calculate the GGO ratio in these two studies[8,12] may be useful in defining noninvasive cancer. On the other hand, several investigators[16–20] used not only lung window images but also mediastinal window images to classify the tumors on TS-CT scan images. Kondo et al[16] used a ratio of the tumor area on the mediastinal window images to that on lung window images in patients with pulmonary adenocarcinoma of ≤ 2.0 cm, and showed that the tumors with a ratio of ≤ 50% had no lymph node metastasis, rare vascular invasion, and no cancer relapse. Okada et al[18] and Shimizu et al[20] used the tumor shadow disappearance rate, which was determined from the product of the maximum dimension of the tumor and the largest dimension perpendicular to the maximum axis on both pulmonary and mediastinal window images on TS-CT scan, as previously described by Takamochi et al.[22] They showed that the tumors with a tumor shadow disappearance rate of ≥ 50% had no lymph node metastasis, rare vascular invasion, and no cancer relapse in patients with lung adenocarcinomas ≤ 2.0 cm in diameter. However, the methods used to classify the tumors in these studies with both pulmonary and mediastinal window images could not completely discriminate the tumor without invasive findings (*ie*, vascular, lymphatic, and pleural involvement) from the other. In contrast, the present study showed that the air-containing-type tumor did not have lymph node metastasis, pleural involvement, vessel invasion, or lymphatic permeation, and did not recur after resection. These results suggest that the air-containing-type tumor should be defined as a noninvasive cancer.

The GGO area is sometimes neither clear nor objective. We sometimes experienced cases in which the border of consolidation and the GGO shadow on the TS-CT scan was unclear, and it was difficult or impossible to measure this size accurately. To select noninvasive cancer more simply and more objectively, we measured the maximum dimensions of tumors on both the lung and mediastinal windows. Our classification has the advantage of simplicity and objectiveness. We have only to compare the greatest dimension of the tumor on lung window images with that on mediastinal images of the TS-CT scan.

Although a number of prognostic indicators have been proposed such as TNM staging, tumor differentiation, molecular expression, and vascular inva-

Table 5—*Relationship Between Recurrence and Both TS-CT Findings and Noguchi Type Tumor in 207 Patients for Whom 3 Years or More Have Passed Since Surgery*

TS-CT Scan Findings	Recurrence		p Value
	No	Yes	
Tumors			0.000
Air-containing type	84	0	
Solid-density type	93	30	
Noguchi type tumor			0.000
Type A or B	66	0	
Type C, D, E, or F	111	30	

Table 6—*Multivariate Analysis of Relapse-Free Survival*

Variables	Hazard Ratio	95% Confidence Interval	p Value
Age	0.968	0.923–1.014	0.170
Gender (male vs female)	2.372	0.986–5.707	0.054
Tumor size	1.062	0.947–1.192	0.305
Pathologic stage (≥ II vs I)	1.795	0.598–5.389	0.297
Noguchi type tumor (type D, E, or F vs type C)	2.169	0.842–5.586	0.109
Pleural involvement (positive vs negative)	2.181	0.951–5.001	0.066
Lymphatic permeation (positive vs negative)	2.819	1.094–7.265	0.032
Vascular invasion (positive vs negative)	0.864	0.289–2.588	0.795
Operation mode (lobectomy vs wedge resection)	0.453	0.188–1.094	0.079

sion, the final results are defined only after surgery. As yet, no definite preoperative indicators have been discovered for the postoperative outcome of patients with adenocarcinomas. This study showed that preoperative TS-CT scan findings had prognostic importance. The air-containing-type tumor defined in this study showed no cancer relapse and was revealed as an independent prognostic factor for relapse-free survival. The identification of prognostic variables, especially before the operation is important to decide on the operative procedure and adjuvant therapy. Although lobectomy and pneumonectomy with systemic mediastinal lymphadenectomy is the standard surgical treatment for non-small cell lung cancer, if noninvasive lung cancers are distinguishable on CT scans, limited surgery can be indicated before the operation. Since patients with the air-containing-type tumor showed neither pathologic invasion nor relapse after surgery, we think it is reasonable that we can treat patients with lesser resection for tumors of this type. Treating patients with limited resection leads to a reduction in operative complications and the maintenance of pulmonary function. The number of both elderly patients with lung cancer and patients with a second lung cancer has been increasing. Lesser invasive techniques such as limited resection and stereotactic radiotherapy will play an important role in the future. Studies[23,24] have shown the results of the attempt to apply limited surgery for small lung tumors ≤ 2.0 cm in diameter, in which a small number of local relapses was seen in patients who underwent limited resections. Our study also showed that 11% of solid-density-type tumors had lymph node metastasis. We think that it is not the size of the tumor but the findings of the CT scan of the tumor that is a good indicator for determining whether to use limited resection. Nakata et al[25] reported the results of limited resection of pure GGO selected by the CT scan, in which no cancer relapse was seen in 33 patients who underwent limited resection. In the selection of a candidate for limited surgery, it is important to select patients with noninvasive cancers that not only have high specificity but also high sensitivity. In our study, among 162 patients with Noguchi type C tumor, which is thought to be advanced carcinoma, 49 patients had air-containing-type tumors (Table 4). This result means that our classification using TS-CT scans can preoperatively determine the presence of type C tumors without invasive findings. A prospective study is needed to clarify whether patients with air-containing-type tumors defined preoperatively on TS-CT scan images are candidates for limited surgery. In conclusion, the presence of air-containing-type tumors in patients with peripheral adenocarcinomas < 2.0 cm in diameter means noninvasive cancer and that such patients are candidates for limited surgery.

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Paradoxical Discrepancy Between the Serum Level and the Placental Intensity of PP5/TFPI-2 in Preeclampsia and/or Intrauterine Growth Restriction: Possible Interaction and Correlation with Glypican-3 Hold the Key

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Abstract

There have been controversies whether maternal serum placental protein 5 (PP5)/tissue factor pathway inhibitor (TFPI)-2 is increased in the patients with preeclampsia and/or intrauterine growth restriction (IUGR). Here, we have estimated the serum PP5/TFPI-2 in these patients by a sandwich enzyme-linked immunosorbent assay with a newly developed monoclonal antibody, coupled with placental immunohistochemical studies of their placentae with semiquantitative scoring.

Serum PP5/TFPI-2 level was significantly elevated only in the patients with preeclampsia alone ($p = 0.033$), while PP5/TFPI-2 was detected significantly less intensely in the placentae of the same patients ($p = 0.035$) in immunohistochemistry, as compared to Controls. A proteoglycan present on the placental villous surface, glypican-3, showed the same pattern of staining as PP5/TFPI-2, and there was a positive correlation (C.I. = 0.506, $p = 0.004$) between the immunohistochemical scores for these. Further experiments using HepG2 cells transfected with *PP5/TFPI-2* suggested that glypican-3 could anchor PP5/TFPI-2 on the placental villi.

A possibility that a decrease in glypican-3 in the placenta increases the outflow of PP5/TFPI-2, which in turn increases its serum level, was proposed. Preeclampsia and IUGR, often regarded to have the same pathological basis in common, showed distinct distributions of PP5/TFPI-2, which could be a clue to elucidate the pathogenesis of preeclampsia and IUGR.

Keywords: Placental protein 5/tissue factor pathway inhibitor-2; Glypican-3; Preeclampsia; Intrauterine; Growth restriction; Syndecan-1

1. Introduction

Preeclampsia and intrauterine growth restriction (IUGR) are difficult to predict clinically, and are some of the severe complications of pregnancy. Although part of the mechanisms underlying these disorders has been elucidated, the ultimate causes of preeclampsia and IUGR remain unknown [1–3].

Placental protein 5 (PP5) is a soluble protein produced in the human placenta and is detected in the serum of the pregnant woman [4]. We previously have found from amino acid sequence comparisons that PP5 is identical to a 29-kDa Kunitz type proteinase inhibitor [5]. The same protein, named tissue factor pathway inhibitor (TFPI)-2, was cloned independently

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as a homologue of TFPI from a human placental cDNA library by others [6].

PP5/TFPI-2 is a potent inhibitor of trypsin, plasmin, plasma kallikrein, factor XIa and factor VIIa/TF complex, and also weakly inhibits amidolytic activity of factor Xa [7]. The expression of PP5/TFPI-2 has been demonstrated in various human tissues other than the placenta [5,8–10], and its contribution to angiogenesis [9–11] and carcinogenesis [12–15] has been the focus of several studies. Recently, the function of PP5/TFPI-2 as a mitogen for vascular smooth muscle cells [16] and retinal pigment epithelial cells [17] has been demonstrated.

Despite its abundant presence in the placenta, the function of PP5/TFPI-2 during pregnancy is not fully understood. We have demonstrated that PP5/TFPI-2 is localized on the surface of microvilli and the endoplasmic reticulum membrane of syncytiotrophoblasts by immunoelectron-microscopy, and that incubation with heparin releases PP5/TFPI-2 from the villous surface of the placenta [18,19].

TFPI is known to bind to glypican-3, a member of the transmembrane heparan sulphate proteoglycans (HSPGs), on the cell surface of hepatocellular carcinoma cell line, HepG2 cells. When HepG2 cells are incubated with heparin, TFPI is released from the cell surface into the culture medium [20]. TFPI possesses a highly positively charged region in its carboxyl terminus, for which heparin competes with glypican-3 to release TFPI [21].

As PP5/TFPI-2 has a similar structural domain to TFPI, we hypothesized that PP5/TFPI-2 might be retained on the surface of the placental villi by proteoglycans such as members of the glypican and syndecan families, and that PP5/TFPI-2 might play a role to maintain intervillous blood flow [19]. Glypican-3 is known to be expressed abundantly in the placenta [22], along with syndecan-1, a member of the HSPGs syndecan family [23].

It has been reported that the maternal serum level of PP5/TFPI-2 is elevated in the patients with severe preeclampsia [24–26]. Some investigators have reported the elevated maternal serum level of the same protein also in the patients with IUGR [25,26], while others have failed to demonstrate the elevation [27,28], using the same rabbit polyclonal antibody raised against a fraction of purified PP5/TFPI-2 as for the radioimmunoassay [29,30]. Another evaluation with new specific monoclonal antibody and with a more specific technique (sandwich ELISA) than radioimmunoassay may serve to clarify the association between the maternal serum PP5/TFPI-2 levels and preeclampsia and/or IUGR. In addition, the mechanism underlying the increase in PP5/TFPI-2 in the maternal serum remains to be elucidated. To date, there have been no reports on the in situ expression of PP5/TFPI-2 in the placentae of the patients with preeclampsia and/or IUGR as compared with their serum PP5/TFPI-2 levels.

Here we have attempted to clarify the maternal serum levels of PP5/TFPI-2, along with the in situ expression of the same protein in the placentae of the patients with preeclampsia and/or IUGR. We have also sought for the association of PP5/TFPI-2 with some proteoglycans in the placentae.

2. Materials and methods

2.1. Placental tissue and serum samples

The experimental protocol was peer-reviewed and approved by the Ethical Committee of Yokohama City University Graduate School of Medicine. Placentae, maternal and umbilical venous sera were collected from the patients who were scheduled to undergo caesarean section. After receiving a detailed explanation, each of the patients who agreed to be enrolled in this study gave written informed consent.

Preeclampsia was diagnosed according to the definition established by the National High Blood Pressure Education Program [31]. IUGR was diagnosed if the estimated weight of the fetus was less than the 10th percentile for its gestational age according to the Japanese standard fetal growth curve [32], and the presence of growth arrest and non-reassuring fetal status were inferred from the fetal monitoring. For each of the patient, the gestational age had been confirmed in the first trimester by ultrasound.

The maternal serum was sampled 10–60 min before the mothers moved to the operation room, before the administration of anesthesia. The maternal serum was also sampled 4 days after delivery. The umbilical venous serum was collected carefully from the cord to avoid contamination with maternal blood. All serum samples were stored at −80 °C until the assay.

Placental tissues were sectioned into samples of approximately 3 cm × 3 cm × whole thickness taken from five different portions, fixed in 10% neutral-buffered formalin and embedded in paraffin for histopathological studies.

2.2. Preparation of mouse monoclonal anti-PP5/TFPI-2 antibody

Monoclonal antibody was raised against a synthetic peptide antigen consisting of 14 amino acid residues, NH_2-DAAQEPTGNNAEIC-COOH, corresponding to the N-terminus of the mature PP5/TFPI-2 protein after cleavage of the putative signal peptide. Specificity of the antigenic peptide to PP5/TFPI-2 was verified by searching the peptide sequence against other proteins with the BLAST program at the National Center for Biotechnology Information, National Institute of Health, Bethesda, MD (http://www.ncbi.nlm.nih.gov/BLAST/). The cysteine residue at the carboxy terminus was conjugated to keyhole limpet hemocyanin.

To use as the standard PP5/TFPI-2 protein for screening of the hybridoma cell clones of new antibodies and for the enzyme-linked immunosorbent assay (ELISA), recombinant PP5/TFPI-2 was prepared as follows. Histidine-tagged *PP5/TFPI-2* cDNA was transfected into the yeast (*Pichia Pastoris*) by using an EasySelect *Pichia* Expression Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. The expressed recombinant PP5/TFPI-2 was affinity purified against the histidine-tag by using a Ni-NTA Spin Kit (QIAGEN, Valencia, CA).

Five-week-old BALB/c mice, gained from Oriental Yeast Co., Ltd., Tokyo, Japan, were immunized with the antigenetic peptide every 2 weeks. Three days after the last injection of 250 μg of the immunogen, the mouse spleen cells were sampled and fused with a mouse myeloma cell line P3U1 using polyethyleneglycol. From the antibody produced by the hybridomas, a clone 28Aa was selected for use in the study by Western blotting against the recombinant PP5/TFPI-2 expressed in the yeast described above. The antibody of the selected clone was purified from the ascites of the BALB/c mice that had been injected intraperitoneally with the hybridoma cells by column chromatography using protein A (Amersham Biosciences Co., Piscataway, NJ).

2.3. Sandwich ELISA

Serum levels of PP5/TFPI-2 were assayed by Sandwich ELISA using the clone 28Aa mouse monoclonal antibody against human PP5/TFPI-2 as described above, and a previously described rabbit polyclonal antibody against human PP5/TFPI-2 [18].

PP5/TFPI-2 antibody clone 28Aa diluted to 10 μg/ml was applied to a 96-well plate (Greiner Bio-one, Longwood, FL). After incubation at 4 °C overnight, the plate was blocked with 1% bovine serum albumin (Sigma) in

phosphate-buffered saline (PBS) at room temperature for 1 h. Serum samples diluted five times or the recombinant PP5/TFPI-2 protein diluted to different concentrations was added to each well. The plate was then incubated at 37 °C for 1 h. After washing, the rabbit polyclonal antibody against human PP5/TFPI-2 diluted to 10 μg/ml was added to the wells, and the plate was incubated at 37 °C for an additional hour. For detection, a horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Ig) G H+L (Molecular Probes, Invitrogen, Carlsbad, CA), diluted to 1:16000 was added to each well. After incubation at 37 °C for 1 h, *O*-phenylenediamine (Sigma) was added for color development. Absorbance at 490 nm was read by a Benchmark Plus spectrophotometer (Bio Rad, Hercules, CA) and the results were analysed by Microplate Manager Ver. 5.2 (Bio Rad).

2.4. Immunohistochemical analyses

Paraffin sections of the placentae were routinely stained with Hematoxylin and Eosin. The samples were also subjected to immunohistochemical staining for PP5/TFPI-2 and glypican-3.

Deparaffinized and rehydrated slides were immersed in 0.01 M citrate buffer, pH 6.0 (Sigma), and heated in a microwave oven for antigen retrieval. The slides were then cooled, washed in PBS, and immersed in 3% H_2O_2 diluted in methanol at room temperature.

The clone 28Aa mouse monoclonal antibody against human PP5/TFPI-2 or a mouse monoclonal antibody against human glypican-3 (clone 1G12, Biomosaics, Burlington, VT), diluted to 5 μg/ml or 40 μg/ml, respectively, was used as the primary antibody. Histofine SAB-PO multikits (Nichirei, Co., Tokyo, Japan) were used to detect the labeled antigens. Histochemically labeled antigens were visualized by reaction with 3,3′-diaminobenzidine (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Immunohistochemical staining for syndecan-1 (CD138) was also performed with a mouse monoclonal antibody against human CD138 (clone B-B4, Serotec, Oxford, UK) (diluted 1:200), as described above except for the antigen retrieval. Adjacent sections were used for immunohistochemical stainings for PP5/TFPI-2, glypican-3, and syndecan-1.

The results of the immunohistochemistry were analysed by using a modified German immunoreactive score [33–35]. The immunostaining intensity was rated as follows: 0, none; 1, weak; 2, moderate; and 3, intense. The quantity of immunohistochemically positive trophoblasts was also graded as follows: 0, none; 1, 1–10%; 2, 11–50%; 3, 51–80%; and 4, 81–100%. A score per slide was calculated as the summation of the areas of intensity multiplied by the quantity of each area. Each slide was evaluated of its score three times by two independent examiners who were blinded to its origin. The average of the scores from all of the slides of the placenta was determined as the representative data for that sample.

2.5. Transfection, immunoprecipitation and Western blotting

HepG2 cells were obtained from the Cell Bank, RIKEN BioResource Center (Tsukuba, Japan), and cultured in RPMI1640 (Kohjin Bio, Co., Itado, Japan) containing 10% fetal bovine serum (Moregate Biotech, Balimba, Australia) under an atmosphere of humidified 5% CO_2. A mammalian expression vector pcDNA3 (Invitrogen) or the vector containing the whole coding region of human *PP5/TFPI-2* cDNA was transfected to HepG2 cells with Lipofectamine 2000 transfection reagent (Invitrogen) under the manufacturer's instruction.

Forty-eight hours after transfection, the cells were lysed at room temperature for 10 min in 1 ml of a lysis buffer (25 mM Tris–Cl, pH 7.5; 100 mM NaCl; 2 mM EDTA (Sigma); 1% Triton X-100 (Sigma)) containing protease inhibitors (Complete Mini, Roch Diagnostics, Indianapolis, IN). After cell debris was removed by centrifugation, each lysate was further pre-cleared with Protein G Sepharose 4 fast flow (Amersham Biosciences).

Immunoprecipitation was carried out with 2.5 μg of the 28Aa mouse monoclonal antibody against human PP5/TFPI-2 and 50 μl of the Protein G Sepharose at 4 °C, and then the Sepharose phase was washed four times with the lysis buffer. Each immunoprecipitate was recovered by adding 50 μl of 2× SDS containing sample buffer and incubating at 70 °C for 10 min. Equal amount of immunoprecipitate (10 μl each) was subjected to SDS PAGE, followed by Western blotting for PP5/TFPI-2 (with the clone 28Aa antibody) or glypican-3 (the clone 1G12 mouse monoclonal antibody against human glypican-3, Biomosaics), respectively. An HRP conjugated sheep anti-mouse IgG (Amersham Biosciences) was used as the secondary antibody, and the signals were detected with the Supersignal West Pico chemiluminescent substrate (Pierce).

2.6. Statistics

Data are expressed as the mean ± standard error (SE). Statistical comparison was performed by either Student's *t* test, Welch's *t* test, Mann–Whitney's *U* test, or analysis of covariance (ANCOVA). The correlation index was calculated by using either Pearson's test or Spearman's test. SPSS software (Basic 11.0, SPSS Inc., Chicago, IN) was used for calculation. Significance was set at $p < 0.05$.

3. Results

3.1. Patients

Fifty-five patients who had been scheduled to undergo caesarean section at 24–39 weeks of pregnancy agreed to the collection of samples for research usage. Four patients were excluded from the study because they had previously taken medication for other pre-existing diseases. Hence, the 51 patients who had not been diagnosed of any pre-existing disease such as hypertension, renal disease, diabetes mellitus, or other chronic disease before pregnancy were enrolled in the study. There were no neonates with congenital or chromosomal abnormalities.

3.2. Maternal serum PP5/TFPI-2 levels in preeclampsia and/or IUGR

Maternal serum samples at delivery were available from the 51 patients, whose obstetrical complications are summarized in Table 1. Nineteen patients had preeclampsia, 10 of whom had preeclampsia only (Group P), and the other nine of whom had been also diagnosed as IUGR (Group P + IUGR). Seven had been diagnosed with IUGR alone (Group IUGR). The other 25 patients did not have the above-mentioned obstetric complications (the Control).

We confirmed from the clinical records that none of the patients in Group P, Group P + IUGR, and Group IUGR had been hypertensive or had proteinuria early in pregnancy, nor had they persisted hypertension or proteinuria at the time of their follow-up visits 1 month after delivery. All of the patients in Group P and Group P + IUGR had received antihypertensive medications for as long as 1–14 days.

The patients with preterm premature rupture of the membrane and premature labor in the Control had mild, if any, pathological changes in the placentae (Blanc stage [36] one, i.e., intervillositis at most), and had no clinical sign of severe chorioamnionitis or maternal systemic infection such as uterine tenderness, foul smelling amniotic fluid, maternal fever more than 38 °C, maternal tachycardia (≥120 beats/min), or maternal leukocytosis (≥20 000/μl).

The clinical features of the study groups are summarized in Table 2. The maternal mean arterial pressure (MAP) and

Table 1
Distribution of the patients

Complications/indications for C/S	Number of the patients	
Preeclampsia (Group P)		10
Non-reassuring fetal status	3	
Incontrollable maternal hypertension/renal insufficiency	6	
Both of the above	1	
Preeclampsia with IUGR (Group P + IUGR)		9
Non-reassuring fetal status	7	
Incontrollable maternal hypertension/renal insufficiency	2	
IUGR (Group IUGR)		7
Non-reassuring fetal status	7	
No above complications (the Control)		25
History of C/S	9	
Breech presentation	5	
Placenta previa	3	
Preterm PROM	4	
Preterm labor	2	
History of uterine surgery	1	
Operated atresia ani	1	
Total		51

IUGR, intrauterine growth restriction; C/S, caesarean section; PROM, premature rupture of the membrane.

urinary protein (UP) were measured at the time of blood sampling. Although none of the patients in Group IUGR had been diagnosed as hypertensive, the maternal MAP was significantly higher not only in Group P and Group P + IUGR ($p < 0.001$ for both Groups), but also in Group IUGR ($p = 0.032$), as compared with the Control. However, the maternal MAP was significantly lower in Group IUGR than in Group P ($p = 0.001$). There were no differences in the maternal MAP and UP between Group P and Group P + IUGR.

The mean gestational age at delivery was significantly younger in Group P ($p = 0.039$), in Group P + IUGR ($p = 0.005$), and in Group IUGR ($p = 0.037$) than in the Control. Even controlling for the gestational age at delivery, the neonatal birth weight was still significantly lower in Group P + IUGR ($p < 0.0001$) and in Group IUGR ($p < 0.0001$) than in the Control.

PP5/TFPI-2 has been reported to be detectable early in pregnancy, and rise to a maximum at gestational weeks 36–37 [29,30]. To adjust for the effect of gestation, we compared the serum PP5/TFPI-2 levels in the maternal samples obtained at delivery by analysis of covariance (ANCOVA) (Fig. 1), after controlling for gestational age at delivery and neonatal birth weight. The detection limit of the sandwich ELISA for PP5/TFPI-2 was 1 ng/ml, and the intra- and inter-assay coefficients of variances were 5.0% and 10.0%, respectively. The analytical recovery was 80%.

The maternal serum PP5/TFPI-2 levels were 530.8 ± 111.3 ng/ml in Group P, 362.1 ± 146.0 ng/ml in Group P + IUGR, 223.9 ± 149.8 ng/ml in Group IUGR, and 233.2 ± 83.8 ng/ml in the Control. The maternal serum PP5/TFPI-2 level was significantly higher in Group P than in the Control ($p = 0.033$), but there were no significant differences in this value between Group IUGR and the Control, and between Group P + IUGR and the Control.

The PP5/TFPI-2 levels in the umbilical serum samples and in the maternal serum samples obtained 4 days after delivery were too low to be measured (data not shown).

3.3. Immunohistochemistry for PP5/TFPI-2, glypican-3, and syndecan-1

Placental samples were available from eight patients in Group P, seven patients in Group P + IUGR, six patients in Group IUGR, and from 24 patients in the Control. We selected 12 placental samples from the patients in the Control who were matched in gestational age at delivery with the patients in the other three study groups randomly. There was no significant difference in the maternal age, body mass index, and umbilical arterial pH among the patients in the three study groups and the Control whose placental samples were subjected to immunohistochemical analysis.

Table 2
Comparison of the characteristics of the study groups

	P ($n = 10$)	P + IUGR ($n = 9$)	IUGR ($n = 7$)	Control ($n = 25$)
Maternal age (years)	31.8 ± 1.9	29.8 ± 2.1	31.0 ± 2.4	31.8 ± 1.0
Maternal BMI	24.6 ± 1.7	24.0 ± 1.7	22.7 ± 1.4	21.8 ± 0.7
Maternal MAP at delivery (mmHg)	118.2 ± 4.4*[a]	110.1 ± 6.3*[b]	88.8 ± 5.5*[c]	78.1 ± 2.0
Maternal UP (mg/dl)	296.3 ± 75.1*[d]	491.8 ± 179.5*[e]	21.4 ± 10.1	13.7 ± 7.5
% of primiparas	50.0	44.4	42.9	52.0
Gestational age at delivery (weeks)	32.5 ± 1.6*[f]	31.1 ± 1.6*[g]	31.1 ± 1.9*[h]	36.0 ± 0.8
% of male babies	30.0	44.4	57.1	48.0
UApH	7.270 ± 0.017	7.232 ± 0.019*[i]	7.295 ± 0.028	7.273 ± 0.018
Neonatal birth weight (g)	1922 ± 311	1124 ± 185*[j]	1344 ± 230*[k]	2550 ± 130

BMI, body mass index; MAP, mean arterial pressure; UP, urinary protein; and UApH, umbilical arterial pH.
Student's t test, otherwise noted.
*[a] $p < 0.001$ (compared to the Control), $p = 0.001$ (compared to Group IUGR), *[b] $p < 0.001$ (compared to the Control), *[c] $p = 0.032$ (compared to the Control), *[d] $p < 0.001$ (compared to the Control), $p = 0.009$ (compared to Group IUGR), *[e] $p < 0.001$ (compared to the Control), $p = 0.01$ (compared to Group IUGR), *[f] $p = 0.039$, *[g] $p = 0.005$, *[h] $p = 0.037$ (compared to the Control), *[i] $p = 0.037$ (compared to the Control, Mann–Whitney's U test), *[j] $p < 0.0001$ and *[k] $p < 0.0001$ (compared to the Control, ANOVA, where gestational age at delivery was set as a covariate.)

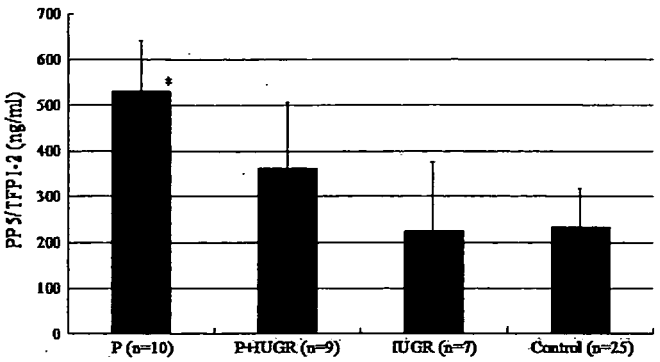


Fig. 1. Comparison of the PP5/TFPI-2 levels in maternal serum samples in the different groups obtained at delivery. Data are expressed as the mean ± SE and are adjusted for gestational age at delivery and the neonatal birth weight. $*p = 0.033$ (ANCOVA, where gestational age at delivery and the birth weight of the neonate are set as covariates).

PP5/TFPI-2 was detected in the cytoplasm of syncytiotrophoblasts, but not in any other type of cell such as cytotrophoblasts, decidual cells, stromal cells, or chorionic vascular endothelial cells (Fig. 2), as we have previously described [18,19]. Glypican-3 showed the same pattern as PP5/TFPI-2, that is, it was present only in the cytoplasm of syncytiotrophoblasts. Syndecan-1 was limited to the surface of the syncytiotrophoblasts.

3.4. Immunohistochemical evaluation

The results of the immunohistochemical analyses are summarized in Fig. 3. The coefficients of variation (CVs) of the scores from each of the two independent examiners were 19.7% and 13.8%, respectively. The CV between the scores from the two examiners was 17.9%. In contrast to the increase in maternal serum levels, PP5/TFPI-2 was detected scarcely in the placenta of Group P (Fig. 2), and so was glypican-3. The scores for PP5/TFPI-2, and also for glypican-3, in the placenta were significantly lower in Group P than in the Control ($p = 0.035$ for PP5/TFPI-2, 0.047 for glypican-3).

The scores for syndecan-1 in the placenta were significantly higher in Group P and Group IUGR ($p = 0.023$ and $p = 0.003$, respectively) than in the Control.

There was a positive correlation between the score for glypican-3 and that for PP5/TFPI-2 among the 33 placental samples examined (C.I. $= 0.506$, $p = 0.004$) (Fig. 4). The score for syndecan-1 correlated with neither that for PP5/TFPI-2 nor that for glypican-3 (data not shown).

3.5. Interaction of PP5/TFPI-2 and glypican-3

HepG2 cells abundantly produced both the core protein (approximately 60 kDa) and the glycated form (observed as a broad band around 97 kDa) of glypican-3, and no detectable amount of PP5/TFPI-2 was observed (Fig. 5, lanes 1 of (A) & (B)). With the antibody against PP5/TFPI-2, only the glycated form of glypican-3 and PP5/TFPI-2 were co-immunoprecipitate from the lysates of the PP5/TFPI-2 expression vector transfectants, and the core protein of glypican-3 was not detectable (Fig. 5, lanes 4 of (A) & (B)). From the lysates of the empty vector transfectants, which were prepared as a negative control, no detectable bands of PP5/TFPI-2 or glypican-3 were observed (Fig. 5, lanes 2 of (A) & (B)).

4. Discussion

First, we found that PP5/TFPI-2 interacts with glypican-3. In immunohistochemistry, glypican-3 was detected in a pattern identical to that of PP5/TFPI-2, with a positive correlation between the immunohistochemical scores for the two. The biochemical interaction of PP5/TFPI-2 with glycated glypican-3 was demonstrated in the HepG2 cells transfected with *PP5/TFPI-2*. These findings strongly support our previous proposal that glypican-3 serves as the anchor for PP5/TFPI-2 on the placental villi [19]. It is known that some proteins anchored to HSPGs can be shed together to the extracellular space [37]. Glypican-3 may not only anchor PP5/TFPI-2 but also play more roles in the secretory pathway of PP5/TFPI-2. Future studies should identify the precise localization of glypican-3 in the syncytiotrophoblasts and whether PP5/TFPI-2 and glypican-3 interact in the maternal serum.

Second, we highlighted the discrepancy that the maternal serum PP5/TFPI-2 level was increased, whereas placental PP5/TFPI-2 was detected significantly less intensely, in Group P as compared to the Control. This is the first study to investigate in parallel the maternal serum level and the placental immunohistochemistry of PP5/TFPI-2. Most glycoproteins that are produced by the placenta and detected in the maternal serum are known to be increased in the maternal serum of the patients with preeclampsia as compared to Controls [38–44]. It is thought that in preeclampsia, increased apoptosis of trophoblasts occurs in early pregnancy and that newly differentiated trophoblasts later in pregnancy overfunction as a compensation [40,42], based either on the assays of the extracts from the placenta at term [42] or on immunohistochemical studies of the placenta [41,43]. It is obvious from our data that the increase in PP5/TFPI-2 in maternal serum in preeclampsia must result from a mechanism different from that regulating other glycoproteins, which are detected strongly in the placenta in preeclampsia as compared to Controls [41,43].

Glypican-3, which was also detected significantly less intensely in the placenta of Group P as compared to the Control, may provide a clue to clarify the discrepancy in PP5/TFPI-2 levels. It is not clear whether the decreased amount of glypican-3 in the placenta in preeclampsia is caused by reduced expression of the protein through unknown mechanisms, and/or by increased cleavage of it. In either case, the amount of PP5/TFPI-2 anchored on villous surface might be decreased due to the smaller amount of glypican-3 on the villi. One could speculate that more PP5/TFPI-2 would flow out from the placenta to the maternal blood, as compared to normal pregnancy, which in turn would increase the level of PP5/TFPI-2 in maternal serum in preeclampsia. However, other mechanisms should be taken into account for the increase in PP5/TFPI-2 in

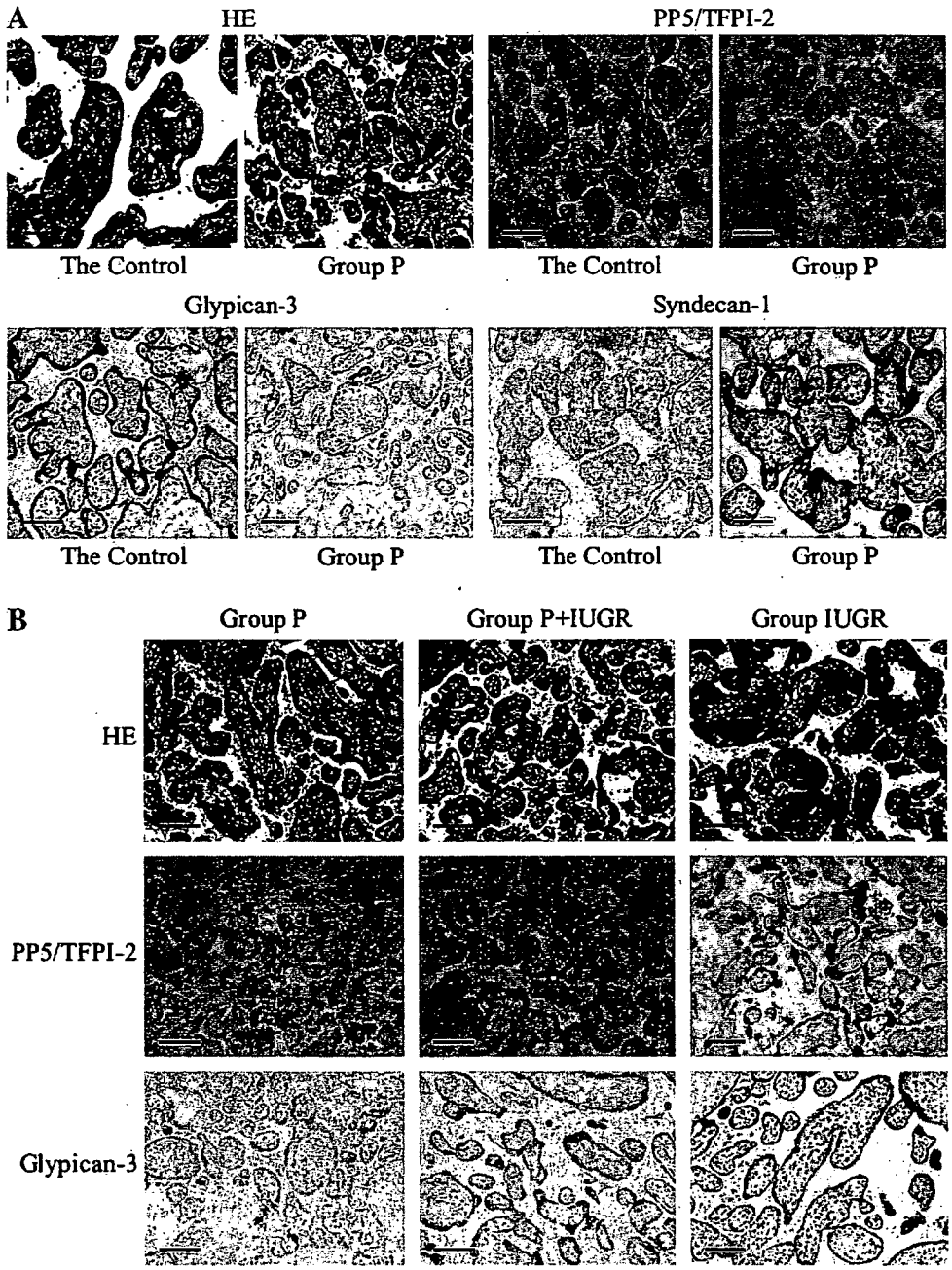
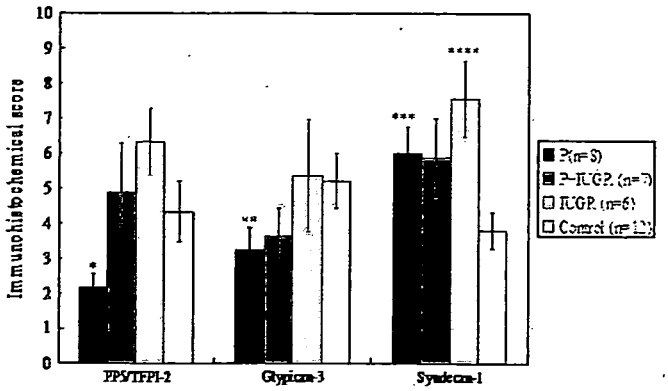


Fig. 2. Examples from the results of immunohistochemical studies. All images original magnification, ×100; scale bar, 100 μm. (A) HE staining, and immunohistochemical staining for PP5/TFPI-2, glypican-3, and syndecan-1 in the placental samples of the Control and Group P. (B) HE staining (the upper lane), and immunohistochemical staining for PP5/TFPI-2 (the middle lane) and glypican-3 (the lower lane) in the placental samples of Group P (the light column), Group P + IUGR (the middle column), and Group IUGR (the right column).

maternal serum, such as the metabolic pathway of the protein. Influences of impaired renal clearance of the glycoproteins, and of antihypertensive drugs in the patients with preeclampsia might not be ignored. As for the influence of renal function, even the clearance of human chorionic gonadotropin, a glycoprotein mainly excreted in urine, is shown to be not different between the patients with preeclampsia and normal Controls [45], which implies minimal influence of renal function. Further studies on the metabolic pathway of PP5/TFPI-2, as well as precise evaluation of the kinetics of PP5/TFPI-2 in preeclampsia, are required.

Third, syndecan-1 was immunohistochemically detected at significantly higher intensities in the placenta in Group P and Group IUGR than in the Control, contrary to another report [46]. This contradiction might be caused mainly by the different methods used for evaluation; for example, we used a semi-quantitative scoring system that focused on both the intensity and the quantity of the stained areas, whereas others had scored only for the intensity.

Fourth, we found that preeclampsia and IUGR, often considered to share the same pathological basis in common, presented distinct distributions of PP5/TFPI-2. In Group IUGR



	P(n=8)	P+IUGR (n=7)	IUGR (n=6)	Control (n=12)
PP5/TFPI-2	2.16±0.39*	4.86±1.43	6.31±0.95	4.33±0.85
Glypican-3	3.25±0.62**	3.64±0.79	5.36±1.62	5.22±0.77
Syndecan-1	6±0.74***	5.89±1.12	7.57±1.07****	3.8±0.53

Fig. 3. Comparison of the immunohistochemical scores for PP5/TFPI-2, glypican-3, and syndecan-1 in the placental samples. Data are expressed as the mean ± SE. Student's t test was used for all comparisons. *$p = 0.035$, compared to the Control, and $p = 0.001$, compared to Group IUGR, **$p = 0.045$, compared to the Control, ***$p = 0.023$, compared to the Control, and ****$p = 0.003$, compared to the Control.

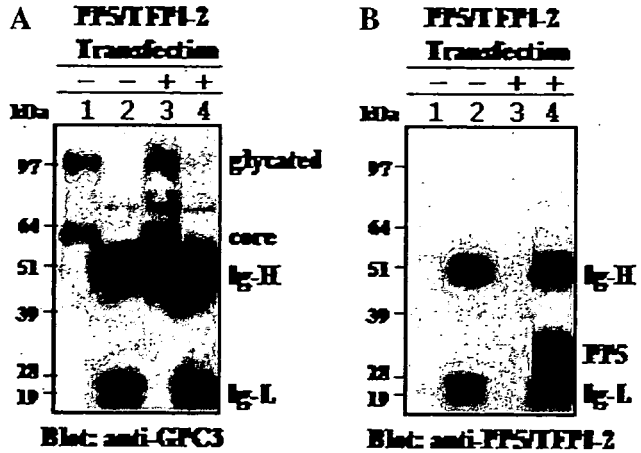


Fig. 5. Interaction of PP5/TFPI-2 and glypican-3 by immunoprecipitation experiments. HepG2 cell lysates from the PP5/TFPI-2 expression vector transfectant or the empty vector one were prepared as described in the text. Ten microliters of each sample before immunoprecipitation was loaded as input. Ten microliters from each 50 μl immunoprecipitant was also loaded. (A) Immunoblotted with anti-glypican-3 antibody and (B) with anti-PP5/TFPI-2. Molecular size from the marker bands was presented on the left side of each panel. Lanes 1 and 3, inputs; Lanes 2 and 4, immunoprecipitants. Ig-H, immunoglobulin heavy chain; Ig-L, immunoglobulin light chain; glycated, the glycated form of glypican-3; and core, the core protein of glypican-3.

and Group P + IUGR, the maternal serum PP5/TFPI-2 levels and placental immunohistochemical intensities of PP5/TFPI-2 were comparable to the Control. Although the patients in Group P and Group P + IUGR had preeclampsia to the same severity, they were not the same in the status of PP5/TFPI-2. The reason of the different status of PP5/TFPI-2 between preeclampsia and IUGR, as well as its relation to the clinical symptoms, is not known. Further studies would provide some available information on the pathogenesis of preeclampsia and IUGR.

Finally, we found that the umbilical serum levels of PP5/TFPI-2 were too low to be measured. The PP5/TFPI-2 levels were decreased in the maternal serum samples obtained 4 days after delivery, in agreement with another report [30].

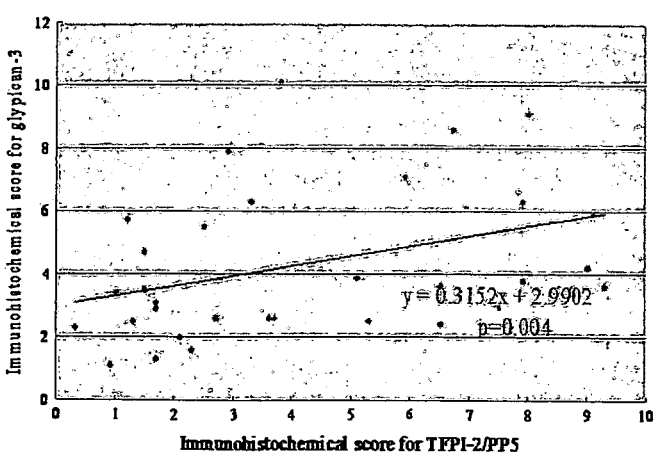


Fig. 4. Correlation between the immunohistochemical scores for PP5/TFPI-2 and those for glypican-3. C.I. = 0.506, $p = 0.004$ (Spearman's correlation test).

The role of PP5/TFPI-2 in pregnancy is not yet fully understood, but it is certain that PP5/TFPI-2 functions within the maternal serum and/or in the placenta, rather than in the fetal side. Our hypothesis has been that PP5/TFPI-2 works as an anticoagulant on the villous surface, which is not verified yet. Another group [47] has shown that the cognate tissue factor initiated coagulation inhibitor TFPI (or TFPI-1) is responsible for inhibiting coagulation in the placenta. Our finding in the present study, demonstrating the loss of PP5/TFPI-2 in the syncytium of the patients with preeclampsia, might imply its anticoagulant feature, because preeclampsia often encounters with elevated coagulation activity. Measuring the parameters of maternal coagulation activation in parallel with the examinations of placental events in situ should be considered as a further step to answer these questions.

In summary, the interaction of PP5/TFPI-2 with glypican-3 has been demonstrated from our studies. In patients with preeclampsia, there was a discrepancy in the PP5/TFPI-2 level in maternal serum, and the immunohistochemical intensity of the protein in the placenta. A decrease in the amount of glypican-3 in the placenta seems to hold the key for the discrepancy, but further studies are necessary to clarify the facts. Preeclampsia and IUGR, often regarded to share the same pathological basis, appeared to be totally distinct in terms of PP5/TFPI-2 distribution.

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