and tolerable this regimen was for epithelial ovarian cancer in Japanese patients. Based on an analysis of the adverse effects seen with the TJ regimen,[5] we have implemented a regimen that administers paclitaxel at 175 mg/m^2 and carboplatin with an area under the curve (AUC) of 5.0 using the formula of Carvert et al.[6] as our first-line chemotheraphy choice. In this study, we compared the mid-term results of use of the TJ regimen with those of conventional platinum-based chemotheraphy from the perspective of both antitumor and adverse effects.

Patients and methods

The subject population comprised 57 women receiving a combination of cisplatin or carboplatin–epirubicin–cyclophosphamide (the CAP group) from 1991 until 1998, and 49 women receiving a combination of paclitaxel–carboplatin (the TJ group) from 1998 until 2002, as the initial chemotherapy for histologically diagnosed FIGO stage Ic–IV epithelial ovarian cancer at Yokohama City University Hospital, Kanagawa Cancer Center, and Yokohama Minami-Kyosai Hospital. The criteria for enrollment were: three courses or more of each regimen, and the acquision of written informed consent; a GOG performance status (PS) of 0–2; FIGO stage Ic–IV epithelial ovarian cancer; start of treatment within 6 weeks of surgery; no history of treatment for ovarian cancer; no serious systemic complications. In addition, well maintained function of the major organs was required, which was defined as: white blood cell (WBC) counts ≥3000/μl; platelet counts ≥100000/μl; serum creatinine ≤2.0 mg/dl; serum bilirubin and aspartate amino transferase values equal to, or less than, double the normal limit for each institution; a creatinine clearance value of ≥50 ml/min. The standard operational procedure included abdominal hysterectomy, bilateral adnexectomy, omentectomy, pelvic lymphadenectomy, and paraaortic lymphadenectomy. The optimal surgery was defined as the presence of residual tumor less than 1 cm in diameter.

After primary tumor reduction surgery, the CAP group was scheduled to receive six courses of cisplatin (70 mg/m^2 intravenously (i.v.)) or carboplatin (AUC 4.0–5.0 i.v.), epirubicin (50 mg/m^2 i.v.), and cyclophosphamide (400 mg/m^2 i.v.) every 4 weeks. The TJ group was scheduled to receive six courses of paclitaxel (175 mg/m^2 i.v. over 3 h) and carboplatin (AUC 5.0 i.v.) every 3 weeks. In addition to a 20-mg drip-infusion of dexamethasone at 7 and 14 h before paclitaxel administration, 50 mg ranitidine hydrochloride was drip-infused and 50 mg diphenhydramine was administered orally 30 min prior to paclitaxel dosing. The carboplatin dose was calculated using the method of Calvert according to the following formula:[6]

$$\text{Carboplatin dose (mg)} = \text{AUC} \times (\text{GFR} + 25)$$

where the glomerular filtration rate (GFR) was estimated by using creatinine clearance values.

All the patients' backgrounds, including age, WHO performance status (PS), FIGO stage/histology, and number of chemotherapy courses were compared between the two groups using a Cox regression analysis. Those patients with a history of serous adenocarcinoma were also evaluated. For the antitumor effect in patients with measurable diseases noted in the computed tomography (CT) scan and/or magnetic resonance imaging (MRI) before chemotherapy, a complete response (CR) was defined as the disappearance of all clinical evidence of malignant disease, a partial response (PR) was defined as a greater than 50% decrease in tumor size, and progressive disease (PD) was identified as a greater than 25% increase in tumor size. The sizes of the measurable lesions were defined using the longest diameter in any one direction. To evaluate patient prognosis, overall survival (OS) and progression-free survival (PFS) were examined. The survival rate was calculated based on all causes of death, and the cumulative survival rate was estimated using the Kaplan–Meier method. Overall survival and PFS were evaluated using the two-sided log–rank test. Statistically significant differences were defined as $P < 0.05$ for all tests. The response rate, PFS, and OS were examined for patients with serous adenocarcinoma by treatment group in order to reveal the outcome of the representative tissue type.

All patients who had completed six courses of treatment were included in the toxicity analysis of using Fisher's exact test. Adverse events were evaluated according to the grading of the National Cancer Institute Common Toxicity Criteria (NCI–CTC, 1998).[7,8] When adverse events of Grade 4 (G4) bone marrow suppression were ongoing, the subsequent course of treatment was postponed until the event resolved. For patients with G4 bone marrow suppression, the doses of anticancer drugs in the next chemotherapy were reduced by 10%–20% of the amounts used in the most recent course of treatment. If other G4 severe adverse events occurred, the protocol was discontinued.

Results

Characteristics of the patients, cycles of chemotherapy, and doses administered

Forty-nine patients were treated with TJ, while 57 received CAP (cisplatin, 54 cases; carboplatin, 3 cases). There were no statistical differences between the two groups in age, PS, FIGO stage, the status of residual disease, or histology (Table 1). The demographics of the serous adenocarcinoma cases, as the most frequent and representative tumor tissue type, were also compared between groups. There were no statistical differences between these two groups (Table 2).

In the TJ group, the mean dose of paclitaxel and the mean carboplatin AUC were 170.6 mg/m^2 and 4.3, respectively, and an average of 6.3 courses of chemotherapy were administered. In the CAP group, cisplatin (54 cases) and the AUC of CBDCA (3 cases) were administered at mean doses of 61.4 mg/m^2 and 4.5, respectively, with an average of 5.8 treatment courses. Three patients in the TJ group and 21 patients in the CAP group discontinued chemotherapy

Table 1. Patient characteristics (all cases)

Characteristic	Paclitaxel + carboplatin TJ group ($n = 49$) (%)	Cyclophosphamide + epirubicin + cisplatin (or carboplatin) CAP group ($n = 57$) (%)
Age (years)		
Median	59.1	56.2
Range	33–74	26–76
WHO performance status		
0	40 (81.6)	52 (91.2)
1	6 (12.2)	4 (7.0)
2	3 (6.1)	1 (1.8)
3	0	0
Residual disease		
Optimal (≦1 cm)	23 (46.9)	34 (59.6)
Suboptimal (>1 cm)	26 (53.1)	23 (40.4)
FIGO stage		
I	15 (30.6)	16 (28.1)
II	5 (10.2)	7 (12.3)
III	23 (46.9)	20 (35.1)
IV	6 (12.3)	14 (24.5)
Cell type		
Serous adenocarcinoma	21 (42.9)	31 (54.4)
Endometrioid adenocarcinoma	7 (14.3)	5 (8.8)
Mucinous adenocarcinoma	4 (8.2)	7 (12.3)
Clear cell adenocarcinoma	12 (24.5)	11 (19.3)
Undifferentiated	5 (10.1)	3 (5.2)

WHO, World Health Organization; FIGO, International Federation of Gynecology and Obstetrics

Table 2. Patient characteristics (serous adenocarcinoma cases)

Characteristic	Paclitaxel + carboplatin TJ group ($n = 21$) (%)	Cyclophosphamide + epirubicin + cisplatin (or carboplatin) CAP group ($n = 31$) (%)
Age (years)		
Median	57.3	55.4
Range	33–70	49–70
WHO performance status		
0	18 (85.7)	29 (93.5)
1	1 (4.8)	2 (6.4)
2	2 (9.5)	0
3	0	0
Residual disease		
Optimal (≦1 cm)	8 (38.1)	14 (45.2)
Suboptimal (>1 cm)	13 (61.9)	17 (54.8)
FIGO stage		
I	3 (14.3)	4 (12.9)
II	3 (14.3)	3 (9.7)
III	12 (57.1)	13 (41.9)
IV	3 (14.3)	11 (35.5)

before completion of the scheduled six cycles of treatment. All three discontinuations in the TJ group were due to disease progression. The discontinuations in the CAP group were due to severe adverse events (7 patients: 2 cases of bone marrow suppression, 5 cases of nausea and vomiting), disease progression (4 patients), or complete remission prior to the completion of six courses of treatment (10 patients).

Response to treatment (Table 3)

The response rate in patients with stage III–IV cancer who had residual tumors was 82.8% (24/29) in the TJ group and 70.6% (24/34) in the CAP group. Complete response (CR) in cases at stage III–IV was achieved in 51.7% (15/29) of the TJ group and 50.0% (17/34) of the CAP group. With respect to the histology of stage III–IV, the response rate for serous

Table 3. Clinical response in stage III–IV cases by treatment group and histological type

Response	TJ group			CAP group		
	Serous (n = 15) No. of pts. (%)	Nonserous (n = 14) No. of pts. (%)	Total (n = 29) No. of pts. (%)	Serous (n = 22) No. of pts. (%)	Nonserous (n = 12) No. of pts. (%)	Total (n = 34) No. of pts. (%)
Complete response	8 (53.3)	7 (50.0)	15 (51.7)	10 (45.5)	7 (58.3)	17 (50.0)
Partial response	5 (33.3)	4 (28.6)	9 (31.0)	6 (27.3)	1 (8.3)	7 (20.6)
No change	2 (13.3)	2 (14.3)	4 (13.8)	2 (9.1)	3 (25.0)	5 (14.7)
Progressive disease	0	1 (7.1)	1 (3.4)	4 (18.2)	1 (8.3)	5 (14.7)
Response rate (%)	86.7	78.6	82.8	72.7	66.7	70.6

Table 4. Frequency of adverse effects (NCI–CTC scale)

Adverse effect	TJ group (%), n = 6					CAP group (%), n = 36				
	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Neutropenia	8.7	0	0	6.5	84.8	2.9	2.9	11.1	37.1	45.7
Leukopenia	0	0	47.8	50.0	2.2	11.1	0	27.8	55.6	2.8
Thrombocytopenia	86.7	8.9	0	4.4	0	75.0	12.5	6.3	6.3	0
Anemia	13.3	35.6	48.9	2.2	0	18.8	28.1	40.6	9.4	3.1
Allergic reaction**	78.2	19.6	0	0	2.2	100	0	0	0	0
Neuropathy*	13.3	60.0	26.7	0	0	77.8	19.4	0	2.8	0
Myalgia/arthralgia*	28.3	39.1	26.1	6.5	0	100	0	0	0	0
Alopecia	0	20.0	80.0	–	–	8.3	33.3	58.3	–	–
Nausea/vomiting	8.7	56.5	32.6	2.2	0	5.6	16.7	58.3	19.4	0
Renal dysfunction*	88.9	4.4	4.4	2.2	0	50.0	41.7	8.3	0	0

NCI–CTC, National Cancer Institute common toxicity criteria
* $P < 0.001$; ** $P < 0.01$

adenocarcinoma was 86.7% (13/15) in the TJ group and higher than 72.7% (16/22) in the CAP group. The response rate for cancer with histology other than serous adenocarcinoma (the nonserous group) was 78.6% (11/14) in the TJ group and 66.7% (8/12) in the CAP group. There was no statistically significant difference in the response rate between the two groups.

Progression-free survival and overall survival

Figure 1 shows the PES and OS curves for patients at stage Ic–IV. The median PFS values were 33.6 months for the TJ group and 47.3 months for the CAP group. No statistically significant difference was found (PFS, $P = 0.29$; OS, $P = 0.71$). Figure 2 shows the PFS and OS curves for patients at stage III–IV. The median PFS was 17.6 months for the patients treated with TJ and 29.6 months for the patients treated with CAP, while the median of the OS was 43.9 months for the TJ group and 44.3 months for the CAP group. These results showed no statistically significant difference (PFS, $P = 0.11$; OS, $P = 0.95$). Figure 3 shows the PFS and OS curves in patients with serous adenocarcinoma. The median PFS in the serous type was 17.6 months for the TJ group and 25.3 months for the CAP group, while the median OS was 43.9 months for the TJ group and 44.3 months for the CAP group (PFS, $P = 0.95$; OS, $P = 0.72$).

Toxicity

Toxicity was evaluated in the 46 cases from the TJ group and 36 cases from the CAP group who had completed six or more courses of chemotherapy (Table 4). In terms of hematological toxicity, neutropenia at G3 or G4 occurred more frequently in those patients undergoing TJ treatment (91.3%) than in those receiving CAP (82.8%), but this difference was not significant. Neutropenia accompanying a fever was rare, and no patients required treatment termination due to that condition. Grade 3 or G4 leukopenia was observed in 52.2% of the patients receiving TJ and 58.4% of those receiving CAP. Grade 3 or G4 thrombocytopenia and hemoglobin level decrease rarely occurred in either treatment group.

In terms of nonhematological toxicity, peripheral neuropathy (TJ, 86.7%; CAP, 22.2%; $P < 0.001$), myalgia/arthralgia (TJ, 71.7%; CAP, 0%; $P < 0.001$), and allergic reaction (TJ, 21.8%; CAP, 0%; $P < 0.01$) occurred significantly more often in the TJ group. However, renal dysfunction (TJ, 11.0%; CAP, 50.0%; $P < 0.001$) occurred more frequently in the CAP group. Alopecia often appeared in both groups (TJ, 100%; CAP, 91.6%; no significant difference). A grade 4 allergic reaction was noted for one TJ patient during the 6th course of chemotherapy while the patient was receiving carboplatin.

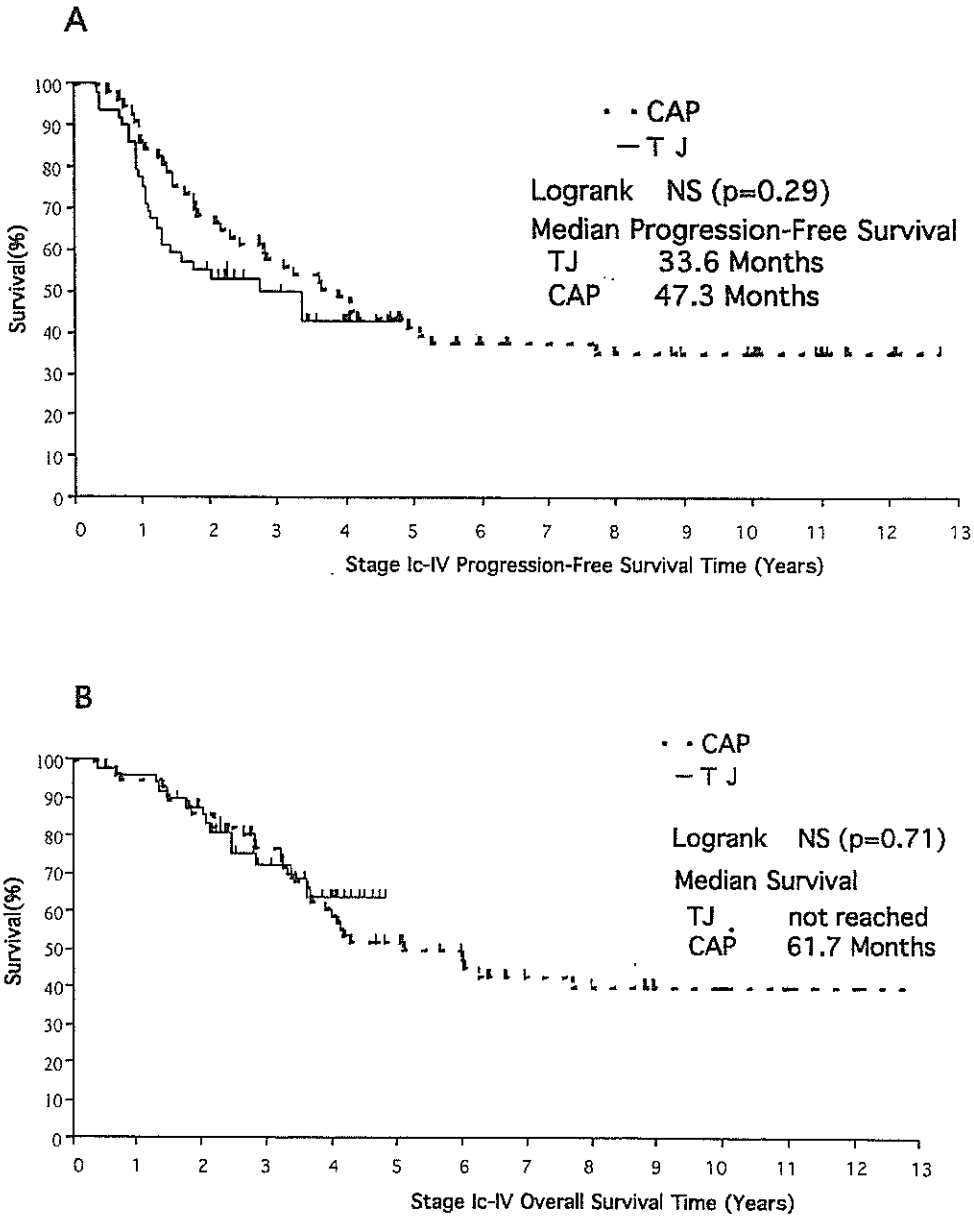

Fig. 1A,B. Kaplan–Meier curve for progression-free and overall survival times in patients with ovarian cancer (stage Ic–IV) by treatment type. Patients received either paclitaxel–carboplatin treatment (TJ; *solid line*) or cyclophosphamide–epirubicin–cisplatin or carboplatin treatment (CAP; *dotted line*). **A** Progression-free survival time was not significantly different between the two treatment types (log–rank, $P = 0.29$). **B** Overall survival time was also not significantly different between the two treatment types. (log–rank, $P = 0.71$)

Discussion

Although randomized clinical trials performed on a large scale in Western countries[1–4] convinced gynecologists of the superiority of the taxan-based chemotherapy regimen (paclitaxel plus cisplatin or carboplatin) compared with the previous standard CP regimen, the validity of substituting the TJ regimen for the CAP regimen was not clear, as CAP had been widely used as the first-line chemotherapy for epithelial ovarian cancer in Japan.

In the TJ group in this study, the response rate and CR in patients with stage III–IV epithelial ovarian cancer were 82.8% and 51.7%, respectively. These data were comparable to the results seen in other countries when advanced cases were treated with TJ, as shown by a 66% response rate reported by Neijt et al.[9] and a 72% response rate noted by Schink et al.[10] With respect to the midterm prognosis for the TJ group in this study, the median PFS for patients at stage III–IV after optimal or suboptimal surgery was 17.6 months, while the median OS was 43.9 months. A study performed in Europe by the AGO[4] revealed a median PFS and OS of 17.2 and 43.3 months, respectively, in patients that received six courses of paclitaxel (185 mg/m^2 i.v. over 3 h) and carboplatin (AUC 6.0) every 3 weeks to treat stage IIb–IV cancer. Despite the differences in the patients' backgrounds and the dosage of paclitaxel and carboplatin, the survival period for our advanced cases treated with the TJ regimen was equivalent to those given in previous reports. In our study, despite the higher response rate seen for patients treated with TJ, the prognosis was not significantly improved in the TJ group compared with those patients

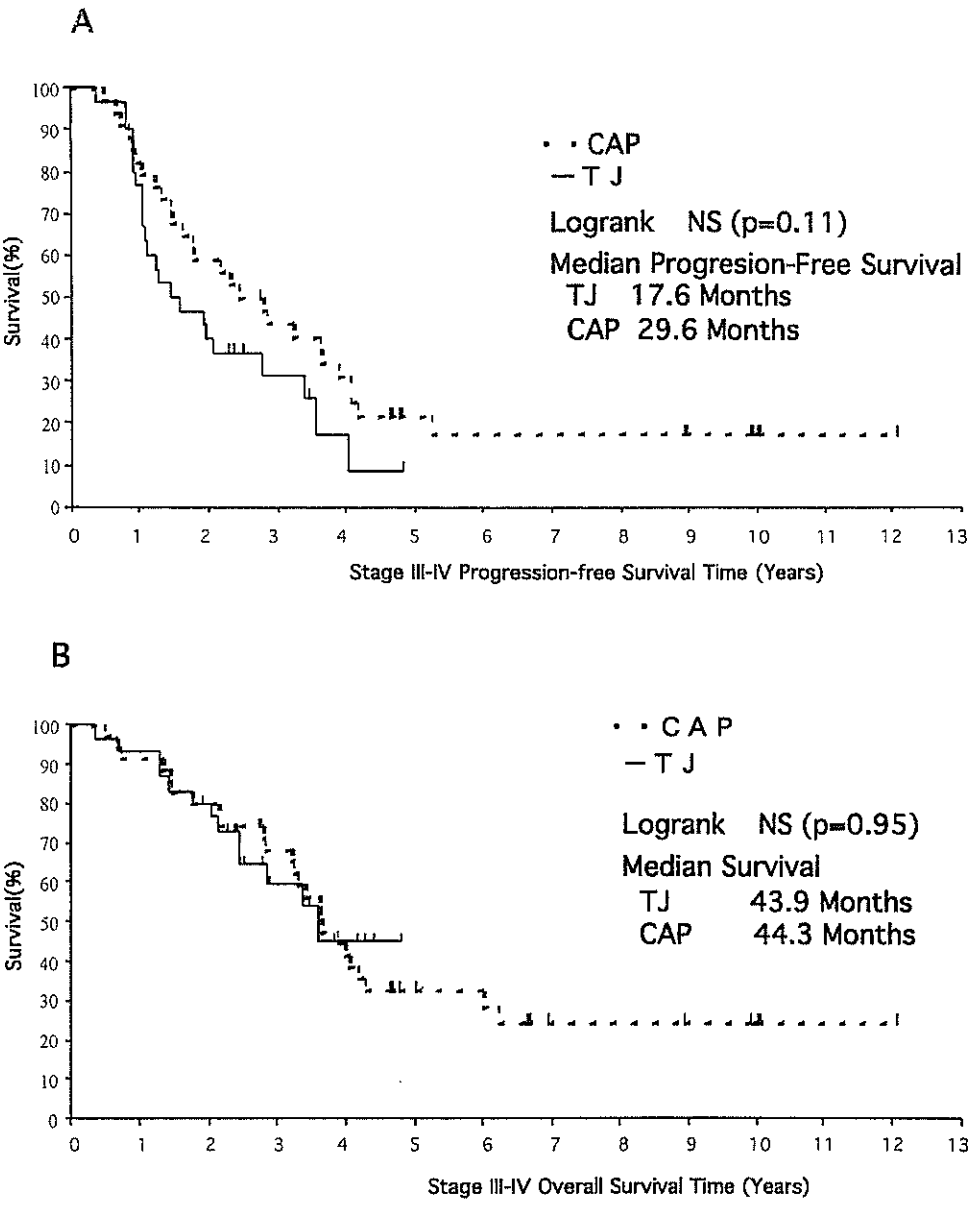

Fig. 2A.B. Kaplan–Meier curve for progression-free and overall survival times in patients with advanced ovarian cancer (stage III–IV) by treatment type. Patients received either paclitaxel–carboplatin treatment (TJ; *solid line*) or cyclophosphamide–epirubicin–cisplatin or carboplatin treatment (CAP; *dotted line*). **A** Progreesion-free survival time was not significantly different between the two treatment types (log–rank, $P = 0.11$). **B** Overall survival time was also not significantly different between the two treatment types (log–rank, $P = 0.95$)

receiving CAP. One explanation for this result may be that the CDDP dose level for the CAP regimen used in our institutes was relatively high for Japanese women (70 mg/m^2 every 4 weeks), and the carboplatin AUC for the TJ regimen was reduced in some patients owing to the occurrence of G4 neutropenia and/or G3–G4 myalgia/arthralgia, resulting in a lower average AUC of 4.3. However, the frequency of G4 neutropenia was equivalent to that in reports from overseas. Based on our own recent experience, G4 neutropenia cases do not necessarily require a reduction in dosage when the patient has no infection-related fever. Therefore, it is necessary to thoroughly evaluate both the prognosis and the adverse effects for patients who receive carboplatin at an AUC of 5.0 or more, and we must also determine an appropriate carboplatin AUC for Japanese women. Regarding the similar median survival times but marginally significant differences in OS time between the TJ and the CAP groups with stage III–IV diseases (see Fig. 2), after long-term observation of additional cases we hypothesize that repeated taxan-based chemotherapy for the persistent or recurrent cancers which were sensitive to the first TJ treatment regimen may produce statistically significant longer survival times compared with those in the CAP group.

There are few reports from other countries on chemotherapy response rates for different ovarian histology types. In this study, we investigated treatment response rates in serous and nonserous tumor groups. In our cases at stage III–IV the response rate was 86.8%, and was highest in the TJ serous group. Previous reports[11–13] had indicated that the TJ regimen was scarcely effective for clear cell adenocarcinoma and mucinous adenocarcinoma. However, we observed a response in 40% (2/5) of our clear cell adenocarcinoma cases and in all three mucinous adenocarcinoma

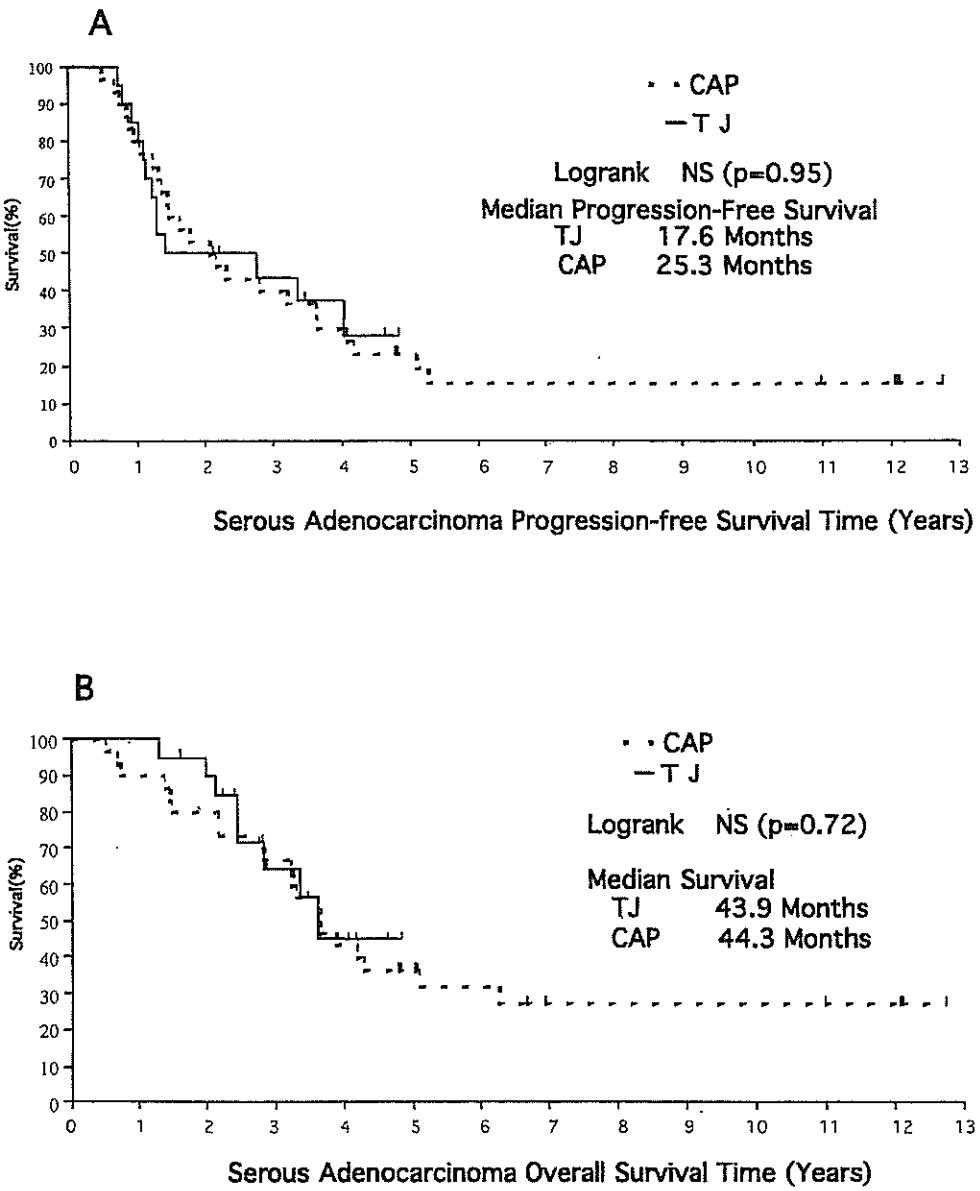

Fig. 3A,B. Kaplan–Meier curve for progression-free survival (PFS) and overall survival (OS) times in patients with serous ovarian cancer by treatment type. Patients received either paclitaxel–carboplatin treatment (TJ; *solid line*) or cyclophosphamide–epirubicin–cisplatin or carboplatin treatment (CAP; *dotted line*). **A** Progreesion-free survival time in patients with serous ovarian cancer (log–rank, $P = 0.95$). **B** Overall survival time in patients with serous ovarian cancer (log–rank, $P = 0.7$)

cases (data not shown). A further accumulation of such cases is required before conclusions can be drawn regarding the treatment efficacy for these cancer histology types.

Peripheral neuropathy, allergic reaction, and myalgia/arthralgia were found significantly more often in the TJ group. Peripheral neuropathy appeared in 86.7% of the TJ group, but no cases developed G3 or more severe neuropathy leading to the termination of treatment. Irreversible renal dysfunction appeared statistically more frequently in the CAP group. Our previous study also revealed that the volume overload by drip infusion is a burden for these patients, even though it was intended to alleviate the renal dysfunction associated with the CAP regimen.[5] The AGO study also reported that the TJ regimen rarely caused gastrointestinal symptoms or severe neurotoxicity, while an excellent quality of life was achieved.[4]

It became clear in this study that the TJ regimen was comparable to the previous CAP regimen in antitumor effect when used to treat epithelial ovarian cancer. In addition, the adverse effects of the TJ regimen were clinically controllable, and it provides the advantage of brief hospitalization or the feasibility of treatment at an outpatient clinic. In our preliminary data, the TJ regimen allowed short-term hospitalization for 2–5 days (mean 3.3 days) compared with the 5–10 days (mean 7.5 days) for hydration in the CAP treatment regimen as the only chemotherapy (data not shown). Chemotherapy in an outpatient clinic has not been common in Japan, but TJ therapy might spread rapidly as one of the regimens suitable for the trend to outpatient chemotherapy. It would be reasonable to select the TJ regimen as the first-line chemotherapy until a newer, even more superior regimen is established. With respect to optimal treatment, a dose lower than that used overseas

may be effective enough in Japanese women, but it is necessary to accumulate additional cases before making a dosing recommendation. In addition, no conclusion has yet been reached as to the appropriate number of courses of the TJ regimen needed to treat advanced cases. Since studies have been carried out to evaluate new drugs such as gemcitabine and topotecan for advanced ovarian cancer,[14,15] and there are reports of the weekly TJ regimen of paclitaxel and carboplatin in divided administration,[16,17] a comparison with the usual TJ regimen is awaited.

Together with our results and previously reported data, we conclude that the TJ regimen maintains patients' quality of life and is able to replace conventional cisplatin-based chemotherapy at present.

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The role of pretreatment squamous cell carcinoma antigen level in locally advanced squamous cell carcinoma of the uterine cervix treated by radiotherapy

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Abstract. Ogino I, Nakayama H, Okamoto N, Kitamura T, Inoue T. The role of pretreatment squamous cell carcinoma antigen level in locally advanced squamous cell carcinoma of the uterine cervix treated by radiotherapy. *Int J Gynecol Cancer* 2006;**16**:1094–1100.

The purpose of this study was to determine the pretreatment serum squamous cell carcinoma antigen (SCC-ag) level as a generally applicable measurement in predicting and estimating the treatment outcome of patients with locally advanced SCC of the cervix. Three hundred fifty-two patients with stage IIB–IVA SCC of the cervix were managed with both external irradiation and high-dose rate intracavitary brachytherapy. A significantly higher median SCC-ag was seen in association with increasing stage, tumor size, and lymph node involvement. The difference in disease-free survival (DFS) between stages IIB and III patients was not statistically significant with SCC-ag level <2 ng/mL. In multivariate analysis, median SCC-ag level (≥6.0 ng/mL) and lymph node metastases had significant independent effects on absolute survival and DFS. A direct linear relationship ($y = -2.932x + 84.896$) existed between the median SCC-ag of groups distributed by pretreatment prognostic factors and the 5-year DFS rate. The 5-year DFS rate as a function of SCC-ag level defined by cervix size, lymph node status, and hydronephrosis was obtained from a formula combining risk scores and the baseline survival function. From the obtained formulas, we can objectively estimate the treatment outcome in patients with locally advanced squamous cell cervical cancer.

KEYWORDS: cervical carcinoma, high-dose rate intracavitary brachytherapy, prognostic factors, radiation therapy, squamous cell carcinoma antigen.

The serum squamous cell carcinoma antigen (SCC-ag) is a tumor-related antigen for which Kato and Torigoe[1] developed a radioimmunoassay method for human cervical SCC. This antigen, which was obtained from SCC of the cervix or liver metastasis tissue, has a molecular weight of approximately 45 kd and is almost a pure glycoprotein[2]. The release of SCC-ag into the serum depends on the infiltrative growth and mass of the tumors[3]. A recent description of two of its genes indicates that SCC-ag may function as a serine/cysteine proteinase inhibitor; its components are known to be involved in the degradation of the extracellular matrix and in tumor invasion and metastasis[4].

Many publications have confirmed its value as a sensitive and specific tumor marker in invasive SCCs of the cervix[3,5–8]. Several studies have indicated serum levels of SCC-ag to be correlated with stage, tumor size, lymph node involvement, residual tumor after treatment, recurrent or progressive disease, and survival in cervical carcinoma[4,9–20]. Although an elevated pretreatment SCC-ag level with different cutoffs using the available positive data was proposed, the positive data were generally not used to estimate the treatment outcomes.

The purpose of our study was to determine the pretreatment SCC-ag level as a generally applicable

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measurement in predicting and estimating the treatment outcome in patients with locally advanced squamous cell cervical cancer.

Materials and methods

Patient population

Between April 1986 and January 2001, 352 patients with FIGO stage IIB–IVA invasive SCC of the intact uterine cervix were managed with both external irradiation of the pelvis and high-dose rate intracavitary brachytherapy at Kanagawa Cancer Center. SCC-ag was measured in all patients before radiotherapy, and we conducted a retrospective review of the patient records. The primary treatment protocol was uniform, and there was no protocol for recurrent patients after primary treatment. Treatment other than radiotherapy was not performed in any patients before recurrence.

Initial clinical assessment

Patients were initially evaluated with a complete history and physical examination as well as a pelvic and rectal examination, blood counts, chemistry profile, chest X-ray, intravenous pyelogram, and computed tomography (CT). Cystoscopy was performed routinely. All patients underwent a biopsy.

CT images were acquired for all patients. The anterior–posterior (AP) cervix diameter was measured objectively by CT on a section at the largest portion of the cervix[21]. Magnetic resonance imaging (MRI) was also used to confirm the location of the cervix in some patients. Lymph nodes greater than 1 cm in greatest diameter were interpreted as being involved.

Serum samples were collected from each patient for serum SCC-ag level determination within 2 weeks before treatment initiation. Serum specimens were stored at −20°C until the assay. The SCC-ag assay was performed within a week after sample collection[1]. Serum SCC-ag levels were determined by radioimmunoassay using an SCC-RIABEAD kit from Dainabot Co., Ltd. (Tokyo, Japan). The sensitivity limit of the assay was 0.1–150 ng/mL.

Radiation treatment

The radiotherapy techniques used in these patients have been reported previously in detail and are described here only briefly[21,22]. All the patients received combination treatment with intracavitary brachytherapy and external irradiation. External irradiation was delivered to the pelvis using a 10-MV linear accelerator. The patient was treated through parallel opposed anteroposterior portals. The external beam daily dose calculated along the central axis at the midplane of the pelvis was 1.8–2 Gy. The median total dose given by the external irradiation was 50 Gy (range: 23.4–66 Gy), and 328 (93%) patients received external doses between 45 and 50.4 Gy. A small block was placed to protect the bladder and rectum after irradiation of 10–51.4 Gy (median 28 Gy) when the cervical tumor burden was small enough to be cured by intracavitary brachytherapy. The treatment schedule included a program of four external beam fractions per week combined with one high-dose rate intracavitary brachytherapy treatment using ^{60}Co per week. The dose per fraction planned at point A was 5–6 Gy per week for 5–6 fractions, and the total dose was adjusted by clinical tumor regression. The median intracavitary brachytherapy dose was 29 Gy (range: 5–36 Gy).

Follow-up

The median follow-up for all patients was 53 months (range: 2–191 months). Clinical examination, Pap smears, serum SCC-ag, and other blood analyses were performed every 3 months for 5 years after the completion of therapy and once a year thereafter. A CT scan of the whole abdomen was done before and after radiotherapy, every year for 2 years, and then individually scheduled according to clinical findings or elevated serum SCC-ag levels. Chest X-rays were performed every 6 months.

Statistical analysis

Statistical analyses were performed using Kruskal–Wallis one-way analysis of variance and the Mann–Whitney test to examine the relationship between SCC-ag levels and other pretreatment factors. These nonparametric tests were used because they do not rely on the normality of data distribution and do not require equality of variance. Because a cutoff level of 2 ng/mL was the most commonly used level in other reports[4,9–12,15], we selected this level to compare the clinical outcome of other studies. Median data were also chosen to identify the optimal cutoff to discriminate low- and high-probability categories. Kaplan–Meier curves were used to assess pretreatment variables as a risk predictor and compared using the log-rank test[23]. A stepwise Cox proportional hazards model was used to determine which parameters influenced the probability of disease-free survival (DFS) and absolute survival (AS) in carcinoma of the cervix treated by radiation therapy[24]. The correlation between the median SCC-ag of groups distributed by pretreatment prognostic

factors and the 5-year DFS rates was examined graphically and also using Pearson's correlation coefficients (R). Factors with a difference at the 0.05 level were considered significant. Statistics were analyzed using SPSS 11.0.

Results

The AS and DFS rates of all patients at 5 years obtained by Kaplan–Meier product-limited methods were 61.3% and 65.7%, respectively. Five-year pelvic control obtained by radiation therapy was 81.4%. The distribution of possible pretreatment prognostic factors is shown in Table 1. A significantly higher median SCC-ag was seen in association with increasing FIGO stages (3.0 ng/mL for stage IIB vs 8.1 ng/mL for stage III vs 19.75 ng/mL for stage IVA, Kruskal–Wallis, $\chi^2 = 39.1$ [$df = 2$], $P < 0.001$). Large tumors, AP cervix size ≥45 mm, were also associated with a significantly higher SCC-ag level (median SCC-ag of 8.85 vs 4.2 ng/mL, Mann–Whitney, $P < 0.001$). The median SCC-ag was significantly greater in patients with lymph node involvement, LN (+), than in those without lymph node involvement, LN (−) (12.7 vs 5.5 ng/mL, Mann–Whitney, $P < 0.001$).

Table 1. Distribution of patient characteristics ($n = 352$)

Characteristic	Distribution of patients
Age (years)	
Median	61
Range	28–90
Stage (FIGO), *n* (%)	
IIB	99 (28)
III	239 (68)
IVA	14 (4)
SCC-ag (ng/mL)	
Median	6.0
Range	0.5–450
AP cervix size (mm)	
Median	45
Range	20–90
Lymph node involvement *n* (%)	
Negative	286 (81)
Positive	66 (19)
Hydronephrosis *n* (%)	
Negative	307 (87)
Positive	45 (13)
Hematocrit (%)	
Median	35.7
Range	15.1–51.8
External dose without block (Gy)	
Median	28
Range	10–51.4
Intracavitary brachytherapy dose (Gy)	
Median	29
Range	5–36

Of the 352 patients, 82 were found to have SCC-ag level <2 ng/mL and 270 were found to have SCC-ag level ≥2 ng/mL. DFS in relation to cutoff of SCC-ag level at 2 ng/mL is shown in Figure 1. The 5-year DFS rate was lower in patients with SCC-ag level ≥2 ng/mL than in patients with SCC-ag level <2 ng/mL (60.0% vs 83.3%; $P = 0.0011$). The incidence of FIGO stages distribution correlated significantly among those 82 patients with SCC-ag level below 2 ng/mL. There were 40 of 99 patients (40%) with stage IIB, 41 of 239 patients (17%) with stage III, and 1 of 14 patients (7%) with stage IVA (Kruskal–Wallis, $\chi^2 = 23.2$ [$df = 2$], $P < 0.001$). When only patients with SCC-ag level below 2 ng/mL were considered, the difference in the 5-year DFS rate between stages IIB and III patients was not statistically significant (83.7% vs 82.5%; $P = 0.49$) (Fig. 2). For patients with SCC-ag level ≥2 ng/mL, the 5-year DFS rate was higher in patients with stage IIB than in patients with stage III diseases (70.6% vs 58.7%; $P = 0.05$) (Fig. 3).

The 5-year Kaplan–Meier estimates of DFS rate according to pretreatment prognostic factors are outlined in Table 2. Age, FIGO stage, SCC-ag level, AP cervix size, LN (+), and hydronephrosis were identified as having a significant effect on the DFS in univariate analysis.

The 5-year DFS rate was lower in patients receiving a larger external dose without block, or larger total intracavitary brachytherapy dose. The 5-year DFS rate was 52% in patients receiving an external dose without block of ≥ 28Gy, 80% in patients receiving an external dose without block of <28 Gy ($P < 0.0001$), 62% in patients with total intracavitary brachytherapy dose of ≥29 Gy, and 70% in patients with total

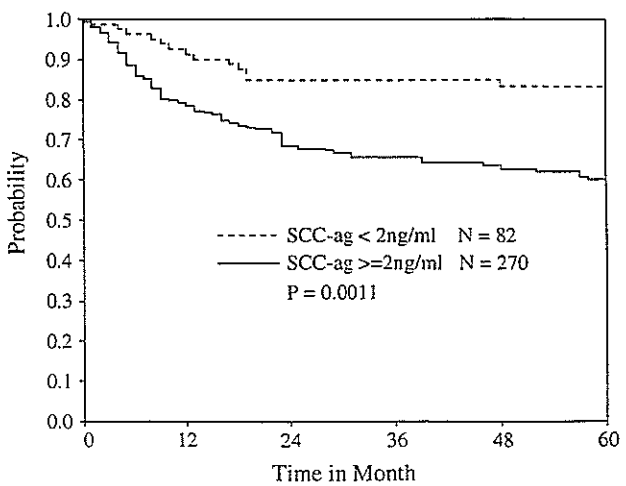

Figure 1. DFS for patients with SCC-ag level ≥2 ng/mL and those with SCC-ag level <2 ng/mL.

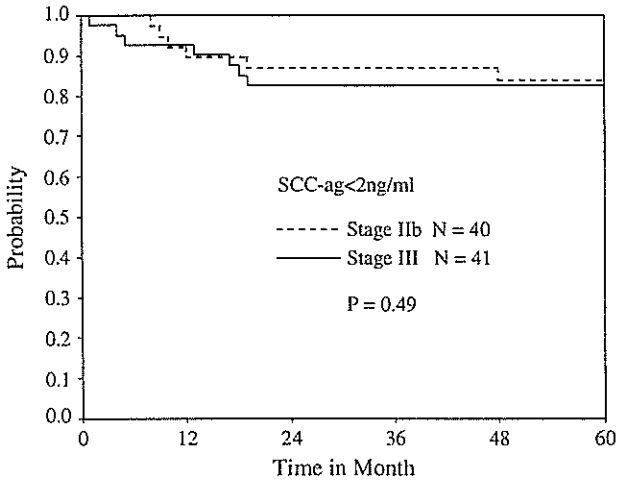

Figure 2. DFS for stages II and III patients with SCC-ag level below 2 ng/mL. Five-year rates: stage II, 84%-and stage III, 82%.

intracavitary brachytherapy dose of <29 Gy (P = 0.044). Radiotherapy doses were excluded from the multivariate analysis because patients with larger tumors had poorer prognosis and were more likely to receive a higher radiation dose. Stepwise Cox multivariate analysis performed with the covariates outlined in Table 2 is shown in Table 3. Parameters carrying significant association with poor outcome in terms of DFS included, in decreasing order of significance, presence of LN (+) ($P < 0.001$), SCC-ag ≥6.0 ng/mL ($P = 0.004$), and hydronephrosis ($P = 0.017$).

For AS, FIGO stage ($P = 0.0013$), SCC-ag level ($P <$ 0.0001), AP cervix size ($P = 0.0058$), LN (+) (P = 0.0004), hydronephrosis ($P = 0.0002$), and hematocrit ($P = 0.0058$) were identified as having a significant

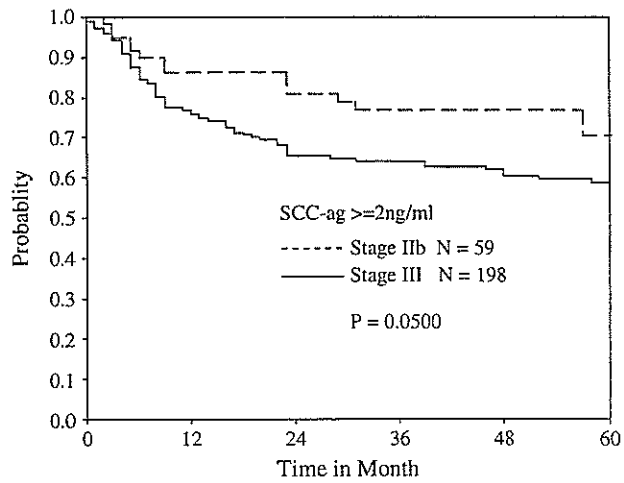

Figure 3. DFS for stages II and III patients with SCC-ag level ≥2 ng/mL. Five-year rates: stage II, 71% and stage III, 59%.

Table 2. DFS at 5 years after radiotherapy

Characteristic	No. of patients	SCC-ag level (ng/mL) median	5-years DFS (%)	P value
Age (years)				
<61	164	5.6	61	0.045
≥61	188	6.95	70	
Stage				
IIB	99	3	76	0.0019
III	239	8.1	63	
IVA	14	19.75	30	
SCC-ag (ng/mL)				
<6.0	175		77	<0.0001
≥6.0	177		53	
AP cervix size (mm)				
<45	172	4.2	76	0.0002
≥45	180	8.85	56	
Lymph node involvement				
Negative	286	5.5	72	<0.0001
Positive	66	12.7	39	
Hydronephrosis				
Negative	307	5.5	70	<0.0001
Positive	45	18.3	33	
Hematocrit (%)				
<35.7	175	7.2	63	0.10
≥35.7	177	5.4	69	

effect in univariate analysis. SCC-ag ≥6.0 ng/mL (P = 0.002) and LN (+) ($P = 0.012$) significantly contributed to AS in stepwise Cox multivariate analysis (Table 4). Multivariate analysis significant testing confirmed the power of the risk associated with SCC-ag ≥6.0 ng/mL in AS and DFS.

A direct linear relationship existed between median SCC-ag of groups distributed by pretreatment prognostic factors and 5-year DFS rates was obtained from Table 2 and is shown in Figure 4. The equation of the direct linear relationship is as follows:

$$y = -2.932x + 84.896 \quad (R = 0.965, P < 0.001)$$

In these expressions, y denotes the percentage of 5-year DFS rate and x denotes median SCC-ag.

Table 3. Significance of pretreatment parameters affecting DFS in stepwise Cox multivariate analysis

	Hazard ratio	95% confidence interval	P value
SCC-ag in ng/mL (<6.0 vs ≥6.0)	1.791	1.201–2.671	0.004
AP cervix size in mm (<45 vs ≥45)	1.442	0.965–2.154	0.074
Lymph node involvement	2.075	1.375–3.133	0.001
Hydronephrosis	1.742	1.102–2.752	0.017

Table 4. Significance of pretreatment parameters affecting AS in stepwise Cox multivariate analysis

	Hazard ratio	95% confidence interval	*P* value
Age in years (<61 vs ≥61)	1.327	0.960–1.834	0.087
Stage (II vs III vs IV)	1.393	0.996–1.947	0.053
SCC-ag in ng/mL (<6.0 vs ≥6.0)	1.717	1.223–2.412	0.002
Lymph node involvement	1.628	1.113–2.382	0.012
Hematocrit (<35.7 vs ≥35.7)	0.747	0.538–1.037	0.081

Table 5. Significance of pretreatment parameters affecting DFS in stepwise Cox multivariate analysis along with the continuous SCC-ag

	Hazard ratio	95% confidence interval	*P* value
SCC-ag in ng/mL (continuous)	1.004	1.000–1.007	0.035
AP cervix size in mm (<45 vs ≥45)	1.541	1.034–2.297	0.034
Lymph node involvement	2.072	1.367–3.141	0.001
Hydronephrosis	1.738	1.068–2.828	0.026

Stepwise Cox multivariate regression analysis of DFS was performed with the previously described covariates, and along with the continuous SCC-ag level the results are shown in Table 5. The risk scores $r(x)$ obtained by stepwise Cox multivariate analysis were combined with the baseline survival function $S(t)$. The formula of survival probabilities is as follows:

$$y = S(t)^{\exp(r(x))}$$

In these expressions, y denotes the percentage of 5-year DFS rate and x denotes the SCC-ag level. $S(t)$ at 5 years was 0.675. The graphs showing the 5-year survival probabilities as a function of SCC-ag within eight groups defined by AP cervix size, lymph node status, and hydronephrosis are shown in Figure 5.

Discussion

FIGO stage, tumor size, and lymph node status have been recognized as important predictors of patient survival. Pretreatment SCC-ag levels have been shown to correlate with clinical FIGO stage, tumor volume, and risk of lymph node metastases.

Strong relationships between pretreatment SCC-ag levels and clinical FIGO stage were reported. In association with increasing stage, a significantly increased proportion of SCC-ag levels divided by different cutoff ranging from 1.5 to 5 ng/mL was reported[12,13,18]. A significantly higher median or mean SCC-ag was also seen in association with increasing stage[10,11].

A good correlation between the pretreatment SCC-ag levels and the tumor size was reported[12,13,17,18,20]. Cervical tumor size was measured using different modalities such as bimanual examination, colposcopy, ultrasonography, and surgically removed specimens. Using a multiple logistic model, Takeshima *et al.*[20] reported that pathologic tumor size and pelvic lymph node metastasis were found to have a significant effect

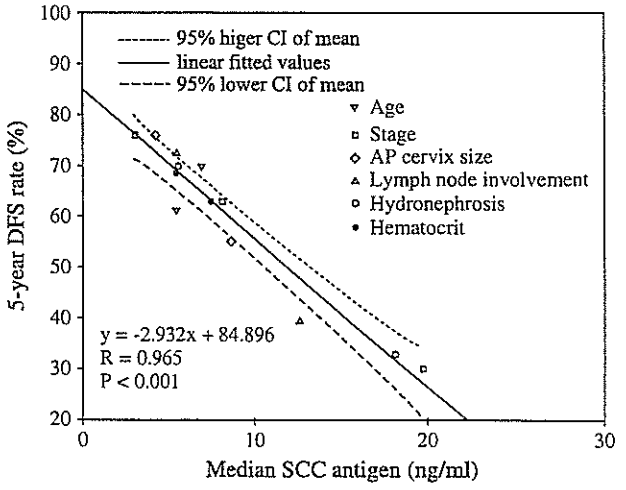

Figure 4. Scatter plot demonstrating high correlation between median SCC-ag of groups distributed by pretreatment prognostic factors and 5-year DFS rates. Parameters with statistical significance and nonsignificance are depicted by open and solid symbols, respectively.

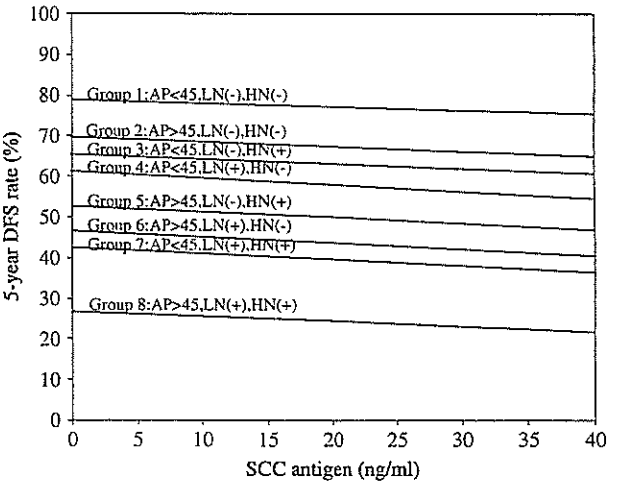

Figure 5. The correlation between SCC-ag level and 5-year DFS rate within each group defined by AP cervix size, lymph node status, and hydronephrosis.
LN(+), with lymph node involvement; LN (−), without lymph node involvement; AP ≥ 45, AP cervix size ≥ 45 mm; AP < 45, AP cervix size <45 mm; HN (+), with hydronephrosis; HN (−), without hydronephrosis.

on the ratio of serum levels above 4 ng/mL in patients with stage IB squamous cell cervical cancer undergoing hysterectomy. Ohara *et al.*[17] reported that there was a significant positive correlation between SCC-ag and tumor volumes measured by T2-weighted MRIs in patients with stage IB–IVA squamous cell cervical cancer treated primarily by radiotherapy.

Elevated pretreatment serum SCC-ag levels were reported as independent predictors for the presence of lymph node metastases[10,12,16,18,20]. Duk *et al.*[12] reported that elevated pretreatment serum SCC-ag levels ≥2.0 ng/mL, lesion size ≥40 mm, and presence of vascular invasion by tumor cells were independent predictors for the presence of lymph node metastases in stage IB–IIA squamous cell cervical cancer patients treated by radical hysterectomy. Bolger *et al.*[10] reported the positive predictive value for lymph node metastases at >8.6 ng/mL SCC-ag is 100% in stage IA–IIB cervical cancer patients with primary surgical treatment. Lin *et al.*[16] reported that around 86% of the patients with SCC-ag levels below 8 ng/mL showed no nodal metastasis, while about 65% of the patients with serum levels above 8 ng/mL exhibited nodal metastasis in stage IB and IIA squamous cell cervical carcinoma undergoing radical hysterectomy and pelvic lymphadenectomy. Only lymph node metastasis had a significant independent impact on SCC-ag levels exceeding 8 ng/mL.

Hong *et al.*[15] reported that the association between increased SCC-ag level and positive lymph nodes detected by CT was significant only when the SCC-ag level was higher than 10 ng/mL after controlling for stage in stage I–IVA squamous cell cervical cancer patient treated primarily by radiotherapy. Assessment for lymph node metastasis usually employs CT or MRI in cervical carcinoma treated primarily by radiotherapy. Because variable sensitivity for lymph node metastasis in imaging analysis using CT (range: 24–65%) or MRI (range: 24–71%) compared with surgicopathologic findings has been reported[25–28], correlation between the levels of SCC-ag and the positive lymph nodes is difficult to evaluate in patients treated by radiotherapy alone, and no surgicopathologic results were obtained.

In our analysis, median SCC-ag was significantly greater, in association with increasing FIGO stage, in those patients with AP cervix size ≥45 mm and in those patients with lymph node involvement.

The most important clinical correlation for pretreatment SCC-ag levels is its ability to predict clinical outcome. There are several reports showing that the serum SCC-ag value is a good prognostic factor in patients with cervical SCC[12,15,19]. Duk *et al.*[12] reported that among clinicopathologic factors (serum SCC-ag level, grade, lesion size, stromal infiltration, vascular invasion, and node status), only pretreatment serum SCC-ag level ≥2.0 ng/mL had a significant independent effect on DFS in stage IB and IIA patients with SCC treated by radical hysterectomy. Strauss *et al.*[19] reported preoperative SCC-ag, using a cutoff value of 3.0 ng/mL, as an independent prognostic factor, both for recurrence-free and overall survival in patients who underwent radical hysterectomy for stages IA–IIB SCC of the cervix. They analyzed prognostic factors including maximal tumor diameter, cervical stroma infiltration, pelvic lymph node invasion, parametrial spread, and tumor grading. Hong *et al.*[15] investigated the prognostic significance of the SCC-ag levels in patients with stages I–IVA SCC of the cervix primarily treated by radiotherapy. They concluded that SCC-ag level higher than 10 ng/mL had a significant impact on DFS in a multivariate analysis. Stage, hemoglobin levels (<10 g/dL), and positive lymph node shown by CT scan were also independent prognostic factors for patient survival.

We previously reported that both CT-measured AP cervical tumor size and CT-detected lymph node metastases had significant independent effects on AS and DFS[21]. The study was performed without analyzing SCC-ag in FIGO stage IIB–IVA cervical carcinoma patients who received radiation therapy between 1983 and 1992. Nonsquamous carcinoma patients were included in the study. In this study, when median SCC-ag was included in multivariate analyses, median SCC-ag and CT-detected lymph node metastases had a significant effect on both AS and DFS. Multivariate analysis confirmed the power of the risk associated with SCC-ag ≥6.0 ng/mL.

Although several authors have reported on the prognostic value of elevated baseline levels of SCC-ag by citing significantly lower survival rates in patients with highly elevated values compared with those with normal baseline values, standard pretreatment SCC-ag level as a pretreatment factor has not been established in clinical practice. Evaluation of the clinical stage by bimanual examination is usually subjective, and it is difficult to objectively predict treatment results in patients with squamous cell cervical cancer treated by radiation therapy alone.

Many authors have suggested a cutoff value of 2 ng/mL[9–12,15]. In this study, the 5-year DFS rate was 83.3% in patients with SCC-ag level <2 ng/mL. Similar 5-year DFS levels in patients with SCC-ag level <2 ng/mL were reported in stage IB and IIA patients with SCC treated by radical hysterectomy[12] and in patients with stage I–IVA SCC of the cervix primarily treated by radiotherapy[15]. The 5-year DFS levels in

their patients with SCC-ag level ≥2 ng/mL were different from ours, but they did not describe the median SCC-ag of their patients.

In our study, a direct linear relationship was found between median SCC-ag of groups distributed by pretreatment prognostic factors and the 5-year DFS rate. We recommend that the median SCC-ag and 5-year DFS rate of pretreatment factors be reported in addition to the treatment outcome. We can use the graph shown in Figure 5 to read the 5-year survival probability for individual patients according to SCC-ag level. From the obtained equations and the graph, we can objectively estimate treatment outcome in both groups of patients and individual patients with locally advanced squamous cell cervical cancer treated by radiotherapy.

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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 1:16 000 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.)

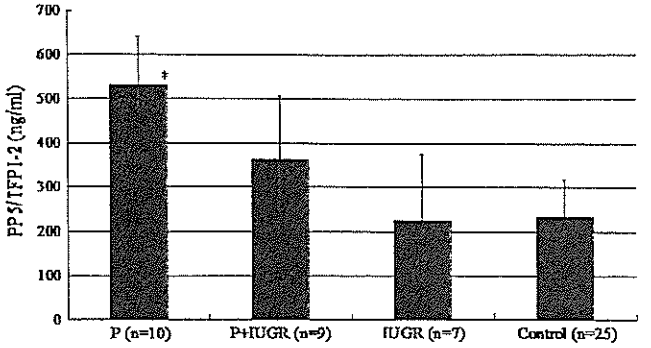

Fig. 1. Comparison of the PP5/TFPI-2 levels in maternal serum samples in the different groups obtained at delivery. Data are expressed as the mean ± SE and are adjusted for gestational age at delivery and the neonatal birth weight. $^*p = 0.033$ (ANCOVA, where gestational age at delivery and the birth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

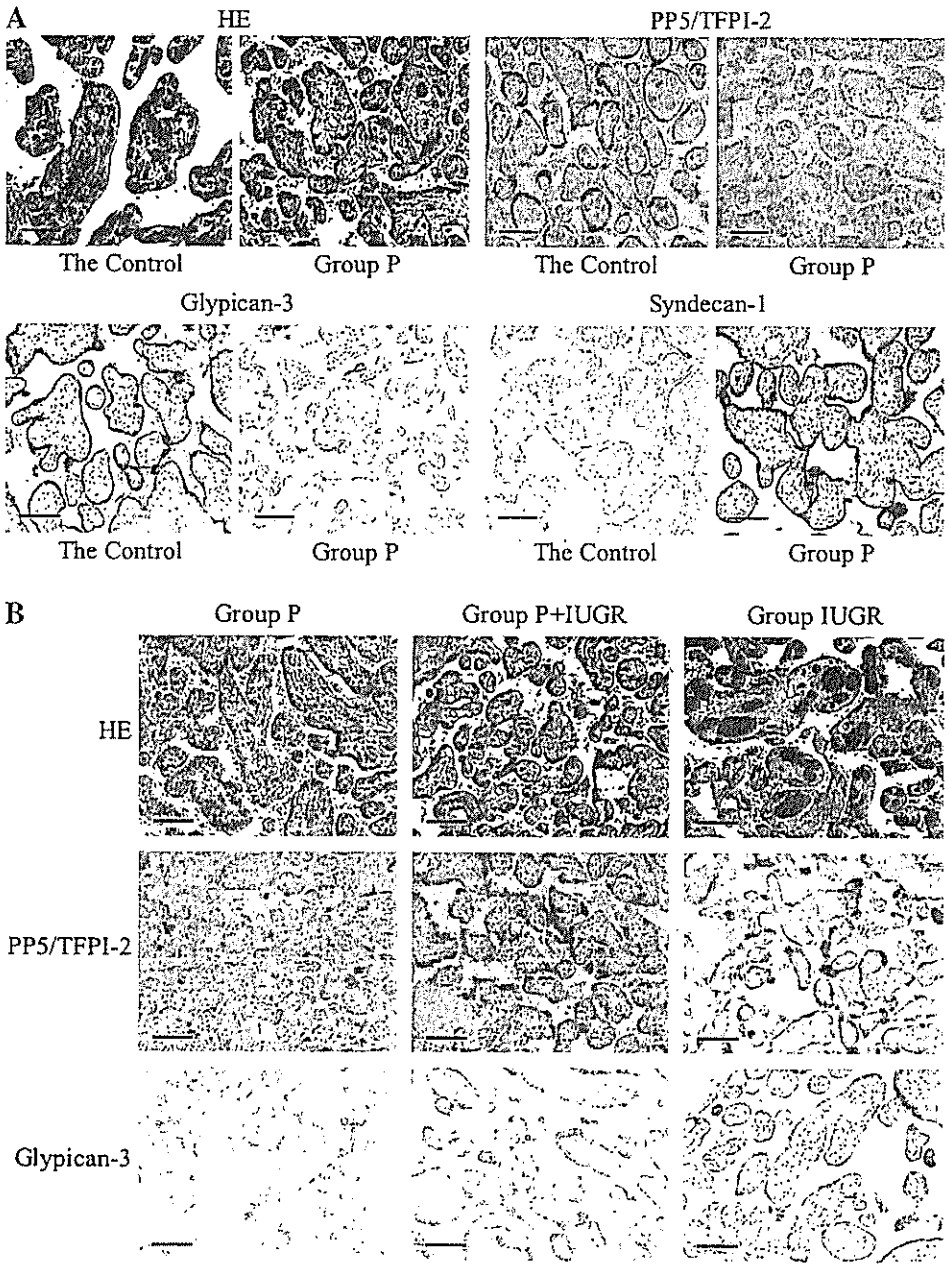

Fig. 2. Examples from the results of immunohistochemical studies. All images original magnification, ×100; scale bar, 100 μm. (A) HE staining, and immunohistochemical staining for PP5/TFPI-2, glypican-3, and syndecan-1 in the placental samples of the Control and Group P. (B) HE staining (the upper lane),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