Expression of TGFß1 and its receptors is associated with biological features of ovarian cancer and sensitivity to paclitaxel/carboplatin

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Abstract. It has been suggested that expression of TGFß1 and its receptors [TGFß receptor type I (TßRI) and TGFß receptor type II (TßRII)] may play a key role in the proliferation and progression of epithelial ovarian cancer. We investigated the biological significance of TGFß1 and its receptors, as well as their association with the tumor response to paclitaxel (PTX) and carboplatin (CBDCA). We studied 24 patients with ovarian cancer, primary peritoneal cancer, or fallopian tube cancer who had undergone surgery and chemotherapy with PTX and CBDCA. Tissues from the primary tumor were examined and the expression of TGFß1, TßRI, and TßRII mRNA was assessed by the RNase protection assay. It was found that TGFß1 mRNA expression was significantly lower in the tumors of patients who had optimal surgery than in the tumors of patients with suboptimal surgery. TGFß1 mRNA expression was also significantly lower in tumors with high sensitivity to PTX and CBDCA than in those with low sensitivity. TßRI mRNA expression was not associated with any clinicopathological factors. Expression of TßRII mRNA was significantly higher in clear cell adenocarcinoma and mucinous adenocarcinoma, while it was lower in serous adenocarcinoma and endometrioid adenocarcinoma. Moreover, it tended to be higher in early-stage tumors compared with advanced tumors. Among TGFß1, TßRI, and TßRII, expression of TGFß1 mRNA was most strongly associated with progression-free survival. When the prognosis of the patients with advanced cancer was compared on the basis of TGFß1 mRNA expression, those whose tumors showed low expression tended to have a better prognosis than those whose tumors showed high expression. It is suggested that TGFß1 mRNA expression is an indicator of tumor sensitivity to standard therapy with PTX and CBDCA, that it can identify biologically aggressive and highly malignant tumors and that it can predict the prognosis of patients with ovarian cancer.

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Introduction

Ovarian cancer is called the 'silent killer', since there is no effective diagnostic method for detecting these tumors at an early stage and more than 50% of patients have advanced disease (≥ stage III) at diagnosis (1). The standard therapy for ovarian cancer is multidisciplinary treatment that combines radical surgery or maximum tumor-debulking surgery with taxane- and platinum-based chemotherapy (2). Ovarian cancer is generally sensitive to anticancer drugs, and approximately 70% of patients can achieve remission with the standard taxane- and platinum-based chemotherapy (3,4). In other words, this therapy is ineffective in about 30% of patients. Moreover, relapse is not uncommon, even if remission is achieved. In fact, approximately 70% of patients with advanced cancer experience relapse within 2 years after the initiation of treatment, and relapsing tumors are often resistant to chemotherapy (4,5). Consequently, the long-term prognosis of patients with advanced ovarian cancer is poor. The biological features of tumors and their sensitivity to anticancer drugs depend on the tumor histology (6-8). Due to the limitations of current standard therapy for ovarian cancer, new treatment strategies to improve the long-term prognosis of patients with this cancer are being examined. Attempts are also being made to develop new biomarkers that can predict biological malignancy and the effectiveness of standard chemotherapy in order to establish tailor-made treatment.

Transforming growth factor-beta 1 (TGFß1) is a multifunctional secreted protein that regulates cell proliferation,

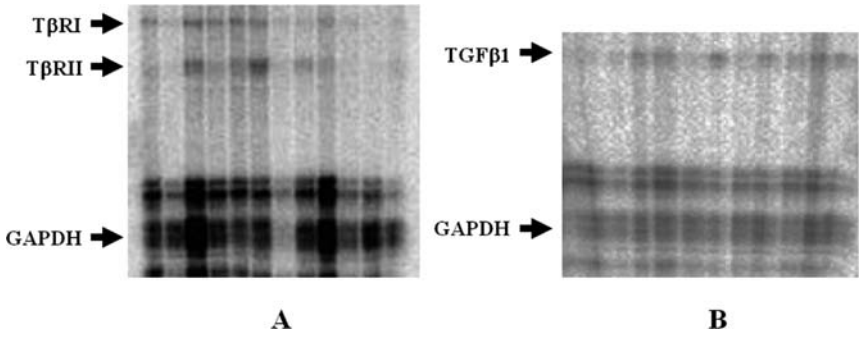

Figure 1. Examples of the expression of TGFß1, TGFß receptor type I (TßRI), and TGFß receptor type II (TßRII) mRNAs (RiboQuant Multi-Probe RNase protection assay system). (A) Expression of TßRI and TßRII. (B) Expression of TGFß1. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

differentiation, and motility, as well as influencing production of the extracellular matrix, neovascularization, and immune function (9-11). In addition, it was recently reported that TGFß1 has an important role in the epithelial-mesenchymal transition (EMT) and strongly influences the metastasis of solid cancers (11-13). Previous studies of various solid cancers have shown that the expression of TGFß1 and its receptors, TGFß receptor type I (TßRI) and TGFß receptor type II (TßRII), is associated with tumor proliferation and progression as well as tumor sensitivity to anticancer drugs, and thus their use as biomarkers for diagnosis and treatment has been proposed (14-18). Although there have been some reports on the expression of TGFß1, TßRI, and TßRII in patients with ovarian cancer, very few studies have examined associations with the prognosis and with sensitivity to anti-cancer drugs.

Therefore, we evaluated the expression of TGFß1, TßRI, and TßRII in patients with ovarian cancer, and assessed associations with the prognosis, tumor biological features, and response to taxane- and platinum-based chemotherapy, in order to examine whether these are potential new biomarkers, for use in the treatment of ovarian cancer.

Materials and methods

The subjects were 24 patients with epithelial ovarian cancer, primary peritoneal cancer, or fallopian tube cancer who underwent surgery and chemotherapy with a taxane (paclitaxel: PTX) and a platinum agent (carboplatin: CBDCA) at the Department of Obstetrics and Gynecology of National Hospital Organization Saitama Hospital (NHO Saitama Hospital). Informed consent was obtained from the patients or their family members. Tissue samples obtained from the primary tumor during initial surgery were studied. Since primary peritoneal cancer and fallopian tube cancer are similar to epithelial ovarian cancer (serous adenocarcinoma) with respect to histopathological features and sensitivity to anti-cancer drugs, they have recently been considered as a single disease entity known as Müllerian adenocarcinoma. Therefore, patients with primary peritoneal cancer and fallopian tube cancer were also included in the present study.

The expression of TGFß1, TßRI, and TßRII mRNA was examined with the RiboQuant Multi-Probe RNase protection assay system (Pharmingen, San Diego, CA). First, total RNA was extracted by using an RNeasy mini kit (Qiagen, Valencia, CA). According to the RiboQuant protocol, two multi-probe template sets (Pharmingen) were used to obtain cDNA for TGFß1 (hCK-3), as well as TßRI and TßRII (hCR-4). Then *in vitro* transcription was performed from the cDNA constructs using T7 RNA polymerase to create ^{32}P-labeled antisense RNA probes and hybridization with total RNA extracted from each tumor. Single-stranded RNA was dissolved with

Table I. Patient profile.

Number of patients	24
Median age (range)	62.6 (42-83)
Median follow-up period (range)	24.4 (3-37)
Diagnosis	
Epithelial ovarian cancer	21
Primary peritoneal cancer	2
Fallopian tube cancer	1
Tumor histology	
Serous adenocarcinoma	12
Endometrioid adenocarcinoma	2
Mucinous adenocarcinoma	6
Clear cell adenocarcinoma	4
FIGO stage	
I	9
II	2
III	9
IV	4
Completeness of PDS for stage III/IV tumors	
Optimal	10
Suboptimal	3
Clinical sensitivity to PTX/CBDCA	
High	11
Low	4

PDS, primary debulking surgery; optimal, residual tumor diameter <1 cm; suboptimal, residual tumor diameter ≥1 cm; PTX, paclitaxel; CBDCA, carboplatin.

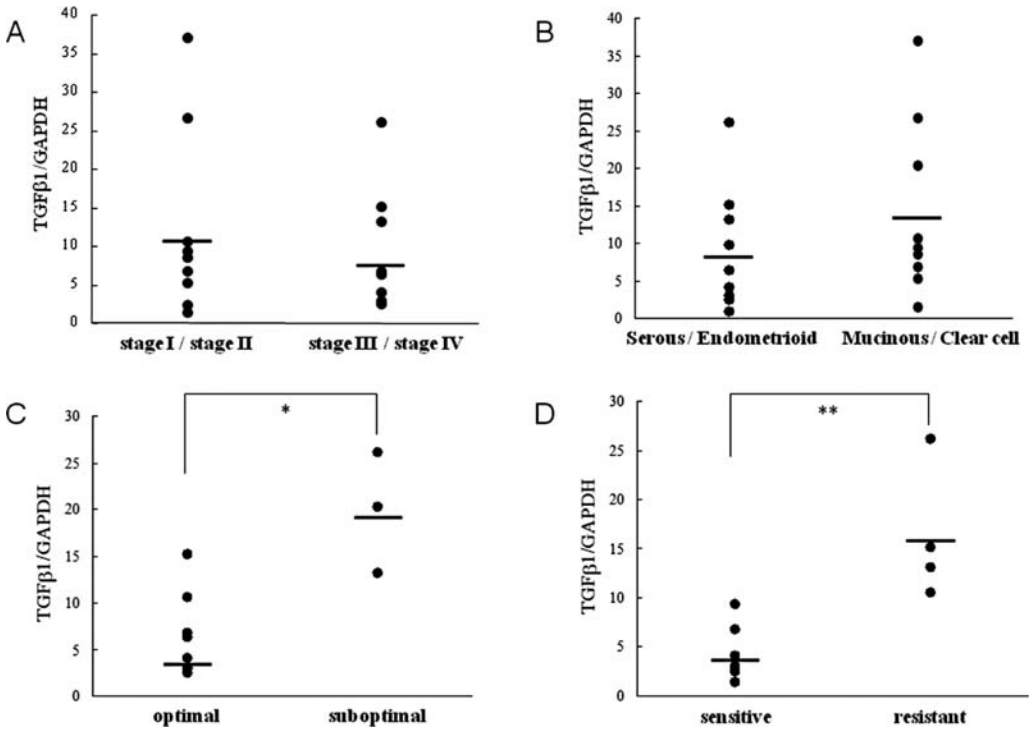

Figure 2. Association between TGFß1 mRNA expression and various clinicopathological factors. Expression of TGFß1 was higher in mucinous adenocarcinoma and clear cell adenocarcinoma, while it was lower in serous adenocarcinoma and endometrioid adenocarcinoma, but there was no significant difference (B). Expression of TGFß1 mRNA was not significantly different between early tumors (stages I and II) and advanced tumors (stages III and IV) (A). Expression of TGFß1 was lower in the tumors of patients with successful (optimal) primary surgery compared with the tumors of patients who had suboptimal surgery, and there was a significant difference ($^{*}p<0.05$) (C). As for sensitivity to PTX and CBDCA, TGFß1 expression was significantly lower in tumors with a high sensitivity (sensitive) than in tumors with a low sensitivity (resistant) ($^{**}p<0.01$) (D).

an RNase cocktail, followed by electrophoresis on 5% denaturing polyacrylamide gel and exposure to an imaging plate.

The mRNA bands were visualized with a FLA3000 (Fujifilm, Tokyo, Japan) and the density of each band was quantified by using Image gauge software (Fujifilm). Then mRNA expression was normalized for that of the endogenous control (glyceraldehyde-3-phosphate dehydrogenase: GAPDH) and quantified. We examined the associations between expression of TGFß1, TßRI, and TßRII mRNA and various biological tumor features or with factors influencing the prognosis of the patients, including tumor histology, the International Federation of Gynecology and Obstetrics (FIGO) stage, the completeness of primary tumor resection (amount of residual tumor), and tumor sensitivity to chemotherapy. The clinical sensitivity of tumors in stages II-IV was determined as follows: tumors that showed a complete response/partial response to standard postoperative chemotherapy with PTX and CBDCA or did not relapse within 6 months after postoperative chemotherapy were defined as 'sensitive', while the others were classed as 'resistant'. Since the number of patients with each tumor histology was limited, serous adenocarcinoma and endometrioid adenocarcinoma (which show similar sensitivity to anticancer drugs) were grouped together, while mucinous adenocarcinoma and clear cell adenocarcinoma were grouped together. Cut-off values were also set for TGFß1, TßRI, and TßRII based on the association with sensitivity to anticancer agents in our study. Then, tumors with a level of expression above the cut-off value were defined as the 'high-expression group' and those with lower expression were classed as the 'low-expression group'.

All clinical information on the patients was obtained from the records of the Department of Obstetrics and Gynecology of NHO Saitama Hospital. For statistical analysis, Student's t-test or the χ^2 test was employed to compare two groups. Kaplan-Meier analysis was used to calculate cumulative survival, and differences were assessed with the log-rank test. The level of statistical significance was considered to be $p<0.05$. The present study was approved by the ethics committee of NHO Saitama Hospital.

Results

Tumor tissues were assayed in all 24 patients. Representative examples of TGFß1, TßRI, and TßRII mRNA expression are presented in Fig. 1. Background data for the 24 patients are listed in Table I. Results are shown as the median ± standard error.

Expression of TGFß1 tended to be high in clear cell adenocarcinoma and mucinous adenocarcinoma (13.2±10.6), while it was low in serous adenocarcinoma and endometrioid adenocarcinoma (7.9±7.3), but there was no significant difference between these tumor types. The expression of TGFß1 was not significantly different between early stage tumors (stages I and II) (11.4±23.7) and advanced tumors (stages III and IV) (8.3±15.1). However, expression of TGFß1 was lower in the

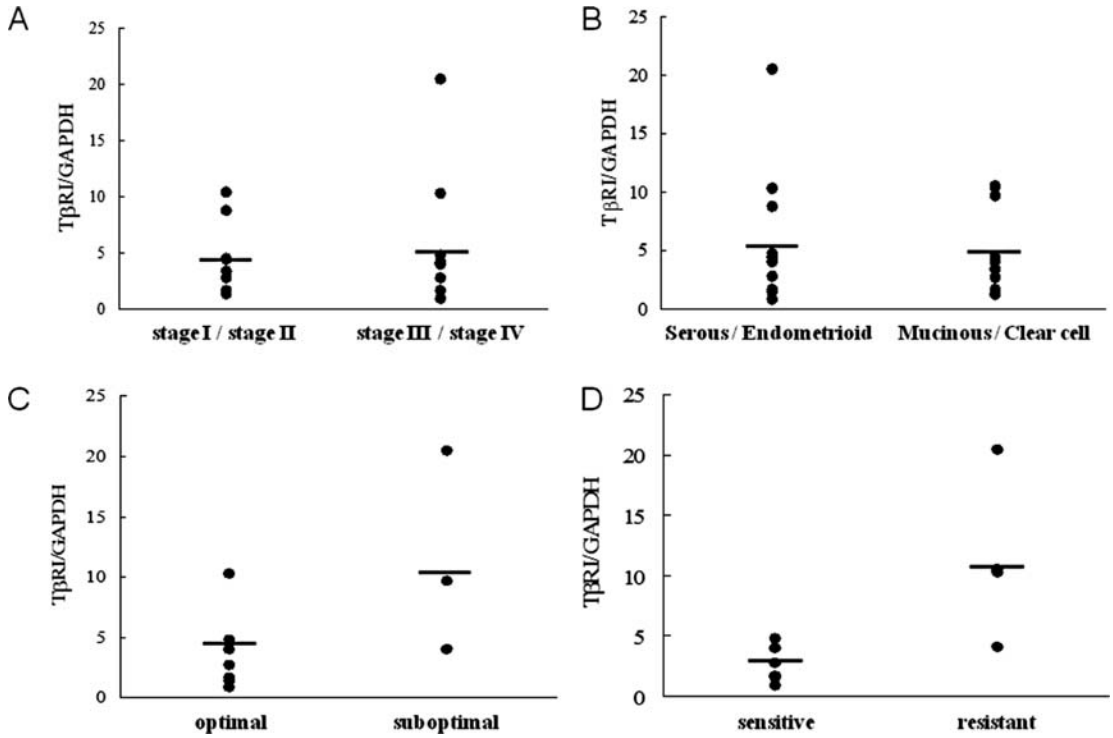

Figure 3. Association between TßRI mRNA expression and various clinicopathological factors. TßRI expression was not associated with tumor stage (A), tumor histology (B), or the completeness of primary surgery (C). However, there was lower expression in the tumors with a high sensitivity (sensitive) to PTX/CBDCA therapy than in tumors with a low sensitivity (resistant) (D).

tumors of patients who had successful (optimal) primary surgery and a residual tumor diameter <1 cm (6.9±4.3) compared with patients who had suboptimal surgery and a residual tumor diameter ≥1 cm (18.5±7.1), and there was a significant difference (p=0.029). As for sensitivity to PTX and CBDCA, the expression of TGFß1 was significantly lower in tumors with a high sensitivity to therapy (3.1±1.8) than in tumors with a low sensitivity (16.2±6.8) (p=0.0011) (Fig. 2).

Expression of TßRI was not associated with tumor histology, stage, or completeness of resection. However, there was lower expression of TßRI in the tumors with a high sensitivity to PTX and CBDCA (2.7±1.4) than in tumors with a low sensitivity (11.3±6.8) (Fig. 3).

Expression of TßII was higher in clear cell adenocarcinoma and mucinous adenocarcinoma (17.1±11.2), while it was lower in serous adenocarcinoma and endometrioid adenocarcinoma (7.3±8.9), and there was a significant difference (p=0.025). Expression of TßII tended to be higher in tumors at an early stage (15.6±12.1) and lower in advanced tumors (8.2±9.3). It was also low in the tumors of patients who had optimal surgery (4.8±3.9), while it was high in the tumors of patients who had suboptimal surgery (17.8±11.7), but there was no significant difference. Regarding sensitivity to PTX and CBDCA, expression of TßII was lower in tumors with a high sensitivity (6.2±6.6) compared to those with a low sensitivity (24.4±15.7) (Fig. 4).

Analysis of the association of TGFß1, TßRI, and TßRII expression with progression-free survival (PFS) revealed that the expression of TGFß1 mRNA was most strongly associated with PFS (r= -0.805, p=0.0004) (Fig. 5). For TGFß1, a cut-off value was set at the median (3.1) level of expression in tumors with a high sensitivity. Then the cumulative overall survival was compared between tumors with higher expression than this cut-off value (high-TGFß1 group) and those with lower expression (low-TGFß1 group). It was found that patients with tumors showing lower expression tended to have a better prognosis, although there was no significant difference (Fig. 6).

Discussion

The standard therapy for ovarian cancer is multidisciplinary treatment that combines radical surgery or maximum tumor debulking surgery with taxane- and platinum-based chemotherapy. Particularly in patients with advanced ovarian cancer, it is extremely important to remove as much of the tumor as possible by appropriate debulking surgery, and whether primary debulking surgery is successful or not has a significant impact on the prognosis. Further improvement of the prognosis can be achieved by appropriate treatment with anticancer drugs after surgery. Nevertheless, there are limitations to surgical resection, and anticancer drugs more effective than standard taxane- and platinum-based chemotherapy have not been released for a decade (19). Consequently, the long-term prognosis of patients with ovarian cancer remains poor. Under these circumstances, it is extremely important to identify patients who are likely to respond or not respond to the current standard therapy, and to establish tailor-made treatment for those who do not respond.

Conventional pharmacodynamic indicators such as diagnostic imaging or changes of serum tumor markers may

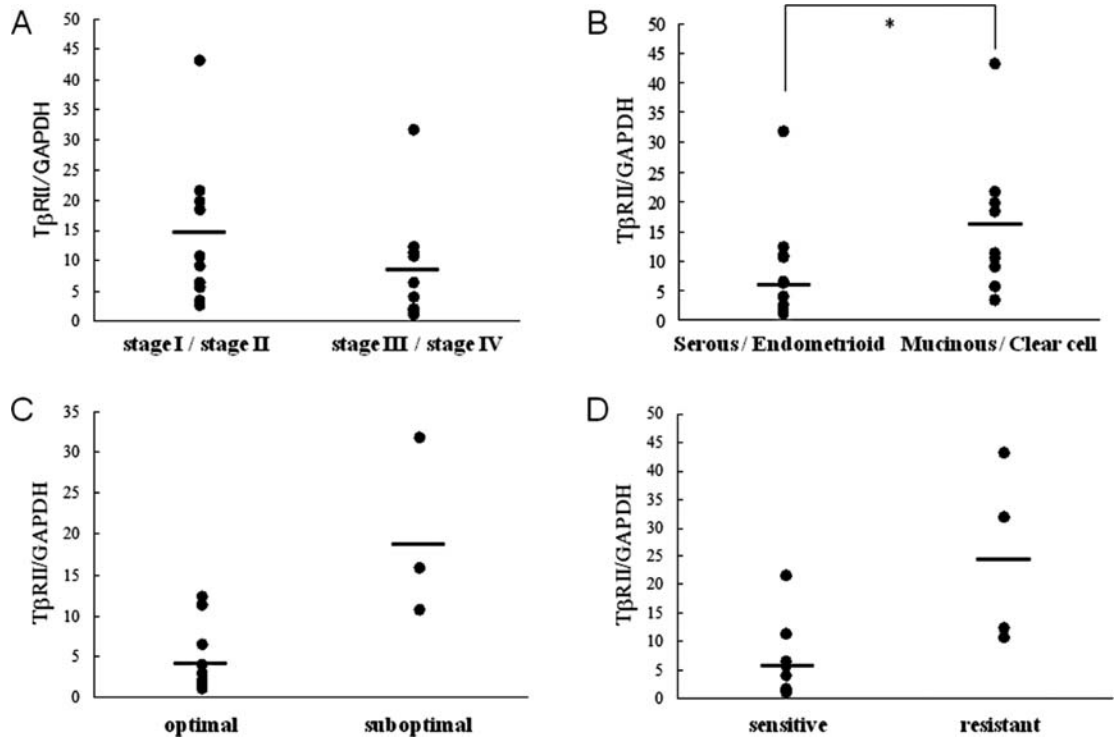

Figure 4. Association between TßRII mRNA expression and various clinicopathological factors. TßII expression was higher in tumors at an early stage (stages I and II) and lower in those at an advanced stage (stages III and IV), but there was no significant difference (A). TßII expression was higher in mucinous adenocarcinoma and clear cell adenocarcinoma, while it was lower in serous adenocarcinoma and endometrioid adenocarcinoma, and there was a significant difference ($^{*}p<0.05$) (B). Moreover, it was low in the tumors of patients who had successful (optimal) primary surgery and high in the tumors of patients who had suboptimal surgery, although there was no significant difference (C). TßII expression was lower in tumors with a high sensitivity to PTX/CBDCA therapy (sensitive) than in tumors with a low sensitivity (resistant) (D).

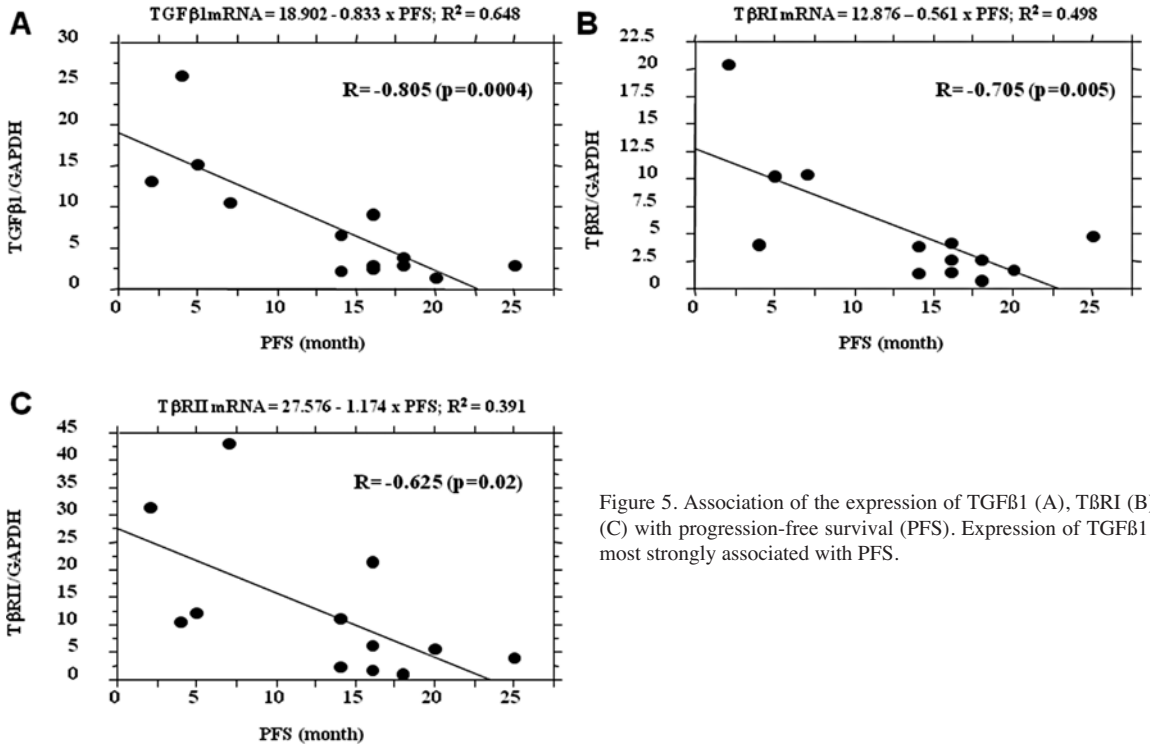

Figure 5. Association of the expression of TGFß1 (A), TßRI (B), and TßRII (C) with progression-free survival (PFS). Expression of TGFß1 mRNA was most strongly associated with PFS.

be useful to determine the short-term tumor response, but are not effective for predicting the long-term prognosis. Thus, new indicators are needed that can predict the prognosis after remission and identify patients who are not responding to standard therapy, in other words, new biomarkers to help determine treatment approaches.

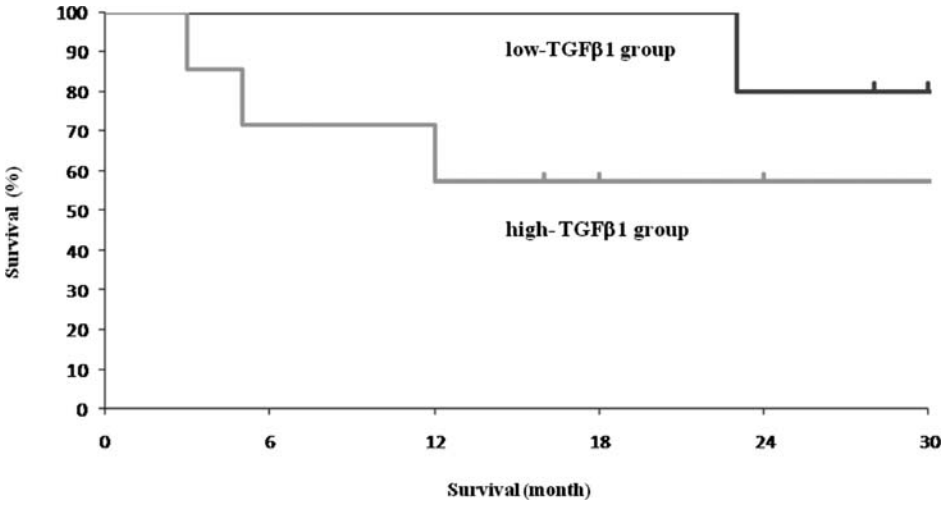

Figure 6. Comparison of the prognosis of patients with advanced cancer (stages III and IV) in relation to the expression of TGFß1. Overall survival was compared between patients whose tumors showed higher expression (high-TGFß1 group) and those whose tumors showed lower expression (low-TGFß1 group). Patients with tumors showing lower expression tended to have a better prognosis, although there was no significant difference.

It is known that TGFß1, its receptors (TßRI and TßRII), and a series of proteins in the downstream signaling cascade such as Smads are associated with the proliferation, progression, and metastasis of solid cancers. It has been reported that expression of TGFß1 is a prognostic factor for various solid cancers, is correlated with sensitivity to anticancer drugs, and is directly involved in the mechanisms of drug resistance. Therefore, TGFß1 expression is an indicator of the aggressiveness or malignancy of solid cancers. Consequently, various molecular targeting therapies for TGFß1 and its receptors are being investigated (20-22), and some have already been introduced clinically (23-29).

Although abnormalities in the expression or function of TGFß1 and its receptors, or molecules in the downstream signaling cascade, have been reported in several studies of ovarian cancer, some authors have reported significant abnormalities, while others have found minor changes and controversy exists (30-34). In addition, very few studies of ovarian cancer have assessed the associations between TGFß1 expression and the prognosis or sensitivity to anticancer drugs. Moreover, the results obtained so far have been inconsistent, with some studies of a positive association between TGFß1 expression and the prognosis (35), while other studies have found no association (36). Nevertheless, it was recently reported that, among proteins associated with the resistance of advanced ovarian serous adenocarcinoma to chemotherapy, the role of the TGFß pathway is very important (37), and that p53 and TGFß1 are key genes involved in the mechanism of resistance of ovarian cancer to platinum agents (38), Consequently, the association of TGFß1 and various molecules in its signaling cascade with the sensitivity of ovarian cancer to chemotherapy has increasingly attracted attention.

In the present study, we used the RiboQuant Multi-Probe RNase protection assay system. Although the need for a radioisotope is a disadvantage of this assay, other procedures can be undertaken easily and the results show excellent reproducibility.

We demonstrated that expression of TGFß1 was significantly lower in tumors with a high clinical sensitivity to PTX/CBDCA therapy than in tumors with a low sensitivity. Thus, it is suggested that there is a correlation between TGFß1 expression and sensitivity to standard therapy with PTX and CBDCA. Moreover, although there was no significant difference, expression of TGFß1 tended to be high in clear cell adenocarcinoma and mucinous adenocarcinoma, while it was lower in serous adenocarcinoma and endometrioid adenocarcinoma. Since it is known that clear cell and mucinous adenocarcinoma are more likely to show resistance to taxanes and platinum (7,8), the results of the present study provide further evidence of a potential association between TGFß1 and the tumor response to chemotherapy. Moreover, although there was no difference in the expression of TGFß1 between early stage tumors and advanced tumors, it was significantly lower in the tumors of patients who had optimal surgery than in the tumors of patients who had suboptimal surgery, suggesting that the higher TGFß1 expression indicates a more biologically aggressive or malignant tumor.

As for the TGFß receptors, TßRI was not associated with any of the clinicopathological factors. Thus, based on the findings of the present study, the biological significance of this receptor for ovarian cancer seems to be low. On the other hand, expression of TßRII was high in clear cell adenocarcinoma and mucinous adenocarcinoma, but was low in serous adenocarcinoma and endometrioid adenocarcinoma, suggesting tissue specificity. In addition, TßRII expression tended to be higher in early tumors compared with those at an advanced stage. This is consistent with the findings of a previous study that showed down-regulation of the gene expression of TßRII in ovarian serous adenocarcinoma at an advanced stage rather than at an early stage (39). Therefore, it can be suggested that TßRII expression is correlated with the progression of ovarian cancer. Clear cell adenocarcinoma and mucinous adenocarcinoma are more likely to be detected at an early stage than at an advanced stage, which also supports the tissue specificity of TßRII expression.

When the associations among TGFß1, TßRI, and TßRII and PFS were analyzed, it was found that TGFß1 mRNA expression was most strongly associated with PFS. Moreover, when the survival of the patients with advanced cancer was compared between those with higher and lower TGFß1 expression, the latter group had a better prognosis. Although a small number of patients was a limitation of the present study, analysis of a larger sample may reveal a significant difference. Therefore, it can be suggested that TGFß1 expression is a useful biomarker for predicting the prognosis of patients with ovarian cancer. In addition, considering that TGFß1 expression is associated with the malignancy of tumors and with sensitivity to taxanes or platinum agents, as mentioned earlier, tailor-made treatment (rather than standard therapy) may be needed for patients with tumors over-expressing TGFß1.

In summary, the present study revealed that TGFß1 can be an indicator of tumor sensitivity to standard chemotherapy (PTX and CBDCA), can identify tumors that are biologically aggressive or highly malignant, and can help to predict the prognosis of ovarian cancer patients. Consequently, it was suggested that TGFß1 may be a useful biomarker for the diagnosis and treatment of ovarian cancer. In addition, TßRII was associated with the histology and progression of ovarian cancer.

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