Jpn. J. Cancer Res. **90**, 1344–1350, December 1999

Expression of Platelet-derived Endothelial Cell Growth Factor/Thymidine Phosphorylase in Human Bladder Cancer

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We investigated the expression of platelet-derived endothelial cell growth factor/thymidine phosphorylase (PD-ECGF/TP) in primary bladder cancer, its association with clinicopathologic findings, and their prognostic value. mRNA was extracted from 20 bladder cancer specimens and 6 normal bladder mucosal tissues. Relative amounts of PD-ECGF/TP mRNA were evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR) and compared with the level of glyceraldehyde-3-phosphate dehydrogenase mRNA (used as an internal standard). PD-ECGF/TP expression was examined by immunohistochemistry in 85 patients who underwent cystectomy for bladder cancer. Serum PD-ECGF/TP levels were measured in 23 patients using a sandwich-type enzyme-linked immunosorbent assay. By RT-PCR analysis, expression of PD-ECGF/TP was found to be 7-fold higher in invasive tumors than in superficial tumors ($P<0.01$) and 9-fold higher than in normal bladder ($P<0.01$). Out of 85 transitional cell carcinoma tissue samples, 69 (81%) were evaluated as PD-ECGF/TP-positive by immunohistochemical staining. PD-ECGF/TP expression correlated significantly with tumor grade ($P=0.001$), depth of invasion ($P=0.012$), and lymphatic invasion ($P=0.01$). No correlation was found between expression of PD-ECGF/TP and the number of tumors, tumor configuration, lymph node involvement, venous invasion, c-erbB-2 expression, or overall survival. We could not detect a significant serum level of PD-ECGF/TP in any patient. The results suggest that PD-ECGF/TP might give valuable information for bladder cancer management, though it may not be a good new tumor marker for bladder cancer.

Key words: Thymidine phosphorylase — Platelet-derived endothelial cell growth factor — Bladder transitional cell carcinoma

It is now well established that malignant tumors depend on neovascularization for their growth and metastasis. There appears to be a quantitative relationship between the degree of angiogenesis and prognosis in several human malignancies including bladder cancer.[1)] Recently, several angiogenic factors have been identified, one of which is thought to be platelet-derived endothelial cell growth factor (PD-ECGF). PD-ECGF has chemotactic activity for endothelial cells *in vitro*, shows angiogenic activity *in vivo*, and differs from other angiogenic factors in that it lacks both heparin-binding domains and a secretion peptide.[2)] It has been shown that PD-ECGF is identical to thymidine phosphorylase (TP), an enzyme involved in pyrimidine nucleoside metabolism.[3)] Interestingly, TP activity has been found to be increased in several types of malignant tumors compared with normal adjacent tissues.[4)] With regard to bladder cancer, there are only a few reports on this protein,[5–8)] and the clinical significance of PD-ECGF/TP in bladder cancer has not been thoroughly established yet.

In this study, we compared PD-ECGF/TP expression in primary bladder cancer tissue with that in normal bladder tissue, and investigated the correlation between PD-ECGF/TP expression and other clinicopathologic variables.

MATERIALS AND METHODS

Four kinds of experiments were conducted. First, the levels of PD-ECGF/TP mRNA and protein were evaluated by reverse transcriptase polymerase chain reaction (RT-PCR) and western blot analysis, respectively. The former method was also employed to estimate the malignant potential of bladder cancer cells. The amount of PD-ECGF/TP in peripheral blood was measured by means of an enzyme-linked immunosorbent assay (ELISA). In order to investigate the prognostic significance of this protein,

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archival paraffin-embedded tissues were utilized for immunohistochemistry. For other purposes, clinical materials were employed as mentioned below.

Patients and samples Twenty fresh tumor specimens and 6 non-neoplastic tissue specimens remote from the tumor were obtained at the time of surgery from 20 patients with transitional cell carcinomas (TCC). Tissues not grossly involved with tumor were stained with hematoxylin and eosin (HE) and microscopically examined to verify that tumor cells were not present. All samples were immediately stored at −80°C until use for RT-PCR and western blot analyses.

Tissue samples from primary TCC were obtained for immunohistochemical study from 85 patients who underwent cystectomy at our hospital between 1979 and 1995. The median length of follow-up was 32 months (range 1–160). The characteristics of these patients' tumors are detailed in Table I.

PD-ECGF/TP serum levels were measured in another group of 23 advanced bladder cancer patients. Patient and tumor profiles are reported in Table I.

A thorough histologic examination was made on all HE-stained preparations, and histologic classifications were performed according to the grading system of the Japanese Urological Association (Japanese Urological Association, 1993).

Analysis of PD-ECGF/TP mRNA expression by RT-PCR The total quantity of RNA was extracted from tissues with the guanidinium thiocyanate/phenol/chloroform technique as reported previously.[9] First-strand cDNA was synthesized from 5 μg of this total RNA using 20 units of RAV-2 reverse transcriptase (Takara, Otsu) and random nonamer primers. Portions (1 μg in amount) of the cDNA were amplified by PCR as described previously.[9] Sense and antisense primers for PD-ECGF/TP mRNA were: 5′-AGGGAGCCAGGGACTTCCCGA-3′ (in exon 2) and 5′-TGGAATGCTTGTCCACAAGCTGC-3′ (in exon 3), respectively. They were synthesized according to published information[8] and amplified a 290 bp fragment. Sense and antisense primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were: 5′-GGATTTGGTCGTATTGGGCGCCT-3′ (in exon 2) and 5′-AGTGAGCTTCCCGTCTAGCTCAG-3′ (in exon 7), respectively. They were synthesized based on the DDBJ, EMBL, and GenBank database sequences (accession number J04038), and themselves amplified a 660 bp fragment. Amplification was performed under the following conditions: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. A preliminary experiment showed that the amount of PCR products increased exponentially from the 24th to the 34th cycle with both primer pairs (data not shown). We therefore performed the PCR for 30 cycles in the case of PD-ECGF/TP and 25 cycles for GAPDH. PCR products were electrophoresed and visualized with ethidium bromide. The intensity and area of each fragment visualized were measured using an Epi-Light UV image analyzer EU-1150II (Aisin Cosmos R&D, Kariya). The intensity of each band of PD-ECGF/TP mRNA was normalized to that of GAPDH mRNA.

Western blot analysis Anti-PD-ECGF/TP mouse monoclonal antibody MoAb 654-1 was kindly supplied by the Nippon Roche Research Center (Kanagawa). This antibody was prepared using human PD-ECGF/TP purified from human colon cancer xenograft HCT116 as an immunogen. The characterization and specificity of this antibody has already been reported.[10] To investigate the presence of PD-ECGF/TP in bladder cancers, western blot analysis was performed as previously described.[11] In all gels, 20 μg of protein was loaded per lane in a sample buffer.

Immunohistochemical staining method Tissue sections (4 μm thick) from paraffin blocks were stained using the streptavidin-biotin-peroxidase complex method.[12] Sections were then counterstained with hematoxylin and mounted. Normal mouse IgG was used instead of primary antibody as a negative control. Slides were examined for antigen expression by a pathologist without knowledge of the clinical data. PD-ECGF/TP expression was considered low when <50% neoplastic cells showed positive staining and high when >50% cells showed positive staining. For comparison, immunostaining of the *c-erbB-2* gene product, which is a receptor-type oncogene product and was reported as a useful tumor marker for bladder cancer, was performed using a specific rabbit polyclonal anti-human *c-*

Table I. Patient and Tumor Characteristics

	Immunohistochemical	Serum
Age (mean±SD)	66.4±9.9	68.2±8.1
(range)	43–85	51–81
Sex		
Male	63	12
Female	22	11
Grade		
1	3	0
2	18	4
3	64	18
X		1
Depth of invasion		
pTa	12	0
pT1	18	4
pT2	19	2
pT3	24	4
pT4	12	13
Follow-up (day)	8–4802	
(median)	951	

erbB-2 gene product (Nichirei, Tokyo) and the streptavidin-biotin detection system as above.[12, 13] With this marker, tumors were considered positive for c-erbB-2 when >10% of cells showed staining.

Measurement of serum TP levels PD-ECGF/TP levels in sera were measured using a sandwich-type ELISA.[10] This ELISA for human PD-ECGF/TP uses MoAb 104B (IgM) and MoAb 232-2 (IgG1), which were generated simultaneously with MoAb 654-1 and are specific for PD-ECGF/TP. The lower limit of sensitivity of this assay is 4.0 units/ml of PD-ECGF/TP. One unit equals the amount of PD-ECGF/TP which phosphorolyzes doxifluridine to 5-fluorouracil (5-FU) at a rate of 1 μg of 5-FU/h.

Statistical analysis The levels of expression of PD-ECGF/TP in normal bladder tissue and bladder cancer mucosa were compared using the Mann-Whitney *U* test. Correlations between expression of PD-ECGF/TP and various clinicopathologic factors were assessed by χ^2 analysis. Survival curves were determined using the Kaplan-Meier method, with a log-rank test used to evaluate differences. A *P* value of 0.05 or less was judged to be statistically significant. The analysis was performed using the Statview program (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Analysis of PD-ECGF/TP mRNA expression by RT-PCR Twenty-six samples (6 normal bladders, 8 Ta tumors, 5 T1 tumors and 7 invasive tumors) were analyzed for PD-ECGF/TP mRNA expression by RT-PCR (Fig. 1A). All of these samples showed some degree of expression of this mRNA. Levels of PD-ECGF/TP mRNA expression were analyzed in bladder tumors of different stages (Fig. 1B). The average amount of mRNA was 4-fold higher in all cancerous tissues than in normal bladder mucosa ($P=0.06$), was 9-fold higher in invasive cancers than in normal bladder mucosa ($P<0.01$) and 7-fold higher in invasive cancers than in superficial cancers ($P<0.01$). There was no statistically significant difference in expression between normal bladder mucosa and superficial cancers ($P=0.3$).

Western blot analysis Fig. 2 shows the protein bands identified by MoAb 654-1 in human bladder cancer tissues. Bladder cancer tissues obtained by cystectomy (lane 1) or by transurethral resection (lanes 2 and 3) were analyzed, and HCT116 cells served as a positive control (lane

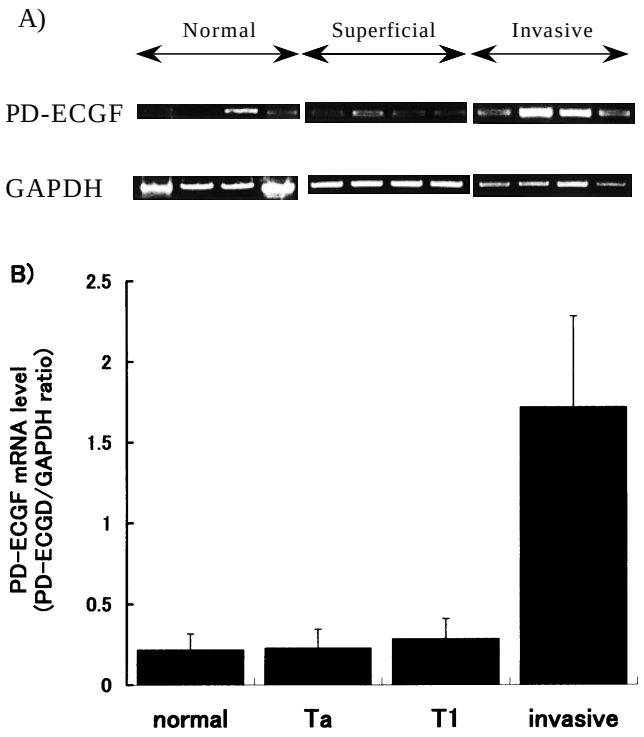

Fig. 1. (A) RT-PCR analysis of expression of PD-ECGF/TP mRNA in human bladder cancer and in normal bladder samples. GAPDH was also amplified as an internal control. (B) PD-ECGF/TP mRNA expression in bladder tumors at different stages. Quantitation was achieved by an image analyzer (normal vs. invasive, $P<0.01$; superficial vs. invasive, $P<0.01$). In each case, the intensity of specific bands was standardized by referring to the amount of GAPDH mRNA (mean±SEM).

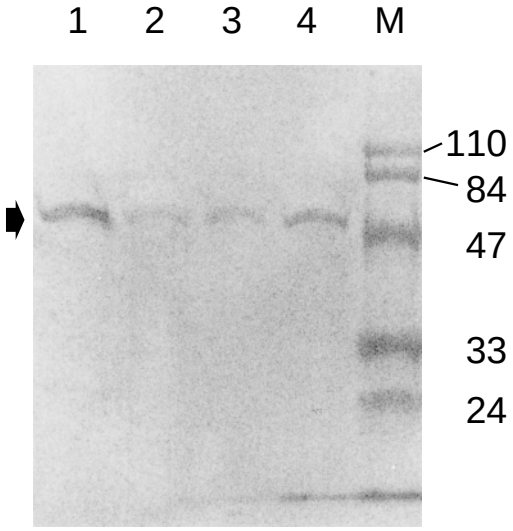

Fig. 2. Characterization of anti-PD-ECGF/TP monoclonal antibody 654-1. Human cancer tissues (20 μg protein) were stained with MoAb 654-1 (2 μg/ml). Lane 1, human bladder cancer (radical cystectomy); lanes 2, 3, human bladder cancer (TUR); lane 4, human colon cancer xenograft HCT116; M, molecular weight markers. The arrowhead indicates bands corresponding to PD-ECGF/TP (55 kDa).

4). All lysates contained detectable amounts of PD-ECGF/TP appearing as a single band of 55 kDa, which corresponds to the molecular weight of PD-ECGF/TP. Thus, it was confirmed that MoAb 654-1 can adequately recognize PD-ECGF/TP protein in bladder cancer tissues.

PD-ECGF/TP expression in human bladder tissues by immunohistochemistry Normal transitional epithelia seemed to lack immunoreactivity with anti-PD-ECGF/TP MoAb 654-1 (Fig. 3a), whereas cancer cells clearly showed a positive reaction (Fig. 3, b and c). PD-ECGF/TP was distributed mainly in the cytoplasm or nuclear compartment (Fig. 3d). The immunodistribution of PD-ECGF/TP was heterogeneous in cancer lesions, and small numbers of non-cancerous positive cells were scattered in the stroma. As these cells demonstrated positive immunostaining for both PD-ECGF/TP and CD68 (PG-MA1 clone, DAKO A/S, Glostrup, Denmark), which is a specific marker antigen for macrophages, it was thought that these cells were macrophages (data not shown).

PD-ECGF/TP expression and clinicopathologic factors Table II shows the relationship between PD-ECGF/TP expression and various clinicopathologic factors of the 85 patients treated by cystectomy. Cancer tissues showed low-positive immunostaining in 16 cases (19%) and high-positivity in 69 cases (81%). PD-ECGF/TP expression in cancer cells showed statistically significant correlations with tumor grade (P=0.001), depth of invasion (P=0.012), and lymphatic invasion (P=0.01). No correlation was observed between PD-ECGF/TP staining and gender (P>0.99), tumor number (P=0.12), tumor configuration (P=0.87), lymph node metastasis (P=0.50), or venous invasion (P=0.72). To obtain more information, PD-ECGF/TP expression was compared with that of the *c-erbB-2* oncogene product. Staining of c-erbB-2 was performed in 76 cases among the total of 85 study patients. Forty-six (61%) tumors were negative for c-erbB-2 and 30 (39%) were positive. There was no relationship between c-erbB-2 expression and overall survival (P=0.98), and nei-

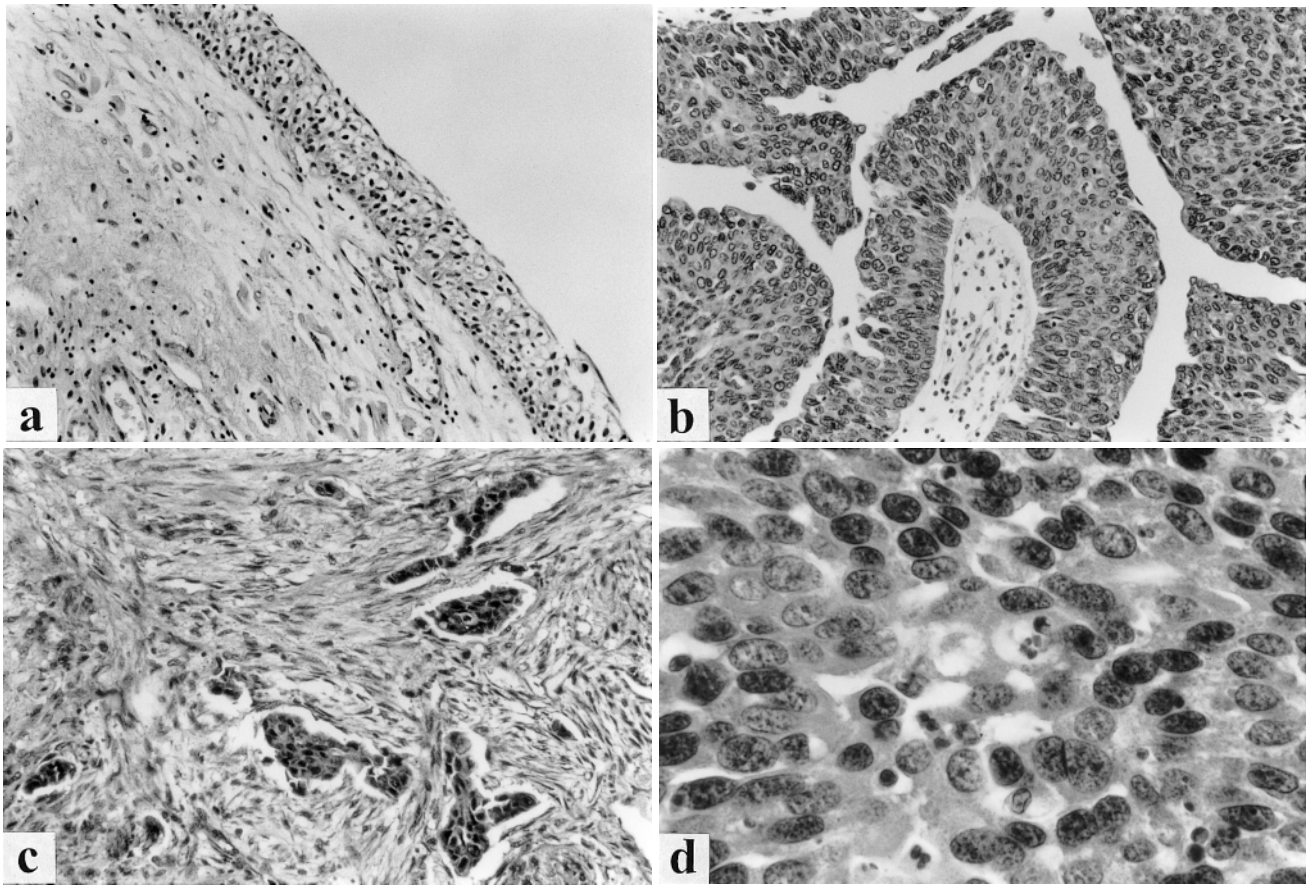

Fig. 3. Immunostaining of PD-ECGF/TP. (a) Negative staining of PD-ECGF/TP in normal transitional cell tissue. (b) Positive staining of PD-ECGF/TP in a papillary carcinoma. (c) Positive staining of PD-ECGF/TP in an invasive transitional cell carcinoma. (d) Tumor cell nuclear and cytoplasmic immunoreactivity.

Table II. Relationship between Tumor Cell PD-ECGF/TP Expression and Other Tumor Variables

Variable	Low-positive	High-positive	$P^{a)}$
Sex			
Male	12	51	
Female	4	18	>0.99
Number			
Solitary	4	32	
Multiple	12	37	0.12
Configuration			
Papillary	5	23	
Solid/mixed	11	46	0.87
Grade			
G1	3	0	
G2/G3	13	69	0.01
Depth of invasion			
pTa-pT1	10	20	
pT2-pT4	6	49	0.01
Lymphnode metastasis			
Negative	13	51	
Positive	2	16	0.50
Lymphatic invasion			
Negative	9	12	
Positive	6	41	0.01
Venous invasion			
Negative	12	45	
Positive	3	9	0.72
c-erbB-2			
Negative	12	34	
Positive	4	26	0.18

a) *P* values were obtained from the χ^2 test (two-sided).

ther was there any correlation between expression of PD-ECGF/TP and c-erbB-2 protein (P=0.18).

The clinical outcome of this group of 85 patients was analyzed (Fig. 4). It was found that there was no significant difference in overall survival between patients with PD-ECGF/TP-high positive cancer and those with PD-ECGF/TP-low positive cancer (P=0.66).

Serum PD-ECGF/TP levels Despite the high grade and/or high stage of cancer found in the group of patients we studied [18 had grade 3 cancers and 13 had pT4 cancers (Table I)], we were unable to detect significant levels of PD-ECGF/TP in these patients' sera.

DISCUSSION

PD-ECGF/TP is a mitogenic and angiogenic factor present in platelets. PD-ECGF/TP levels are markedly increased in tumor tissues compared with normal tissue in a variety of tumor types.[4, 14, 15] In one study, PD-ECGF/TP activity was determined by an enzyme assay, and its expression was found to correlate with both stage progression and a higher grade of bladder cancer.[5] By RNase protection assay (n=43) and western blot analysis (n=52), O'Brien *et al*. demonstrated that PD-ECGF/TP expression in invasive tumors was higher than in superficial tumors and in normal bladder. Furthermore, they found that there was no statistically significant difference in expression between superficial tumors and normal bladder.[6, 7] Sawase *et al*. reported that 47% (23/49) of bladder cancer patients expressed PD-ECGF/TP and the PD-ECGF/TP positive rate in cancer cells correlated with tumor grade and stage.[8] However, O'Brien *et al*. indicated that PD-ECGF/TP expression was positive in only 27% (28/105) of cases and that there was a significant correlation between PD-ECGF/TP expression and tumor grade, but no correlation between expression and tumor stage.[7]

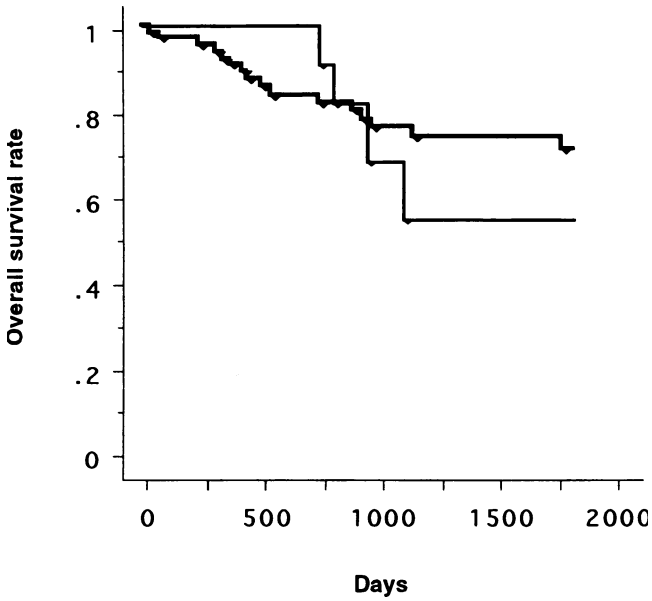

Fig. 4. Overall survival curve of patients with transitional cell carcinomas stratified by tumor PD-ECGF/TP expression. —— TP high expression (n=69), —— TP low expression (n=16), P=0.66.

We also confirmed the expression and existence of this protein at the levels of mRNA and protein by RT-PCR, western blotting, and immunohistochemistry. We demonstrated the increased expression of PD-ECGF/TP protein in bladder cancer tissues compared to non-cancerous tissues by immunohistochemistry (Fig. 3). This same protein was up-regulated in TCC, while normal transitional cells were not immunoreactive with MoAb 654-1. Moreover, high-positive expression of PD-ECGF/TP was observed in 81% (69/85) of bladder carcinomas obtained by cystectomy. The expression was correlated significantly with tumor grade, depth of invasion, and lymphatic invasion, but not with other analyzed parameters (Table II). Based

on three independent reports, the correlation between PD-ECGF/TP and tumor grade can be regarded as reliable. However, further investigation will be necessary to establish more definitively the relationship with other malignant potential-related factors.

Our data confirmed that PD-ECGF/TP expression was not correlated with overall survival in invasive bladder cancer patients. Similarly, O'Brien *et al*. reported that there was no relationship between PD-ECGF/TP expression in tumor cells and overall survival or relapse-free survival.[7] We also found no correlation between PD-ECGF/TP and recurrence-free survival in superficial tumors (data not shown). However, Sawase *et al*. demonstrated that the tumor-free interval of PD-ECGF/TP-positive superficial bladder cancer patients was significantly shorter than that seen in the negative patients.[8]

In that particular study, all patients with superficial tumors were treated with intravesicular chemotherapy following transurethral resection. This therapeutic method may have great significance. It has recently been shown that several cytostatics, such as Taxol, Taxotere, mitomycin C (MMC) and cyclophosphamide (CAP), increased the levels of human PD-ECGF/TP in tumors. These cytostatics simultaneously increased the levels of TNFα, which is an up-regulator of PD-ECGF/TP.[16] In urinary bladder cancer, MMC and Bacillus Calmette-Guérin (BCG) are often used for intravesicular chemotherapy. BCG has also been reported to induce TNFα in bladder tumor tissue.[17] While we showed that there was a low level of PD-ECGF/TP mRNA in superficial bladder cancers (Fig. 1B), it is possible that PD-ECGF/TP activity could be induced by drugs such as MMC or BCG. This might make the recurrence rate actually increase in some cases, though MMC or BCG is well known as an effective drug for intravesical chemotherapy. Such potential effects should at least be considered.

Previous studies in 1977 demonstrated that some cancer patients have increased serum levels of PD-ECGF/TP compared to healthy controls.[18] However, the serum levels of PD-ECGF/TP in 23 patients with advanced bladder cancer whom we investigated were not increased at all. This protein is known to lack a signal sequence necessary for cellular secretion. As for bladder cancer patients, it appears that the PD-ECGF/TP level does not increase in blood. This protein seems to work mainly at local sites. The biological and clinical significance of this protein in human bladder cancer remains to be established.

ACKNOWLEDGMENTS

This work was supported in part by a grant from Nippon Roche K. K. We thank Mr. Masafumi Suzaki of the Central Research Laboratory, Shiga University of Medical Science for his valuable technical assistance.

(Received August 19, 1999/Revised September 24, 1999/Accepted October 5, 1999)

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