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Expression of platelet-derived endothelial cell growth factor/thymidine phosphorylase in human gallbladder lesions

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Abstract

The aim of this study was to investigate the expression of platelet-derived endothelial growth factor (PD-ECGF) in human gallbladder carcinomas to elucidate its role in angiogenesis and tumour progression. To this end, 56 archival surgical specimens of gallbladder lesions were examined for PD-ECGF/thymidine phosphorylase (TP) expression by immunohistochemistry and the PD-ECGF/TP protein level was assessed in five fresh specimens of gallbladder carcinoma by enzyme-linked immunosorbent assay (ELISA). Hyperplastic epithelial cells and adenoma cells showed no or faint staining with PD-ECGF/TP. Out of 43 gallbladder carcinomas, 27 (63%) showed moderate to strong immunoreactivity in the cytoplasm and nuclei of the tumour cells. PD-ECGF/TP immunoreactivity in stromal infiltrating cells was detected in 43% (3/7) hyperplasias, 17% (1/6) adenomas and 86% (37/43) carcinomas. PD-ECGF/TP protein levels in carcinoma tissues were higher than those in corresponding normal mucosa. PD-ECGF/TP expression did not correlate with angiogenesis, but significantly correlated with depth of invasion, lymph node metastasis, and tumour stage. These results overall suggest that PD-ECGF/TP produced by both cancer cells and infiltrating cells is associated with tumour progression in human gallbladder carcinoma.

Keywords: PD-ECGF; Gallbladder carcinoma; Angiogenesis

1. Introduction

Angiogenesis is essential for tumour growth and metastasis and depends on the production of angiogenic regulators by tumour cells and/or host stromal cells [1,2]. Recent studies have shown that increased angiogenesis is associated with a worse prognosis in certain solid tumours such as breast, colon and gastric carcinomas and melanoma [3–6]. Identification of specific angiogenic factors may provide a target for antineoplastic therapy. We have previously shown that vascular endothelial growth factor (VEGF) expression was associated with the angiogenesis of gallbladder carcinoma, however, VEGF expression did not significantly correlate with any clinicopathological parameters [7]. Most gallbladder tumours with high vessel counts expressed high levels of VEGF, some did not. Therefore, it is unlikely that a single angiogenic factor regulates the angiogenic process.

A variety of growth factors and cytokines are reported to regulate one or more key events of angiogenesis [2,8–12]. Platelet-derived endothelial cell growth factor (PD-ECGF) is considered to be one of these factors. PD-ECGF was initially cloned as a novel angiogenic factor distinct from other known endothelial cell growth factors by virtue of its unique sequence homology, and its angiogenicity has been studied in several *in vivo* and *in vitro* assay systems [13–18]. PD-ECGF has recently been shown to be identical with thymidine phosphorylase (TP) [19]. PD-ECGF/TP catalyses the reversible phosphorylation of thymidine to thymine and 2-deoxyribose-1-phosphate, and is possibly involved with drug metabolism [20]. PD-ECGF/TP expression has been observed in cancer cells and their associated stromal infiltrating cells in several organs [21], and correlation between PD-ECGF/TP expression, angiogenesis and any clinicopathological parameters is reported in several tumour systems [22–25].

In this study, we examined the expression of PD-ECGF/TP in tumour-like lesions, adenomas and carcinomas of gallbladder by immunohistochemistry and enzyme-linked immunosorbent assay (ELISA). We also attempted to

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determine whether PD-ECGF/TP is associated with vessel counts and clinicopathological parameters.

2. Materials and methods

2.1. Archival surgical specimens of gallbladder lesions

For immunohistochemistry, a total of 56 surgically resected primary gallbladder lesions, including 7 hyperplasias, 6 adenomas, and 43 carcinomas (12 papillary adenocarcinomas, 13 well differentiated adenocarcinomas, 12 moderately differentiated adenocarcinomas, 6 poorly differentiated adenocarcinomas) which had not received any induction chemotherapy or radiotherapy before the operation, were studied. They were formalin-fixed and paraffin-embedded. The definition of histological typing and grading were made according to the *WHO International Histological Classification of Tumours* [26]. The pTNM pathological classification [27] was used for depth of invasion, regional lymph node metastasis, distant metastasis and stage grouping. Informed consent was obtained from all subjects.

2.2. Immunohistochemistry

Immunohistochemical staining was performed by the immunoperoxidase technique. Paraffin sections (4 μm) of formalin-fixed tissues were dewaxed in five changes of xylene rinsed in graded ethanol and finally rehydrated in three changes of phosphate-buffered saline (PBS). Antigen retrieval was performed by tripsinisation to unmask tissue antigens (with the exception of PD-ECGF/TP). For PD-ECGF/TP, microwave treatment was carried out to unmask the antigen, since this gave a more reproducible reaction when compared with trypsin digestion. Endogenous peroxidase was blocked by incubating the sections with 0.3% hydrogen peroxide in methanol for 30 min. After rinsing gently with PBS three times, non-specific reactions were blocked by incubating the sections in a solution containing 5% normal horse serum. The sections were then incubated with appropriate dilutions of the primary antibody at 4°C overnight. The antibodies used were a mouse monoclonal IgG antibody for CD34 (NU-4A1, Nichirei, Japan) at a 1:2 dilution, a mouse monoclonal IgG antibody for PD-ECGF/TP (gift from Y. Tanaka, Roche Institute, Kamakura, Japan) at a 1:200 dilution, and a mouse monoclonal IgG antibody for CD68 (Dako Glostrup, Denmark) at a 1:50 dilution. The sections were counterstained with Mayer's haematoxylin. Negative controls were performed in PBS for the primary antibody.

For determining the type of cells expressing PD-ECGF/TP in the stroma, immunostaining was performed for CD68 which is predominantly expressed on macrophages [28]. A double-staining procedure was performed as follows. The sections were first reacted with the mouse monoclonal IgG antibody for PD-ECGF/TP using the immunoperoxidase technique, then reacted with the mouse monoclonal IgG antibody for CD68 using the immunoalkaline phosphatase technique as described above. Brown coloration indicated positive PD-ECGF/TP, and red indicated macrophages.

2.3. Grading

Immunoreactivity was according to the classification of Fujii and colleagues [29]. To evaluate the staining intensity in both epithelial and infiltrating cells the following grading system was used.

The immunoreactivity in epithelium for PD-ECGF/TP protein and the immunoreactivity in infiltrating cells for PD-ECGF/TP was graded as − to + + + according to the number of cells stained and the intensity of the reaction in individual cells. Grades were defined as follows: −, almost no positive cells; +, 10–30% of the cells showing weak to moderate immunoreactivity; + +, 30–60% of the cells showing moderate immunoreactivity and/or 10–30% of the cells showing intense immunoreactivity; + + +, over 60% of the cells showing intense immunoreactivity. According to this grading protocol, three independent investigators examined all the immunostained specimens randomly to decide the grades as objectively as possible. There was less than 10% difference in assignments among the three investigators.

2.4. Vessel counting

Vessel counts were assessed in areas of the tumour containing the highest numbers of capillaries and small venules by light microscopy after staining for CD34 (endothelial cell) [30]. Scanning tumour sections at low power (×40 and ×100) identified areas containing the highest number of capillaries and small venules. After the area of highest neovascularisation was identified, individual vessel counts were performed at ×200 magnification (×20 objective and ×10 ocular) [31]. According to this grading protocol, two independent investigators performed all counts.

2.5. ELISA

Frozen tissues of gallbladder lesions ($n=5$) and the corresponding normal mucosa ($n=4$) were used for this assay. PD-ECGF/TP protein levels were measured by the ELISA method at the Nippon Roche Research Center. Complete details of the method have been previously reported by Nishida and colleagues [32].

2.6. Statistical analysis

Statistical analysis was performed using the Kendall tau b correlation analysis. A probability level of

$P < 0.05$ was considered statistically significant. The analyses were performed with STATISTICA™ statistical software (StatSoft™, USA).

3. Results

3.1. PD-ECGF/TP immunoreactivity in human gallbladder lesions

The expression of PD-ECGF/TP protein in gallbladder lesions, including 7 hyperplasias, 6 adenomas and 43 carcinomas was initially examined by immunohistochemical analysis. Histological diagnosis of the cases and immunoreactivity for PD-ECGF/TP in epithelial (tumour) cells and stromal infiltrating cells are summarised in Table 1. Representative pictures are shown in Fig. 1 (original magnification, ×400). Normal and hyperplastic gallbladder epitheliums were not stained with PD-ECGF/TP antibody. The immunoreactivity for PD-ECGF/TP in adenoma cells was also very faint. In hyperplasia and adenoma cases, very few infiltrating cells showed weak immunoreactivity for PD-ECGF/TP (Fig. 1A, B). In contrast, carcinoma cases revealed moderate to strong immunoreactivity of PD-ECGF in the cytoplasm and nucleus of the carcinoma cells and/or their stromal infiltrating cells (Fig. 1C, D). Out of 43 gallbladder carcinomas, 27 (63%) showed positive immunoreactivity to PD-ECGF/TP protein in tumour cells. The PD-ECGF/TP immunoreactivity in stromal infiltrating cells was detected in 3/7 (43%) hyperplasias, 1/6 (17%) adenomas and 37/43 (86%) carcinomas. The majority of PD-ECGF/TP positive cells in stroma were stained positive for CD68 (specific for macrophages) in double staining (Fig. 1E).

There were statistical differences in PD-ECGF/TP positivity in both epithelial cells and their infiltrating cells amongst the different histological types and grades (Fig. 2a, b).

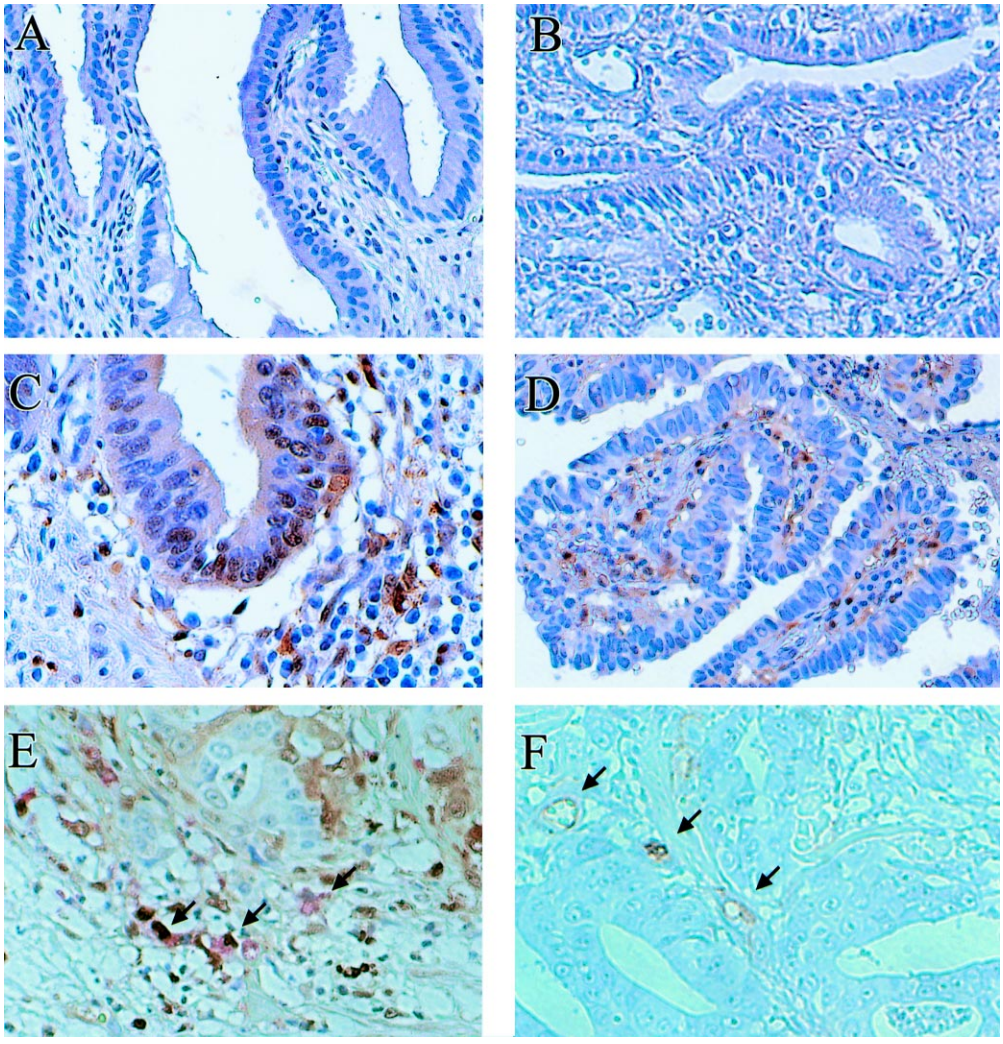

Fig. 1. Immunohistochemical staining for platelet-derived endothelial cell growth factor/thymidine phosphorylase (PD-ECGF/TP) (A–D), in human gallbladder hyperplasia (A), adenoma (B), and carcinoma (C, D). In double staining, the majority of PD-ECGF/TP positive infiltrating cells (E, arrow) were stained with CD68. CD34-positive cells were shown (F, arrow). Original magnification, ×400.

Table 1
PD-ECGF/TP expression and histological diagnosis

Histological diagnosis[a]	Epithelial cells					Infiltrating cells				
	TP expression				Positivity for TP% (No. of cases)	TP expression				Positivity for TP% (No. of cases)
	–	+	++	+++		–	+	++	+++	
Hyperplasia	7	0	0	0	0 (0/7)	4	3	0	0	43 (3/7)
Adenoma	6	0	0	0	0 (0/6)	5	1	0	0	17 (1/6)
Papillary adenocarcinoma	6	5	1	0	50 (6/12)	3	1	5	3	75 (9/12)
Adenocarcinoma, well differentiated	6	6	1	0	54 (7/13)	2	4	1	6	85 (11/13)
Adenocarcinoma, moderately differentiated	2	2	7	1	83 (10/12)	1	2	2	7	92 (11/12)
Adenocarcinoma, poorly differentiated	2	3	1	0	67 (4/6)	0	0	3	3	100 (6/6)

PD-ECGF, platelet-derived endothelial cell growth factor; TP, thymidine phosphorylase.
[a] According to [26].

3.2. Relationship of immunoreactivity for PD-ECGF/TP between cancer cells and infiltrating cells

Since both carcinoma cells and stromal infiltrating cells express PD-ECGF/TP protein, we next examined which cell is dominant for PD-ECGF/TP production. As shown in Table 2, 67% (29/43) of the carcinoma cases showed more intense PD-ECGF/TP immunoreactivity in infiltrating cells as compared with carcinoma cells, whereas only 7% (3/43) of the cases showed less intensity for PD-ECGF/TP in infiltrating cells. Using the Kendall tau b correlation analysis, we found that PD-ECGF/TP immunoreactivity in infiltration cells was higher than that in carcinoma cells (the coefficient of correlation; Kendall tau = 0.29, $P < 0.05$).

Table 2
The relationship of PD-ECGF/TP immunoreactivity in carcinoma cells and in infiltrating cells

TP immunoreactivity	%	No. of cases
Carcinoma cells > infiltrating cells	7	(3/43)
Carcinoma cells = infiltrating cells	26	(11/43)
Carcinoma cells < infiltrating cells	67	(29/43)

Significant correlation was detected by Kendall tau b correlation analysis. The coefficient of correlation: Kendall tau = 0.29, $P < 0.05$. For abbreviations see Table 1.

3.3. Relationship between PD-ECGF/TP immunoreactivity and its protein levels

To confirm the enhanced expression of PD-ECGF/TP in tumour tissues and a tumour-like lesion, we measured PD-ECGF/TP protein levels of fresh surgical specimens of gallbladder lesions and corresponding normal mucosa by ELISA. As shown in Table 3, PD-ECGF/TP protein levels were higher in the tumour tissues than normal gallbladder mucosa. There was a tendency of PD-ECGF/TP protein levels to increase with stage as well as PD-ECGF/TP immunoreactivity in the infiltrating cells.

3.4. Correlation between PD-ECGF/TP expression and tumour vascularity

To prove that PD-ECGF/TP is one of the important angiogenic factors for human gallbladder carcinoma, immunohistochemistry was performed against CD34 (Fig. 1F). Vessel counts ranged from 28 to 236 with a median of 92. Unexpectedly, the vessel counts were not correlated with PD-ECGF/TP immunoreactivity in gallbladder carcinoma cells (Kendall tau = 0.06, $P = 0.56$) or in infiltrating cells (Kendall tau = 0.15, $P = 0.17$) (data not shown).

Table 3
Relationship between PD-ECGF/TP immunoreactivity and its enzymatic activity in five gallbladder lesions

Histological diagnosis[a]	Stage[b]	TP expression in infiltrating cells	Protein level (Unit/mg protein)	
			Gallbladder lesion	Corresponding normal tissue
Adenomyomatous hyperplasia	–	+	37.5	24.4
Adenocarcinoma, well differentiated	I	+	46.5	18.5
Papillary adenocarcinoma	II	++	90.4	22.0
Papillary adenocarcinoma	II	+++	138.9	20.3
Papillary adenocarcinoma	IVA	+++	210.5	Not done

[a] According to [26].
[b] According to [27].
For abbreviations see Table 1.

3.5. Correlation between PD-ECGF/TP expression and clinicopathological features

The gallbladder carcinomas were subclassified according to the criteria of the *UICC TNM Classification of Malignant Tumors* [27]. PD-ECGF/TP expression was compared with the clinicopathological features of the tumours. As there were no cases of pN2 and only one case of distant metastasis in this study, it was not possible to analyse statistically the relationship between PD-ECGF/TP expression and category pN2, and distant metastasis. Using the Kendall tau b correlation analysis, we found that PD-ECGF/TP immunoreactivity in carcinoma cells and infiltrating cells significantly correlated with depth of invasion, regional lymph node metastasis, and tumour stage (Table 4).

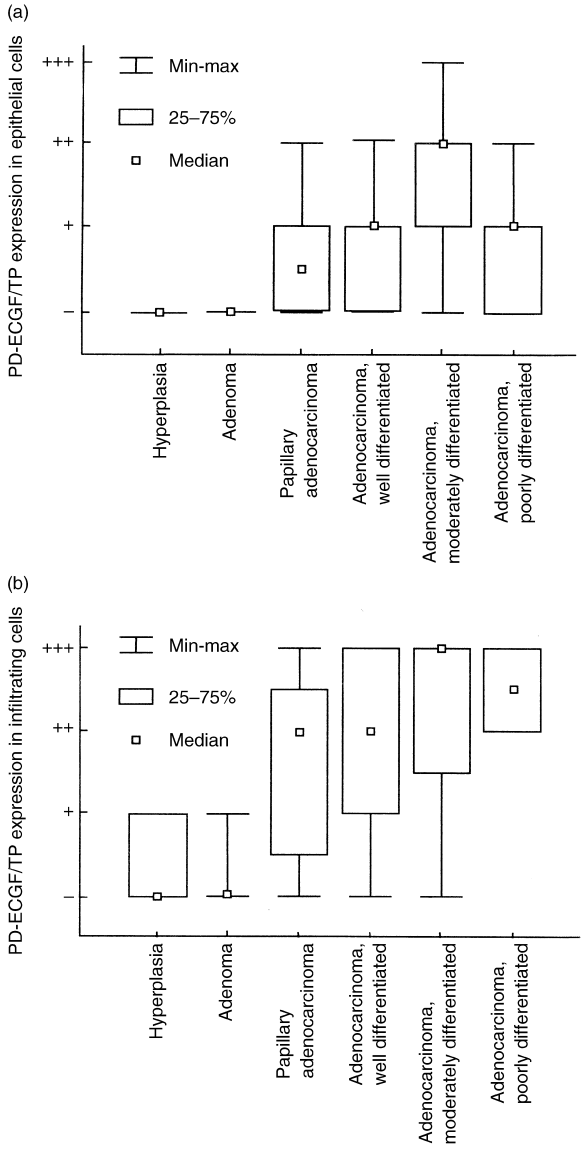

Fig. 2. Significant correlation was observed between histological diagnosis and platelet-derived endothelial cell growth factor/thymidine phosphorylase (PD-ECGF/TP) expression in epithelial cells (Kendall tau = 0.47, $P < 0.05$) (a). There was significant correlation between PD-ECGF/TP expression in infiltrating cells and histological diagnosis (Kendall tau = 0.46, $P < 0.05$) (b).

4. Discussion

Experimental findings have shown that tumour growth is dependent on angiogenesis [2]. When tumours reach a few millimetres in diameter, capillaries penetrate, permitting rapid tumour growth. These new vessels facilitate the entry of tumour cells into the vasculature and their subsequent metastasis. Therefore, angiogenesis is associated with an increased probability of metastasis [33,34]. The induction of angiogenesis is mediated by positive and negative regulators released by both tumour and host cells. Elucidation of the factors governing tumour angiogenesis may help clarify this phenomenon. Several angiogenic factors (positive regulators) have been identified including VEGF, bFGF, IL-8 and PD-ECGF/TP [8,9,35,36]. We have previously reported that VEGF is expressed by gallbladder carcinomas and its expression level is directly correlated with vascularity of the tumours. However, VEGF expression level is not significantly associated with tumour stage and patients' prognosis [7].

In the present study, we examined the expression of PD-ECGF/TP in gallbladder tumours to elucidate its role in angiogenesis and tumour progression. PD-ECGF/TP is expressed by both carcinoma cells and stromal infiltrating cells. The expression level of PD-ECGF measured by ELISA correlated well with PD-ECGF immunoreactivity in the infiltrating cells. The immune cells are known to express other angiogenic factors, including bFGF, transforming growth factor-alpha (TGF-α), VEGF and IL-8. The production of PD-ECGF by tumour-associated macrophages has previously been reported in colon and gastric carcinomas by Takahashi and colleagues [37,38]. Although the exact mechanism by which PD-ECGF induces tumour angiogenesis is presently unknown, stromal infiltrating cells may be involved in the progression of gallbladder carcinoma.

PD-ECGF/TP and VEGF were both isolated as endothelial growth factors. However, their characteristics seem to be different. VEGF is a selective mitogen for endothelial cells and acts on endothelial cells to increase microvascular permeability [39,40]. In contrast, PD-ECGF/TP stimulates the chemotaxis of endothelial cells *in vitro* and possesses angiogenic activity *in vivo* [17,35]. Numerous studies have demonstrated that PD-ECGF/TP expression is significantly correlated with the vessel count in several solid tumours [22–25,41–43]. Takebayashi and colleagues showed increased expression in colorectal carcinoma compared with normal mucosa [24]. Toi and colleagues demonstrated that PD-ECGF/TP and VEGF are frequently coexpressed in

Table 4
PD-ECGF/TP immunoreactivity and clinicopathological factors

Variable[a]		TP expression				Kendall tau	*P* value	TP expression				Kendall tau	*P* value
		Carcinoma cells						Infiltrating cells					
		–	+	++	+++			–	+	++	+++		
pT	1	8	1	1	0			6	2	1	1		
	2	6	10	5	1			0	4	8	10		
						0.36	< 0.05[b]					0.53	< 0.05[b]
	3	2	4	2	0			0	1	2	5		
	4	0	1	2	0			0	0	0	3		
pN	0	14	5	3	0			6	6	5	5		
						0.49	< 0.05[b]					0.51	< 0.05[b]
	1	2	11	7	1			0	1	6	14		
Stage	I	7	1	0	0			6	2	0	0		
	II	6	2	0	0			0	3	4	1		
	III	3	11	8	1	0.59	< 0.05[b]	0	2	7	14	0.71	< 0.05[b]
	IVA	0	1	2	0			0	0	0	3		
	IVB	0	1	0	0			0	0	0	1		

[a] According to [27].
[b] Significant correlation was detected by Kendall tau b correlation analysis.

highly vascularised human breast carcinoma specimens [41]. O'Brien and colleagues reported that there are at least two distinct angiogenic pathways involved in different stages of bladder carcinoma; one is the PD-ECGF/TP pathway in invasive carcinoma, and the other is the VEGF pathway in early superficial carcinoma [44]. We did not find significant correlation between the PD-ECGF/TP expression and vessel count in human gallbladder carcinoma. This is consistent with the findings of Fujieda and associates [45], who reported that there was no association between PD-ECGF expression and vessel count in oral and oropharyngeal carcinoma, suggesting that tumour angiogenesis is not simply controlled by the presence of PD-ECGF/TP, but may be mediated by several angiogenic factors. In this and our previous studies [7] (VEGF series was included almost PD-ECGF/TP series), we speculated that VEGF is the more dominant angiogenic factor compared with PD-ECGF/TP in human gallbladder carcinoma.

Regulation of PD-ECGF/TP expression is now being elucidated. The expression of PD-ECGF/TP by carcinoma cells was dependent on both the pH and oxygen concentration of media, and acidic conditions (from pH 6.3 to pH 6.7) and hypoxic exposure (0.3% of O_2 concentration) were the optimal conditions for PD-ECGF/TP expression [46]. Recent studies have shown that the microenvironment in tumour tissue is more acidic than in normal tissue. Moreover, acidic medium stimulated the invasive activity of the cancer cells through elevation of collagenase type IV expression [47]. Since the expression and activity of PD-ECGF/TP is enhanced by acidic pH, PD-ECGF/TP expression levels may be associated with depth of invasion in gallbladder carcinomas. Additional studies are required to clarify whether PD-ECGF/TP is involved in tumour invasion.

In conclusion, this study has demonstrated that PD-ECGF/TP is expressed by both carcinoma and infiltrating cells, and its expression level is associated with advanced disease in human gallbladder carcinoma. To the best of our knowledge these are the first data showing the correlation between PD-ECGF/TP expression and clinicopathological parameters of the gallbladder carcinoma. It is possible that PD-ECGF/TP may be used as a target for anticancer therapy in the future.

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References

1. Folkman J. How is blood vessel growth regulated in normal and neoplastic tissue? G. H. A. Clowes Memorial Award Lecture. *Cancer Res* 1986, **46**, 467–473.
2. Folkman J. What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 1990, **82**, 4–6.
3. Weidner N, Folkman J, Pozza F, *et al*. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast cancer. *J Natl Cancer Inst* 1992, **84**, 1875–1887.
4. Weidner N. Intratumor microvessel density as a prognostic factor in cancer. *Am J Pathol* 1995, **147**, 9–19.
5. Graham CH, Rivers J, Kerbel RS, Stankiewicz KS, White WL. Extent of vascularization as a prognostic indicator in thin (< 0.76 mm) malignant melanomas. *Am J Pathol* 1994, **145**, 510–514.

6. Tanigawa N, Amaya H, Matsumura M, *et al*. Extent of tumor vascularization correlates with prognosis and hematogenous metastasis in gastric carcinomas. *Cancer Res* 1996, **56**, 2671–2676.
7. Yamamoto S, Fujii K, Kitadai Y, Yamamoto So, Tsuchida A, Kajiyama G. Expression of vascular endothelial growth factor in human gallbladder lesions. *Oncol Rep* 1998, **5**, 1065–1069.
8. Bicknell R, Harris AL. Novel growth regulatory factors and tumour angiogenesis. *Eur J Cancer* 1991, **27A**, 781–784.
9. Leung DW, Cachianes G, Kuang W-J, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 1989, **246**, 1306–1309.
10. Schluze-Othoff K, Fruhbeis B, Overwien B, Hilbig B, Sorg C. Purification and characterization of a novel human angiogenic factor (h-AF). *Biochem Biophys Res Commun* 1987, **146**, 945–952.
11. Van Hinsbergh VWM, Kooistra T, Emeris JJ, Koolwijk P. Regulation of plasminogen activator production by endothelial role in fibrinolysis and local proteolysis. *Int J Radiol* 1991, **60**, 261–272.
12. Höckel M, Sasse J, Wisseler JH. Purified monocyte-derived angiogenic substance (angiotropin) stimulates migration, phenotyping changes, and "tube formation" but not proliferation of capillary endothelial cells *in vivo*. *J Cell Physiol* 1987, **133**, 1–13.
13. Haraguchi M, Miyadera K, Sumizawa T. Angiogenic activity of thymidine phosphorylase and PD-ECGF. *Proc Am Assoc Cancer Res* 1993, **53**, 5680–5682.
14. Toi M, Hoshina S, Taniguchi T, Yamamoto Y, Ishitsuka H, Tominaga T. Expression of platelet-derived endothelial cell growth factor/thymidine phosphorylase in human breast cancer. *Int J Cancer* 1995, **64**, 79–82.
15. Moghaddam A, Zhang H-T, Fan TPD, *et al*. Thymidine phosphorylase is angiogenic and promotes tumor growth. *Proc Natl Acad Sci USA* 1995, **92**, 998–1002.
16. Finnis C, Dodsworth N, Pollit CE, Carr G, Sleep D. Thymidine phosphorylase activity of platelet derived endothelial cell mitogenicity. *Eur J Biochem* 1993, **212**, 201–210.
17. Ishikawa F, Miyazono K, Hellman U, *et al*. Identification of angiogenic activity and the cloning and expression of platelet-derived endothelial cell growth factor. *Nature* 1989, **338**, 557–562.
18. Takahashi H, Uchino J, Ogita M, *et al*. Pyrimidine nucleoside phosphorylase activity and biological characteristics of breast cancer. *Int J Oncol* 1995, **7**, 1139–1144.
19. Miyadera K, Dohmae N, Takio K, *et al*. Structural characterization of thymidine phosphorylase purified from human placenta. *Biochem Biophys Res Commun* 1995, **212**, 1040–1045.
20. Griffiths L, Straford IJ. Platelet-derived endothelial cell growth factor thymidine phosphorylase in tumor growth and response to therapy. *Br J Cancer* 1997, **76**, 689–693.
21. Fox SB, Westwood M, Moghaddam A, *et al*. The angiogenic factor platelet-derived endothelial cell growth factor/thymidine phosphorylase is up-regulated in breast cancer epithelium and endothelium. *Br J Cancer* 1996, **73**, 275–280.
22. Tanigawa N, Amaya H, Matsumura M, *et al*. Tumor angiogenesis and expression of thymidine phosphorylase/platelet derived endothelial cell growth factor in human gastric carcinoma. *Cancer Lett* 1996, **108**, 281–290.
23. Takebayashi Y, Miyadera K, Akiyama S, *et al*. Expression of thymidine phosphorylase in human gastric carcinoma. *Jpn J Cancer Res* 1996, **87**, 288–295.
24. Takebayashi Y, Akiyama S, Akiba S, *et al*. Clinicopathologic and prognostic significance of an angiogenic factor, thymidine phosphorylase, in human colorectal carcinoma. *J Natl Cancer Inst* 1996, **88**, 1110–1117.
25. Koukourakis MI, Giatromanolaki A, O'Byrne KJ, *et al*. Platelet-derived endothelial cell growth factor expression correlates with tumor angiogenesis and prognosis in non-small-cell lung cancer. *Br J Cancer* 1997, **75**, 477–481.
26. Albores-Saavedra J, Henson DE, Sobin LH. *Histological Typing of Tumors of the Gallbladder and Extrahepatic Bile Ducts*. 2nd edn. Berlin, Springer WHO International Organization International Histological Classification of Tumors, 1991.
27. Sobin LH, Wittekind Ch. *UICC TNM Classification of Malignant Tumors*. 5th edn. Tokyo, Kanehara Ltd, 1997.
28. Martinez PL, Platt N, McKnight AJ, DaSilva RP, Gordon S. Macrophage membrane molecules: markers of tissue differentiation and heterogeneity. *Immunobiology* 1996, **195**, 407–416.
29. Fujii K, Yasui W, Kuniyasu H, *et al*. Expression of cripto in human gallbladder lesions. *J Pathol* 1996, **180**, 166–168.
30. Nobuhiko T, Hirokazu A, Masayuki I, *et al*. Extent of tumor vascularization correlates with prognosis and hematogenous metastasis in gastric carcinomas. *Cancer Res* 1996, **56**, 2671–2676.
31. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis correlation in invasive breast carcinoma. *N Engl J Med* 1991, **324**, 1–8.
32. Nishida M, Hino A, Mori K, Matsumoto T, Yoshikubo T, Ishitsuka H. Preparation of anti-human thymidine phosphorylase monoclonal antibodies useful for detecting the enzyme levels in tumor tissues. *Biol Pharm Bull* 1996, **19**, 1407–1411.
33. Liotta LA, Kleinerman J, Saidel G. The significance of hematogenous tumor cell clumps in the metastatic process. *Cancer Res* 1976, **36**, 889–894.
34. Nagy JA, Brown LF, Senger DR, *et al*. Pathogenesis of tumor stroma generation: a critical role for leaky blood vessels and fibrin deposition. *Biochem Biophys Acta* 1988, **948**, 305–326.
35. Haraguchi M, Miyadera K, Uemura K, *et al*. Angiogenic activity of enzymes. *Nature* 1994, **368**, 198.
36. Kitadai Y, Haruma K, Sumii K, *et al*. Expression of interleukin-8 correlates with vascularity in human gastric carcinomas. *Am J Pathol* 1998, **152**, 93–100.
37. Takahashi Y, Bucana CD, Liu W, *et al*. Platelet-derived endothelial cell growth factor in human colon cancer angiogenesis. Role of infiltrating cells. *J Natl Cancer Inst* 1996, **88**, 1146–1151.
38. Takahashi Y, Bucana CD, Akagi Y, *et al*. Significance of platelet-derived endothelial cell growth factor in the angiogenesis of human gastric cancer. *Clin Cancer Res* 1998, **4**, 429–434.
39. Connolly DT, Heuvelman DM, Nelson R, *et al*. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J Clin Invest* 1989, **84**, 1470–1478.
40. Dvorak HF, Siossat TM, Brown LF, *et al*. Distribution of vascular permeability factors in tumors: concentration in tumor blood vessels. *J Exp Med* 1991, **174**, 1275–1278.
41. Toi M, Inada K, Hoshino S, Suzuki H, Kondo S, Tominaga T. Vascular endothelial growth factor and platelet-derived endothelial cell growth factor are frequently coexpressed in highly vascularized human breast cancer. *Clin Cancer Res* 1995, **1**, 961–964.
42. Saeki T, Tanada M, Takashima S, *et al*. Correlation between expression of platelet-derived endothelial growth factor (thymidine phosphorylase) and microvessel density in early-stage human colon carcinomas. *Jpn Clin Oncol* 1997, **27**, 227–230.
43. Imazono Y, Takebayashi Y, Nishiyama K, *et al*. Correlation between thymidine phosphorylase expression and prognosis in human renal cell carcinoma. *J Clin Oncol* 1997, **15**, 2570–2578.
44. O'Brien T, Cranston D, Fuggle S, Bicknell R, Harris WL. Different angiogenic pathways characterized superficial and invasive bladder cancer. *Cancer Res* 1995, **55**, 510–513.
45. Fujieda S, Sunaga H, Tsuzuki H, Tanaka N, Saito H. Expression of platelet-derived endothelial cell growth factor in oral and oropharyngeal carcinoma. *Clin Cancer Res* 1998, **4**, 1583–1590.
46. Giffiths L, Dachs GU, Bickell R, Harris AL, Stratford IJ. The influence of oxygen tension and pH on the expression of platelet-derived endothelial cell growth factor/thymidine phosphorylase in human breast tumor cell growth *in vitro* and *in vivo*. *Cancer Res* 1997, **57**, 570–572.
47. Kato Y, Nakayama Y, Umeda M, Miyazaki K. Induction of 103-kDa gelatinase/type IV collagenase by acidic culture conditions in mouse metastatic melanoma cell line. *J Biol Chem* 1992, **267**, 11424–11430.