Placenta growth factor not vascular endothelial growth factor A or C can predict the early recurrence after radical resection of hepatocellular carcinoma

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Abstract

The purpose of this study was to evaluate the relationship between the expression of PlGF in tumor tissue and clinical outcomes in HCC patients. Tumor PlGF and vascular endothelial growth factor (VEGF)-A and VEGF-C mRNA were analyzed. Results demonstrated that patients with PlGF expression levels higher than median tended to have early recurrence compared to patients with PlGF expression lower than median ($P = .031$). In patients with AJCC stage II–III disease, this difference was even more significant ($P = .002$). In contrast, VEGF-A and VEGF-C could not predict early recurrence-free survival. Since PlGF expression correlated with early recurrence of HCC, PlGF may be an important prognostic indicator in HCC.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. HCC is a hypervascular tumor mainly supplied by hepatic arteries [1] and characterized by a propensity for vascular invasion. HCC is associated with a high

metastatic potential. Early postoperative recurrence in the liver remnant or growth of tumor at distant sites as a result of metastasis is a common phenomenon following resection of HCC [2]. Angiogenesis is now recognized as playing a pivotal role in hepatocarcinogenesis and regarded as one of the marker for invasiveness and metastasis in HCC.

Tumor angiogenesis is regulated by a variety of pro- and anti-angiogenic factors. Among them, vascular endothelial growth factor A (VEGF-A) has been studied most extensively and is considered to

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be the most important proangiogenic factor in HCC [3,4]. Similarly, vascular endothelial growth factor C (VEGF-C), which may play a role in the maintenance of lymphatic endothelium and/or in lymphangiogenesis, is also reported to be an angiogenic factor in some human cancers [5–7]. VEGF-C expression in HCC has also been reported [8]. The correlation between VEGF expression and the prognosis of HCC, however, remains unclear. Li et al. [9] and Ng et al. [3] both reported that expression of VEGF is related to invasion and metastasis of HCC [3,9]. Yamaguchi et al. [1], however, found that VEGF expression was lower in advanced HCC with increased tumor vasculatures [1]. These observations suggest that other angiogenic factors may also contribute to angiogenesis stimulation in HCC.

Placenta growth factor (PIGF) is a dimeric glycoprotein, structurally and functionally related to VEGF [10]. PIGF appears to be crucial for pathological angiogenesis in adults [11] and is not produced by the majority of normal human tissues [12]. A prognostic implication of tumor PIGF expression level independent of conventional pathologic prognosticators has been demonstrated in a variety of cancers. For example, in human gastric cancer, PIGF expression level was significantly correlated with serosal invasion, positive lymph node metastases, tumor stages, and patient survival [13]. PIGF levels were also significantly associated with histological grade, total tumor vascularity, and prognosis in patients with renal cancer [14]. Exposure of melanoma cells to PIGF resulted in a specific proliferative response [15]. PIGF expression was also shown to correlate with disease progression and patient survival in colorectal cancer [16].

To date, PIGF expression in HCC and any correlation between PIGF and prognosis remains unknown. Therefore, the aim of this study was to evaluate the expression of PIGF in postoperative HCC patients following primary resection of the liver to determine if a correlation between PIGF expression and early postoperative recurrence of HCC existed.

2. Materials and methods

2.1. Patients

Seventy-one consecutive patients who underwent primary liver resection for HCC at the National Taiwan University Hospital were included in this study. Patients who had received preoperative chemotherapy or embolization

were excluded. All patients received routine chest X-ray, abdominal ultrasound (US), computed tomography (CT) and hepatic angiography prior to the liver resection. Intra-operative US was routinely performed in all the patients. Tumor complete resection (defined as microscopically free of tumor at the section margin, and no evidence of space occupying lesions in the remaining liver as detected by image studies) were successfully performed in all 71 patients. Neither preoperative therapy nor postoperative adjuvant chemotherapy was administered. The tumor tissue and 62 patient-matched non-tumor tissue samples were collected and snap frozen in liquid nitrogen immediately after excision from the patients under the approval of the Institutional Review Boards and each patient's written informed consent. Clinical data were obtained from the medical records. Tumor differentiation, venous invasion, maximum tumor diameter, and non-tumor part of the excised liver were all examined by an experienced pathologist. The pathologist was blinded to all clinical information.

The patients were followed up at least once every 3 months. Serum alpha fetoprotein (AFP) level and abdominal US were employed to screen for tumor recurrence. Abdominal CT and/or magnetic resonance image (MRI) were performed if AFP levels elevated or a new space occupying lesion was detected by US. Hepatic angiography was performed in the even that recurrent HCC (i.e., new hypervascular lesion found during the arterial phase of hepatic angiography) was suspected. Patients that developed HCC nodules that developed within 1 year of the primary surgery, near the site of the original tumor or if nodules shared the same portal blood supply as the primary tumor, were considered as having early intrahepatic recurrence [17,18].

2.2. Quantitative real time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA of the tumor and non-tumor tissue samples were extracted by Trizol Reagent (Invitrogen, USA) and an RNeasy Mini Kit (Qiagen, Germany). The isolated RNA was quantified by OD260nm and qualified by a Bioanalyzer (Agilent Technology, USA). RNA concentration was determined by a spectrophotometer (GeneQuant II, Pharmacia Biotech, England) and an equal quantity of total RNA was used from different samples for RT-PCR. Real-time RT-PCR was performed with SuperScript II Reverse Transcriptase (Invitrogen). In brief, 1 µg total RNA were mixed with 1 µL dNTP (Viogene, Taiwan) and 1 µL oligo-dT primer (MDBio, Inc.) for 5 min at 65 °C. Then, RNA was reverse-transcribed following the manufacturer's protocol. Real-time PCR was performed using the iCycler iQ Real-Time detection system (Bio-Rad, Hercules, CA) with SYBR-Green I (stock solution

25,000×) as fluorescent dye enabling real time detection of PCR products according to the manufacturer's protocol. The oligonucleotide primers for real-time PCR designed by Beacon Designer 2 software (PREMIER Biosoft International, Palo Alto, CA) were: 5'-d AAT GCC TCG CTC CCT TCA AGA C and 5'-d GGT CTG TGG CTG GCT TCT CTC for human PIGF; 5'-d GCA GAA GGA GGA GGG CAG AAT C and 5'-d GGC ACA CAG GAT GGC TTG AAG for human VEGF-A; and 5'-d CAC ACT TCC TGC CGA TGC and 5'-d GTT CGC TGC CTG ACA CTG for human VEGF-C; and 5'-d GGT GGT CTC CTC TGA CTT CAA C and 5'-d TCT CTC TTC CTC TTG TGT TCT TG for human GAPDH. The cycling conditions were 95 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The specificities of all primers were confirmed by melt-curve detection following real-time PCR reaction. All samples were amplified in a linear amplification range established using a serial cDNA dilution and varying the number of cycles. PCR products were electrophoresed onto a 1.2% agarose gel containing ethidium bromide and visualised under UV light. The relative intensities of the bands were quantified by densitometric analysis. For quantification, the target gene was normalized to the internal standard gene GAPDH. The relative amount of tissue PIGF mRNA, standardised against the amount of GAPDH mRNA, was expressed as $-\Delta C_T = -[C_{T(PIGF)} - C_{T(GAPDH)}]$. The ratio of the number of PIGF mRNA copies to the number of GAPDH mRNA copies was then calculated as $2^{-\Delta C_T} \times K$, where K is a constant. The relative amount of tissue VEGF-A and VEGF-C were also expressed in the same way.

2.3. ELISA (enzyme linked immunoabsorbant assay)

Protein lysate from each specimen was collected from 40 mg of tissue, (mechanically homogenized with a mortar and pestle in liquid nitrogen) using the Total Protein Extraction Kit (BioChain Institute, Inc., Hayward, CA, USA). Protein lysate was maintained at -80°C until time of analysis. Concentrations of PIGF in tumor tissues were quantified using a "Quantikine" human PIGF immunoassay (R&D systems, Inc., Minneapolis, MN, USA). Four milligram total protein lysate was incubated in duplicate overnight at 4°C on microtitre plates coated with a murine monoclonal antibody against human PIGF. Unbound protein was washed off and an enzyme linked polyclonal antibody specific for PIGF was added to "sandwich" the PIGF immobilized during the first incubation. A substrate solution of horseradish peroxidase was added, and color developed in proportion to the amount of antibody bound to the PIGF. Absorbance of the color was read at 450 nm. A standard curve, consisting of known

amounts of PIGF, was prepared and concentrations of PIGF in the unknown samples were determined from this standard curve.

2.4. Evaluation of intratumorous microvessel density

Intratumorous microvessel density (MVD) was assessed by light microscopy using the counting method introduced by Weidner et al. [19]. CD34 was used to identify and count intratumorous vessels. Tumor sections for MVD assessment were performed on peripheral areas adjacent to the tumor margin in all cases. Tumorous and non-tumorous tissue sections were scanned at low magnification ($40\times$ and $100\times$) to identify the areas that demonstrated the most intense vascularisation (i.e., hot spots). Individual microvessels were counted in five fields at $200\times$ magnification ($20\times$ objective lens and $10\times$ ocular lens; $0.7386\text{ mm}^2/\text{field}$). The final MVD was calculated as the mean value obtained from the counts of the five fields. MVD was expressed as mean $\pm$ SD (vessels/ 0.7386 mm^2). Any immunostained endothelial cells or endothelial cell clusters that were clearly separated from the adjacent microvessels, tumour cells or other connective tissue elements were considered to be a single and countable microvessels. The identification of vessel lumens was not necessary for a structure to be defined as a microvessel, and identification of red blood cells was not required to define the presence of a vessel lumen. The evaluation of MVD was performed by an individual with no knowledge of the clinicopathological data.

2.5. Statistical analysis

The clinical and pathologic characteristics of the patients in relation to PIGF, VEGF-A and VEGF-C were compared by the Student's t -test for continuous variables and χ^2 test with Yates' correction for categorical variables. Correlation between continuous variables was performed with linear regression analysis. Overall, cumulative recurrence free survival rates were obtained using the Kaplan–Meier method. The differences in recurrence free survival between the groups were compared using the log-rank test. The starting point for calculating recurrence free survival was the date of surgery, and the endpoint was the date of recurrence. To evaluate the prognostic influence of PIGF, VEGF-A and VEGF-C in the entire cohort and in subgroups of patients stratified by AJCC stage, the median value in each group was used as the cutoff value for comparison. Pearson Product Moment Correlation was used to show the correlation between PIGF, VEGF levels and degree of vascularization in HCC. For all statistical analyses, a software package S-Plus 6.1 (Venables, 2002) was used. A P value $< .05$ was considered to be statistically significant.

3. Results

3.1. Correlation between tumor PIGF, VEGF-A and VEGF-C expression and clinicopathologic features

At the time of analysis, 14 of 71 patients (19.7%) had developed early intrahepatic metastasis. Among the clinicopathologic factors listed in Table 1, tumor size ($P = .004$), AFP ($P = .002$), AJCC stage ($P = .000$), presence of venous invasion ($P = .001$), and presence of satellite nodules ($P = .000$) were significant prognostic factors for early recurrence-free survival by univariate analysis. When comparing tumor PIGF, VEGF-A and VEGF-C between patient subgroups categorized according to various clinicopathologic parameters, significantly higher VEGF-A and VEGF-C expression were associated with poorer differentiated HCC (Table 2). No significant differences were identified when evaluating PIGF expression according to the clinicopathologic parameters.

3.2. The prognostic implications of PIGF, VEGF-A and VEGF-C on recurrence-free survival after resection of HCC

The prognostic implications of tumor PIGF, VEGF-A and VEGF-C expression on early recurrence was evaluated by comparing the 1 year recurrence-free survival between patients with lower than median and those with higher than median expression levels of these angiogenic factors in the tumor tissues. When the entire cohort of 71 patients was analyzed, tumor PIGF expression was a significant predictive factor of early recurrence-free survival ($P = .0311$, Fig. 1A). In contrast, neither VEGF-A nor VEGF-C had any significant prognostic implication on recurrence-free survival ($P = .380$ and $P = .595$, Fig. 1B and C, respectively).

In order to prove that the PIGF expression level is the independent factor for the early recurrence of HCC, we applied the multiple logistic regression analysis on the response variable: recurrence status and the eight explanatory variables: PIGF, VEGF-A and VEGF-C expression levels and the other five clinical factors (i.e., tumor size, AFP, AJCC stage, presence of venous invasion, and presence of satellite nodules) which are significant prognostic factors in the univariate analysis. Here, we used 53 HCC samples consisting of complete early recurrence versus non-early recurrence status after more than the 1 year follow-up period. Logistic regression together with stepwise model selection was used to select the prediction model by the AIC criteria, and the final model consisting of two variables (PIGF and tumor size) was obtained:

$$\lambda = -2.971 + 1.049 \text{ PIGF} + 0.392 \text{ size} + 0.139 \text{ PIGF} * \text{size}$$

$$\pi = \exp(\lambda) / (1 + \exp(\lambda))$$

where π is the probability of “early recurrence”. Early recurrence (defined as recurrence time in <12 months) was predicted when π is greater than or equal to 0.5. The standard errors of the logistic regression coefficients are 0.909 for constant, 0.520 for PIGF, 0.149 for tumor size, and 0.074 for PIGF*tumor size, respectively. Moreover, the cross term PIGF*tumor size in the model representing the inter correlation between PIGF and tumor size demonstrate that the PIGF expression level and tumor size are the major factors for the early recurrence of HCC.

3.3. PIGF is a prognostic factor for HCC early recurrence in patients with AJCC stage II–III HCC, not in stage I

Further analyses were performed for patients with either AJCC stage I tumor or AJCC stage II–III tumor to evaluate whether tumor PIGF had a prognostic value in each subset of patients. Tumor PIGF expression was a significant predictive factor of recurrence-free survival among the 29 patients with AJCC stage II–III HCC, with worse recurrence-free survival among those with a higher than median PIGF expression compared with those with a lower than median PIGF expression ($P = .002$, Fig. 2A1). However, no difference was observed in the recurrence-free survival of AJCC stage I patients regarding PIGF expression ($P = .568$, Fig. 2A2).

When the prognostic implication of VEGF-A and VEGF-C were analyzed, no significant correlation was found between these angiogenic factors and the early recurrence-free survival in either AJCC stage I or AJCC stage II–III patients (Fig. 2B1, B2, C1 and C2).

3.4. PIGF expression levels in the non-tumor tissue

Non-tumor tissue was available in 62 patients. The mean PIGF expression level ($-\Delta C_{T(\text{PIGF})}$) in the non-tumor tissue of patients with chronic active hepatitis was significantly higher compared to the expression of PIGF in the patients without chronic active hepatitis (4.36 ± 5.11 versus 1.35 ± 1.12 , $P = .032$). The mean $-\Delta C_{T(\text{PIGF})}$ for tumor tissue vs non-tumor tissue in patients with chronic active hepatitis was 2.41 ± 3.09 versus 4.36 ± 5.11 ($P = .32$). The mean $-\Delta C_{T(\text{PIGF})}$ for tumor tissue versus non-tumor tissue in patients without chronic active hepatitis was 2.52 ± 2.03 versus 1.35 ± 1.12 ($P = .21$). No correlation between PIGF expression in tumor tissue and the patient-matched non-tumor part was identified.

3.5. PIGF expression level correlate significantly with the degree of vascularization in advanced HCC

MVD was found to be significantly higher in patients with AFP >400 ng/mL (AFP >400 ng/mL versus AFP ≤400 ng/mL: 61.9 ± 29.9 versus 38.0 ± 24.2 , $P = .002$);

Table 1

Comparison of the 1 year recurrence free survival between Clinical and pathological features in the 71 hepatocellular carcinomas studied

Variable	No. of patients (%)	1 year recurrence free survival (%)	P Value
Age			
≤60 years	27 (38.0)	71.9	0.149
>60 years	44 (62.0)	85.7	
Sex			
Male	52 (73.2)	77.9	0.343
Female	19 (26.8)	88.5	
Underlying hepatitis			
HBV	37 (52.1)	74.7	0.465
HCV	25 (35.2)	83.3	
Non-B, non-C	6 (8.5)	NA	
Both B and C	3 (4.2)	NA	
Cirrhosis			
Presence	56 (78.9)	81.3	0.730
Absence	15 (21.1)	78.6	
Serum α -fetoprotein			
>400 ng/mL	20 (28.2)	60.0	0.002*
≤400 ng/mL	51 (71.8)	89.0	
Tumor size			
>5 cm	23 (32.4)	61.7	0.004*
≤5 cm	48 (67.6)	89.2	
Tumor capsule			
Presence	18 (25.4)	NA	0.131
Absence	53 (74.6)	76.3	
Tumor number			
Single	65 (91.5)	82.1	0.341
Multiple	6 (8.5)	66.7	
Edmonson grade			
I	11 (15.7)	NA	0.101
II	40 (57.1)	86.8	
III	19 (27.1)	68.4	
Venous invasion			
Presence	26 (36.6)	61.5	0.001*
Absence	45 (63.4)	92.5	
Microsatellite nodules			
Presence	6 (8.5)	16.7	0.000*
Absence	65 (91.5)	86.9	
AJCC stage			
I	42 (59.2)	91.8	0.000*
II	11 (15.5)	NA	
III	18 (25.4)	50.0	

Abbreviation: HBV, hepatitis B; HCV, hepatitis C.

* $P < .05$ by log-rank test.

grade II–III disease (grade II–III versus grade I: 87.9 ± 23.3 versus 49.5 ± 27.5 ; $P = .006$); microscopic vascular invasion (presence of VI versus absence of VI: 63.2 ± 30.0 versus 40.8 ± 25.3 ; $P = .002$) and satellite nodules (presence of satellite nodules compared to absence of satellite nodules: 56.9 ± 30.6 versus 31.0 ± 10.2 ; $P = .006$). When a scatter plot of MVD versus the expression levels of VEGF-A, VEGF-C, and PlGF

were prepared, a non-significant, albeit borderline, correlation ($P = .077$) was identified between PlGF and MVD (Fig. 3A). In contrast, no correlation was identified between VEGF and MVD (Fig. 3B and C). Further analysis revealed that the MVD positively and significantly correlated with tumor PlGF expression levels in tumors >5 cm ($R = 0.47$, $P = .0314$), but not in tumors ≤ 5 cm ($R = -0.066$, $P = .57$, Fig. 3A1 and A2).

Table 2
Correlation of tumor PIGF, VEGF-A and VEGF-C expression levels with clinicopathological features of HCC

Variable	PIGF	VEGF-A	VEGF-C
Age			
≥60 years	1.43 ± 1.90	2.72 ± 2.45	1.27 ± 0.96
<60 years	2.33 ± 3.50	3.32 ± 3.43	1.76 ± 1.49
Sex			
Male	1.88 ± 2.77	2.92 ± 2.74	1.55 ± 1.31
Female	1.46 ± 2.31	3.04 ± 3.22	1.20 ± 0.86
Underlying hepatitis			
HBV	1.66 ± 2.89	3.44 ± 3.32	1.53 ± 1.39
HCV	1.81 ± 2.27	2.49 ± 2.21	1.42 ± 1.00
Cirrhosis			
Presence	1.84 ± 2.87	2.88 ± 2.91	1.48 ± 1.29
Absence	1.50 ± 1.61	3.23 ± 2.72	1.38 ± 0.89
Serum α -fetoprotein			
>400 ng/mL	2.14 ± 1.98	2.38 ± 2.48	1.51 ± 1.44
≤400 ng/mL	1.63 ± 2.87	3.17 ± 2.99	1.44 ± 1.11
Tumor size			
≥5 cm	2.82 ± 3.65	2.71 ± 2.52	1.51 ± 1.27
<5 cm	1.27 ± 1.82	3.06 ± 3.03	1.44 ± 1.18
Tumor capsule			
Presence	2.42 ± 3.74	2.90 ± 2.44	1.22 ± 0.83
Absence	1.55 ± 2.13	2.97 ± 3.01	1.54 ± 1.31
Tumor number			
Single	1.83 ± 2.76	2.90 ± 2.87	1.39 ± 1.14
Multiple	1.08 ± 0.70	3.49 ± 2.91	2.24 ± 1.66
Edmonson grade			
I	2.79 ± 4.97	1.27 ± 0.75 ^{&}	0.72 ± 0.31 [#]
II–III	1.55 ± 1.90	3.30 ± 3.02 ^{&}	1.61 ± 1.28 [#]
Venous invasion			
Presence	2.00 ± 2.42	2.57 ± 2.95	1.52 ± 1.12
Absence	1.64 ± 2.78	3.17 ± 2.81	1.42 ± 1.26
Microsatellite nodules			
Presence	1.45 ± 0.93	2.81 ± 2.96	2.07 ± 1.57
Absence	1.80 ± 2.76	2.96 ± 2.87	1.40 ± 1.16
AJCC stage			
I	1.68 ± 2.87	3.15 ± 2.88	1.37 ± 1.25
II–III	1.90 ± 2.32	2.66 ± 2.84	1.59 ± 1.15

Note. The relative amount of tissue PIGF, VEGF-A and VEGF-C mRNA, standardised against the amount of GAPDH mRNA, was expressed as $-\Delta C_T = -[C_{T(\text{PIGF})} - C_{T(\text{GAPDH})}]$. The ratio of the number of PIGF mRNA copies to the number of GAPDH mRNA copies was then calculated as $2^{-\Delta C_T} \times K$, where K is a constant. Data are expressed as mean ± SD.

Abbreviation: HBV, hepatitis B; HCV, hepatitis C.

[&],[#] $P < 0.001$ by Student's t -test.

3.6. PIGF protein levels correlated with mRNA levels

PIGF protein levels correlate well with mRNA expressions levels from the same tissue (Fig. 4).

3.7. Correlation between expression levels of PIGF, VEGF-A and VEGF-C

When scatter plots of VEGF-A, VEGF-C, and PIGF expression levels were created, a positive correlation was observed between expression levels of VEGF-A and VEGF-C (Fig. 5A). No significant correlation was observed between expression levels of PIGF and either VEGF in tumor tissues (Fig. 5B and C).

4. Discussion

The study reported herein is the first to quantify PIGF expression in human liver tumor tissue samples from HCC patients in order to evaluate the relationship between PIGF expression and post-operative early recurrence of HCC.

PIGF has been shown to be a key molecule in the angiogenic switch in pathological conditions, including cancer [20]. Studies using epidermal tumor cell lines suggest that tumor development occurred in a strong angiogenesis-prone scenario in which PIGF and Ang-2 acted cooperatively with VEGF in a ras-dependent manner [21]. Antibody against PIGF receptor Flt-1 can suppress tumor neovascularization [22] and inhibits the growth and metastasis of tumors [23].

Angiogenesis is particular important in the clinical management of HCC because HCC is characterized by its high degree of neovascularization [24], high recurrence rate, and frequent blood-born metastases [25]. The importance of angiogenic activity in the progression and metastasis of early and advanced HCC, however, may be variable [26]. For example, angiogenic activity in the expanding edge of HCC may be required for a rapid phase of radial growth. With the advancement of the disease, however, HCC becomes arterialized; microvessel formation may diminish as the arterial vasculature becomes better established with the progression of HCC. As a result, angiogenic factors may also play variable roles in establishing the prognosis of HCC in different stages of disease.

In the present study, we demonstrated that higher PIGF expression levels in HCC tissue correlated with a poorer prognosis. In addition, the correlation between PIGF and prognosis was even more significant in AJCC stage II–III tumors, compared to AJCC stage I patients. A possible explanation is that advanced tumors which continue to produce PIGF may continue to

Fig. 1. Recurrence-free survival curves of all 71 HCC patients. (A) Survival curves for patients with placenta growth factor (PIGF) expression levels higher or lower than the median value; (B) Survival curves for patients with vascular endothelial growth factor A (VEGF-A) expression levels higher or lower than the median value and (C) Survival curves for patients with vascular endothelial growth factor C (VEGF-C) expression levels higher or lower than the median value.

Fig. 2. Recurrence-free survival curves in HCC patients. (A1) Survival curves for the AJCC stage II–III disease group with PIGF expression levels higher or lower than the median value. (A2) Survival curves for the AJCC stage I disease group with placental growth factor (PIGF) expression levels higher or lower than the median value. (B1) Survival curves for the AJCC stage II–III disease group with vascular endothelial growth factor-A (VEGF-A) expression levels higher or lower than the median value. (B2) Survival curves for the AJCC stage I disease group with VEGF-A expression levels higher or lower than the median value. (C1) Survival curves for the AJCC stage II–III disease group with VEGF-C expression levels higher or lower than the median value. (C2) Survival curves for the AJCC stage I disease group with VEGF-C expression levels higher or lower than the median value.

Fig. 3. Correlation between expression levels of vascular endothelial growth factor (VEGF), placental growth factor (PIGF) and Intratumorous microvessel density (MVD). (A) scatter plot of the expression levels of PIGF versus intratumorous microvessel density MVD; (B) scatter plot of the expression levels of VEGF-A versus MVD; (C) scatter plot of the expression levels of VEGF-C versus MVD; (A1) scatter plot of the expression levels of PIGF versus MVD in tumors >5 cm diameter; (A2) scatter plot of the expression levels of PIGF versus MVD in tumors ≤5 cm diameter.

expand and contribute to the metastasis of HCC through either induction of angiogenesis [27] or by enhancing survival of cancer cells [28].

Early recurrence-free survival was high (91.8% in our study) in stage I HCC patients after curative resection. Thus, the requirement of adjuvant

Fig. 4. Correlation between mRNA expression and protein levels of PIGF in tumor tissue.

treatment may be higher for patients with more advanced HCC. These results suggest that PIGF expression levels may be an important prognostic marker for stage II–III HCC patients after complete tumor resection in order to select patients in need of postoperative adjuvant therapy.

It has been well established that PIGF is up-regulated in inflammatory conditions. In the present study, the mean expression level of PIGF was higher in non-tumor tissue of patients with chronic active hepatitis than in patients without chronic active hepatitis. No correlation of PIGF expression between the tumor tissue and the patient-matched non-tumor section was identified. In addition, it was noted that patients with advanced HCC (but not in patients with early stage HCC) higher PIGF expression level correlated with more angiogenetic activity (higher MVD), and poorer disease free survival indicating that PIGF may not be involve in the early development and growth of HCC. On the contrary, PIGF may play an important role in the progression and metastasis of advanced HCC irrespective of the background liver disease. We suggest that PIGF may be an important therapeutic target in this group of patients.

In colon cancer tissues, a positive correlation was observed between expression levels of PIGF and VEGF ($r = 0.747$, $P < 0.001$) [16]. This phenomenon, however, was not observed in the HCC patients included in the present study. It was reported that VEGF up-regulates PIGF expression via the mitogen-activated protein kinase signaling pathway and partially via protein kinase C [29]. In Addition, exogenous erythropoietin also increased production and secretion of PIGF from some pediatric tumor

cells [30]. It was recently discovered that PIGF switches on its own genetic program of angiogenic genes, separate to that of VEGF [31]. Based on the results reported here, we suggest that other factors may influence the expression of PIGF expression in HCC tissue in addition to VEGF.

In this study, a positive correlation was identified between expression levels of VEGF-A and VEGF-C ($r = 0.334$, $P = 0.004$). Both VEGF-A and VEGF-C mRNA expression were significantly higher in the more poorly differentiated HCC (Table 2). VEGF-A and VEGF-C expression and production has been reported in HCC cells, and the expression of both factors may be up-regulated in inflammatory conditions [1,8]. Significantly stronger VEGF-C immunohistochemical staining was reported in poorly differentiated HCC compared to well or moderately differentiated HCC [8]. Recently, Wada et al. reported that VEGF expression correlated positively with MVD, and MVD of well-differentiated HCC were significantly lower than those of moderately and poorly differentiated HCC [32]. An immunohistochemical study of VEGF receptors (VEGFR1 or VEGFR2) distribution in HCC tissue demonstrated that the expression pattern of VEGF receptors influences the histological differentiation of HCC [33]. These results suggested that VEGF-A and VEGF-C expression may be induced by inflammatory cytokines simultaneously and that they may be cooperatively involved in tumor angiogenesis of more poorly differentiated HCC via binding with different VEGF receptors.

As HCC tumor size increased, portal tracts decreased in number, and artery-like vessels gradually increased in both number and size.

Fig. 5. Correlation between expression levels of angiogenic factors. (A) scatter plot of the expression levels of vascular endothelial growth factor-A (VEGF-A) versus VEGF-C; (B) scatter plot of the expression levels of placental growth factor (PlGF) versus VEGF-A and (C) scatter plot of the expression levels of PlGF versus VEGF-C.

Hypervascularity became easily visible when nodules were >2 cm in diameter. However, with increasing tumor size, VEGF positivity may gradually decrease. According to Yamaguchi et al., 36.8% of

nodules >3.0 cm were VEGF-negative [1]. El-Assal et al. reported that, HCC >5 cm in diameter were less vascular than smaller or medium-sized lesions [34]. These results indicate that VEGF may be less

important in the angiogenesis of advanced HCC. Other angiogenesis factors may play important roles in the angiogenesis and metastasis in this kind of HCC. This supposition was supported in the present study in that we reported that PlGF not VEGF expression levels correlated significantly with early recurrence of HCC, especially in patients with advanced disease.

Previous reports also demonstrated that loss of PlGF activity impaired angiogenesis in pathological conditions such as ischaemia, inflammation, and cancer [22,35,36]. From these results, one might presume that PlGF can influence the angiogenesis of advanced HCC thereby leading to early recurrence following surgical resection of HCC.

In summary, this study clearly demonstrated that PlGF expression levels in tumor tissues are correlated with the early recurrence of HCC, particularly in patients with AJCC stage II–III tumors. Therefore, PlGF may be used as a postoperative prognostic indicator and may guide selection and administration of adjuvant therapy in HCC patients following the surgical resection of HCC.

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