Placenta growth factor expression is correlated with survival of patients with colorectal cancer

S-C Wei, P-N Tsao, S-C Yu, C-T Shun, J-J Tsai-Wu, C H H Wu, Y-N Su, F-J Hsieh, J-M Wong

Background: Overexpression of vascular endothelial growth factor (VEGF) correlates with vascularity, metastasis, and proliferation in colorectal cancer but the role of its homologue, placenta growth factor (PlGF), is unknown. The aim of this study was to evaluate expression and clinical implications of PlGF in colorectal cancer.

Methods: We investigated 74 tumour/non-tumour pairs of colorectal cryosections. Clinical staging was based on the UICC-TNM classification. Expression levels of mRNA for PlGF and VEGF were analysed with quantitative real time reverse transcription-polymerase chain reaction. Proteins were analysed by immunohistochemical staining and enzyme linked immunosorbent assay. Analysis of the differences in PlGF and VEGF levels between tumour and non-tumour tissues in the same patient were performed by paired t test; differences between localised and advanced disease patients by the Mann-Whitney, χ², and Fisher’s exact tests and survival curves by the Kaplan-Meier method.

Results: Expression levels for both growth factors were significantly higher in tumour than in non-tumour tissues (p < 0.001). The ratio of PlGF expression in tumour to non-tumour in the advanced disease group was significantly higher than for the localised disease group (p = 0.009). Patients with more tumour PlGF mRNA had shorter survival (p = 0.028). The majority of PlGF was expressed in tumour cells.

Conclusions: Our results suggest that PlGF expression correlates with disease progression and patient survival and may be used as a prognostic indicator for colorectal cancer.

Materials and Methods

Materials
 Seventy four tumour and non-tumour pairs of colorectal cryosections were included from samples collected from September 2000 to June 2003, following the rules set forth by the ethics committee of the National Taiwan University Hospital. All tissues were freshly frozen or immersed in OCT, and kept at −80°C until use. Expression levels of PlGF or VEGF were analysed by quantitative reverse transcription-polymerase chain reaction (RT-PCR) and the ratio of expression levels in the tumour tissue to those in non-tumour tissue was calculated. Clinical staging of cancers was determined based on the UICC-TNM classification. All patients received identical treatment programmes in this study. No preoperation chemotherapy or radiotherapy had been given while stage III and IV patients were subjected to postoperative chemotherapy with 5-fluorouracil and

Abbreviations: VEGF, vascular endothelial growth factor; PlGF, placenta growth factor; LCM, laser capture microdissection; PBS, phosphate buffered saline; ELISA, enzyme linked immunosorbent assay.

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A
ngiogenesis, the process of new blood vessel formation, is a critical step in tumour growth and progression and is regulated by tumour cell derived growth factors that act specifically on vascular endothelial cells.1 It has been shown that rapid exponential growth of tumours does not begin until neovascularisation occurs. Many angiogenic factors have been identified that regulate angiogenesis in colon cancer. Vascular endothelial growth factor (VEGF) has been shown to be upregulated in the progression from non-metastatic to metastatic colon cancers.2–7 Shiraishi et al showed that levels of VEGF were highest in carcinomas followed by those in adenomas, with the lowest in non-neoplastic mucosa.8 Takahashi et al demonstrated that expression levels of VEGF and vessel counts in primary metastatic tumours were higher than those in non-metastatic neoplasms and these two factors directly correlated with the extent of neovascularisation and degree of proliferation.3

Furthermore, high levels of VEGF expression and elevated vessel counts were found to correlate with recurrence and metastasis of colon cancer, suggesting that VEGF expression could serve as an indicator of colorectal cancer prognosis. However, most studies showed that VEGF expression is not a good predictor of prognosis.3,12,13 Placenta growth factor (PlGF), a dimeric glycoprotein with 53% homology to VEGF,10,11 binds to VEGF receptor 1, but not to VEGF receptor 2, and may function by modulating VEGF activity.12 Exogenous PlGF stimulates angiogenesis and induces vascular permeability when co-injected with VEGF.13–15 The angiogenic activity of PlGF may be initiated by displacement of VEGF from the VEGF receptor 1 sink, thus increasing the VEGF available for activation of VEGF receptor 2.11,15 Absence of PlGF has had no significant effect on vascular development and normal embryogenesis, as demonstrated in PlGF knockout mice, but such a deficiency could reduce collateral vascular growth under pathological conditions, such as ischaemia, inflammation, and cancer.15

PIGF expression has been reported in hypervascular renal cell carcinomas and in some thyroid and germ cell tumours.16 Donnini et al demonstrated that PlGF was highly upregulated in a subset of human meningiomas.17 Recently, Adini et al found that overexpression of PlGF leads to tumour growth as well as vascular formation, induces expression of survival genes, and inhibits apoptosis in vitro. They proposed that PlGF contributes to tumour angiogenesis by providing functions needed for endothelial cell survival.18 However, the correlations between PlGF, angiogenesis, metastasis, and prognosis in colon cancer are not clear. The goal of our study was to evaluate correlations among these factors.

MATERIALS AND METHODS

Materials
 Seventy four tumour and non-tumour pairs of colorectal cryosections were included from samples collected from September 2000 to June 2003, following the rules set forth by the ethics committee of the National Taiwan University Hospital. All tissues were freshly frozen or immersed in OCT, and kept at −80°C until use. Expression levels of PlGF or VEGF were analysed by quantitative reverse transcription-polymerase chain reaction (RT-PCR) and the ratio of expression levels in the tumour tissue to those in non-tumour tissue was calculated. Clinical staging of cancers was determined based on the UICC-TNM classification. All patients received identical treatment programmes in this study. No preoperation chemotherapy or radiotherapy had been given while stage III and IV patients were subjected to postoperative chemotherapy with 5-fluorouracil and
leucovorin. Stages I and II were collectively termed as the localised disease group and stages III and IV as the advanced disease group in this study. Follow up duration was defined as the period between the operation date and day of the last visit, according to the patient’s chart.

Quantitative RT-PCR

Total RNA was isolated using an RNA extraction kit (Qiagen Inc., California, USA) from tissue homogenised with Trizol (Invitrogen Inc., Carlsbad, California, USA), according to the manufacturer’s instructions. For confirming expression levels of PlGF in tumour tissue, cells were collected from 15 stage III/IV patients by laser capture microdissection (LCM), as previously reported. Briefly, 5 mm sections tissue were mounted on silicon coated slides. Slides were cleared in xylene and dehydrated in graded alcohols. After staining with haematoxylin-eosin, LCM using a PixCell I system (Arcturus Engineering, Mountain View, California, USA) was used to separate tumours and normal cells. LCM parameters included a laser power of 90 mW, laser pulse duration of 1.2 ms, and laser spot size of 7.5–15 μm in diameter. A cap devised by Arcturus Engineering was used for the transfer film. RNA was extracted and isolated using the PicoPure RNA Isolation kit (Arcturus Engineering) according to the manufacturer’s instructions.

Primers and probes were designed using the Primer Express program (Perkin-Elmer Applied Biosystems, California, USA) and purchased from the same vendor. The primers used, based on the cDNA sequence of PlGF, were as follows: forward primer 5'-GGG AAT GGT GGG CGT TTG TTG T-3'; reverse primer 5'-TCC CCA GAA CGG ATC TTT AGT TTG-3'. The sequence of the probe used to detect and quantify the RT-PCR product was 5'-AGA CCG CCA ATG TCA CCA TGC AGC-3'. The primers and probe used for VEGF mRNA were as follows: forward primer 5'-TAC CTC CAT GCC AGA AGT TG-3'; reverse primer 5'-GAT GAT GCC CTC TCT CTC-3'; probe 5'-TCC CAG GCT CCA CCC ATG GC-3'. The primers and probe used for the GAPDH (internal control) mRNA were as follows: forward primer 5'-AGA GGA GTG GAA GGT CGG AGT-3'; reverse primer 5'-GAA GTG GAT GGT GAT GGG ATT TC-3'; probe 5'-CAG CTA CCA GCC TCG ATC CAG C-3'.

Samples to produce the standard curve for real time RT-PCR were prepared by serial dilution of a specific RNA sample to cover the range 100 ng to 0.1 ng. Aliquots of the serially diluted samples were saved and stored at –80°C until use. Expression levels of the target gene were measured using quantitative real time RT-PCR in the ABI Prism 7700 Sequence Detection System (Applied Biosystems) following the manufacturer’s protocol. The amplification mixture (25 μl) contained 10 ng of sample RNA (5 μl), 2× Master Mix without UNG (12.5 μl), 40× MultiScribe and RNase inhibitor mix (0.625 μl), 9 μM forward and reverse primers (5 μl), and 2 μM probe (2.5 μl) (Perkin-Elmer Applied Biosystems). Thermal cycling parameters were as follows: one cycle of 30 minutes at 48°C, one cycle of 10 minutes at 95°C for deactivation, and 40 cycles at 95°C for 15 seconds and at 60°C for one minute for the melting, annealing, and extending phases of the PCR reaction, respectively. Each assay included standard curve samples in duplicate, a no template control, and approximately 10 ng of sample total RNA in triplicate. Any sample with a coefficient of variation above 10% was retested. Fluorescence emitted by the reporter dye (FAM-6-carboxy-fluorescein, fluorescence emission peak at 518 nm) was detected online in real time with the ABI Prism 7700 Sequence Detection System. Threshold cycle (Ct) is the fractional cycle number at which the fluorescence generated by cleavage of the probe exceeds a fixed level above baseline. For a chosen threshold, a smaller starting copy number results in a higher Ct value. In this study, we chose GAPDH mRNA as an internal control. The relative amount of tissue PlGF mRNA, standardised against the amount of GAPDH mRNA, was expressed as $-\Delta\Delta CT = - [CT_{PlGF} - CT_{GAPDH}]$. The ratio of the number of PlGF mRNA copies to the number of GAPDH mRNA copies was then calculated as $2^{\Delta CT} \times K$, where K is a constant.

Immunohistochemical staining

Serial sections of frozen colon tissues were mounted on poly-L-lysine coated slides and allowed to dry for 30 minutes before fixation in 100% acetone for 15 minutes. Tissue sections were washed for five minutes, three times, in phosphate buffered saline (PBS), immersed in 2% H2O2 in methanol for 25 minutes to inactivate endogenous peroxidase, and blocked with 1% bovine serum albumin. Blocked sections were incubated with antibodies against PlGF, VEGF, or Flt-1 (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) at dilutions of 1:25, 1:50, and 1:25, respectively, for two hours at room temperature, washed with PBS, reacted with biotinylated secondary antibody with anti-goat antibody (dilution of 1:500; Santa Cruz Biotechnology, Inc.) for PlGF, and antirabbit antibody (dilution of 1:500; Vector Laboratories Inc, Burlingame, California, USA) for VEGF and Flt-1 for one hour, followed by incubation with streptavidin conjugated horseradish peroxidase (peroxidase substrate kit, DAB; Vector Laboratories Inc.) which was used to reveal localisation of the antibodies, and tissues were counterstained with Mayer’s haematoxylin.

ELISA (enzyme linked immunoabsorbant assay)

Protein lysate from each specimen was prepared using 10 mg tissue cut into tiny pieces, suspended in cell lysis buffer (0.15 M NaCl; 0.1 M Tris, pH 8.0; 1 mM EDTA, pH 8.0; 1 mM PMSF) and mechanically homogenised with a polytron PT 3000 (30 000 rpm for one minute). Protein lysate was kept at –80°C until use. Concentrations of PI GF in tumour and non-tumour tissues were quantified using a “Quantikine” human PI GF immunoassay (R&D Systems, Inc., Minneapolis, Minnesota, USA). Diluted protein lysate was incubated in triplicates overnight at 4°C on microtitre plates coated with a murine monoclonal antibody against human PI GF. Unbound proteins were washed off, and an enzyme linked polyclonal antibody specific for PI GF was added to “sandwich” the PI GF immobilised during the first incubation. A substrate solution for horseradish peroxidase was added, and colour developed in proportion to the amount of antibody bound PI GF. Absorbance of the colour was read at 450 nm. A standard curve, consisting of known amounts of PI GF, was carried through the above procedure, and concentrations of PI GF in the unknown samples were determined from this standard curve. Concentrations of PI GF were expressed as pg/mg of protein.

Data analysis

Differences in PI GF and VEGF mRNA levels, and PI GF protein levels between tumour and non-tumour tissues in the same patient were analysed using a paired t test while differences between localised disease and advanced disease patients were analysed using the Mann-Whitney, χ2, and Fisher’s exact tests. Correlations between PI GF or VEGF expression levels and clinical stages, and PI GF expression levels between mRNA and protein, were analysed by Spearman’s correlation coefficient. A survival curve was obtained using the Kaplan-Meier method. Differences in cancer specific survival time for patients with tumours with high or low expression levels of PI GF or VEGF were analysed using the Breslow test.
RESULTS
Basic data
A total of 74 colorectal cancer patients, 39 males and 35 females, were included in the study (table 1). There were 14 rectal cancers and 60 colon cancers. However, there were no significant differences in PlGF, VEGF expression levels (either tumour tissue or tumour to non-tumour tissue), age, sex, or cancer specific survival between the rectal and colon cancer groups. Age at first diagnosis ranged from 26 to 94 years (mean 68.4). Follow up for these 74 patients ranged from 1 to 47 months (mean 26.0). During follow up, 14 patients died of colorectal cancer. According to the UICC-TNM classification, there were 14 stage I patients, 27 stage II patients, 22 stage III patients, and 11 stage IV patients. Among these patients, there were three with synchronous colorectal cancers and six with metachronous colorectal cancers.

Both PlGF and VEGF mRNA expression were upregulated in colorectal cancer and the extent of upregulation correlated with disease progression
Expression levels of PlGF and VEGF in tumour tissues were higher than in non-tumour tissues (p < 0.001 and p = 0.001, respectively). In addition, in 15 patients, RNA extracted from tumour cells collected by LCM also confirmed the finding that expression levels of PlGF in tumour cells were higher than in non-tumour cells. Mean (SEM) –ΔCT for tumour tissue was −3.22 (0.35) compared with −0.25 (0.23) for non-tumour tissue (p < 0.001). Tumour to non-tumour ratios for PlGF and VEGF levels were much higher for the three differentiated tumours and there were no statistically significant differences in PlGF or VEGF expression among these three groups.

PIGF, but not VEGF, mRNA expression is a prognostic marker for colorectal cancer
In this study, –ΔCT value of PlGF for the tumour samples ranged from −6.56 to 4.58, with a median value of 0.87, whereas values for VEGF ranged from −2.72 to 4.34 (median 0.79). Patients were divided into low and high expression groups, based on whether they were above or below the median value. Patients in the high PlGF mRNA expression group were more likely to have advanced disease than those in the low expression group (stage IV, p = 0.047) (table 1). The probability of cancer specific survival was significantly lower in the high PlGF mRNA expression group (mean survival 35.8 months (95% confidence interval (CI) 30.5–41.1) than that of the low PlGF mRNA expression group (mean survival 43.3 months (95% CI 39.8–46.7); Breslow test, p = 0.040) (fig 2A). In contrast, the difference between the probabilities of cancer specific survival in the high VEGF mRNA expression group and the low VEGF mRNA expression group was not statistically significant (p = 0.41) (fig 2B).

<table>
<thead>
<tr>
<th>Localised disease</th>
<th>Advanced disease</th>
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<tr>
<td>Stage I (n = 14)</td>
<td>Stage II (n = 27)</td>
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<tr>
<td>Sex</td>
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<td>Male</td>
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<td>Female</td>
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<td>Age (y)</td>
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<td>Follow up period (months)</td>
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<td>Survival†</td>
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<td>Yes</td>
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<td>No</td>
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<td>–ΔCT of PlGF</td>
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<td>T/NT ratio*</td>
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†Age and follow up period are presented as means (SEM).
*Comparison of localised and advanced disease groups. Sex and survival were analysed using the χ² method and Fisher’s exact test; age, follow up period, –ΔCT of PlGF, and –ΔCT of VEGF were analysed using the Mann-Whitney test.
*p<0.05.
Placenta growth factor predicts colon cancer

For further comparison, patients with stages I and II were categorised as the localised disease group and patients with stages III and IV as the advanced disease group. Table 1 also shows that PlGF expression levels, the ratio between PlGF levels in tumour tissues and PlGF levels in non-tumour tissues, and cancer specific survival were significantly different between the localised disease and advanced disease groups (p = 0.013 and 0.001, respectively). Differences in sex, age, follow up period, VEGF expression, and ratio between VEGF levels in tumour tissues and VEGF levels in non-tumour tissues were not statistically significant. As deaths were mostly observed for the advanced disease group, we tried to determine whether there was any independent factor for predicting outcome in these advanced disease patients. We found that there was a trend for PlGF expression as a predictor of survival as the probability of survival was lower in the high PlGF mRNA expression group (mean survival 30.7 months) than in the low PlGF mRNA expression group (mean survival 36.5 months; Breslow test, p = 0.12) (fig 2C). However, the difference was not significant due to the small sample size and short follow up period.

PIGF, VEGF, and Flt-1 localisation in colorectal cancer

Immunohistochemical staining analysis (fig 3) showed that both PIGF and VEGF were expressed mainly in tumour cells. In contrast, Flt-1, the receptor for PIGF and VEGF, was expressed in both tumour cells as well as in endothelial cells.
PIGF protein levels correlated with mRNA levels
Protein levels of PIGF in tumour tissues ranged from 26.6 to 2498.4 pg/mg (mean (SEM) 281.3 (38.8) pg/mg). In corresponding non-tumour tissues, protein levels of PIGF ranged from 26.6 to 358.4 pg/mg (mean (SEM) 128.7 (8.5) pg/mg). PIGF protein levels in tumours were significantly higher than those of corresponding non-tumour tissues (p<0.001) (fig 4A). PIGF protein expression correlated well with mRNA from the same tissue (r = 0.308, p<0.001) (fig 4B).

DISCUSSION
Angiogenesis is required for tumour growth and metastasis.20–22 Recently, PIGF has been shown to be a key molecule in the angiogenic switch under pathological conditions, including cancer.15 In the present study, we demonstrated that PIGF expression levels were significantly higher in tumour tissues compared with non-tumour tissues. The ratio between PIGF levels in tumour tissues and PIGF levels in the non-tumour tissue correlated well with the stage of colon cancer. Furthermore, higher PIGF expression in the tumour, as well as a higher ratio of PIGF levels in tumour tissues to PIGF levels in non-tumour tissues, correlated with a poorer prognosis. It has been proposed that PIGF stimulates angiogenesis through displacement of VEGF from the Flt-1 sink, thereby increasing the fraction of VEGF available for activation of Flk-1.13–15 Alternatively, PIGF can modulate the function of VEGF by regulating intermolecular and intramolecular crosstalk between Flt-1 and Flk-1.23 Moreover, PIGF alone can trigger its own signalling, independent of the VEGF/Flk-1 pathway, and can trigger Flt-1 dependent functions such as proliferation, apoptosis, and angiogenesis.23 In addition, loss of PIGF activity impaired angiogenesis in pathological conditions such as ischaemia, inflammation, and cancer.15 These results suggest that PIGF may be a key molecule in regulating the angiogenic switch under pathological conditions. These data are consistent with our results that PIGF expression levels are significantly higher in tumour tissues than in non-tumour tissues and that the ratio between PIGF levels in tumour tissues and PIGF levels in non-tumour tissues correlates with colon cancer stage.

Several studies have demonstrated that VEGF and PIGF express correlation with tumour growth and angiogenesis.24–37 In our study, we showed that expression levels of PIGF and VEGF in tumour tissues were higher than in non-tumour tissues of the same colorectal cancer patients. These results suggest that both PIGF and VEGF may play roles in colorectal cancer formation and tumour progression, consistent with the hypothesis of Carmeliet et al that PIGF exerts a synergistic effect on VEGF driven angiogenesis under pathological conditions.25 VEGF stimulates growth of new vessels that are fragile, leaky, and prone to regression. PIGF can recruit smooth muscle cells/precursors, and thereby stimulate vessel maturation and stabilisation.24–25 Therefore, PIGF and VEGF should be upregulated in parallel during angiogenesis to stimulate the formation of mature non-leaky vessels. PIGF expression could be regulated by oxygen tension and cytokines.26 However, the mechanism of increased PIGF expression in colorectal cancer remains unclear.

Elevated expression of VEGF in tumour tissues of colorectal cancer patients was observed in many studies. There was generally no correlation between elevated VEGF expression and patient survival,24–25 with only one exception, that of Lee et al who reported that VEGF correlated with survival. However, it did not emerge as an independent risk factor in a multivariate analysis.24 Our studies also showed that VEGF expression increased in tumour tissues but did not correlate with survival. In contrast, PIGF expression levels not only increased in colorectal cancer but also correlated with survival. This indicates that PIGF, in addition to its synergistic effect on VEGF driven angiogenesis, may have its own distinct effect on colorectal cancer. The clinical implication is that PIGF may be useful as a prognostic indicator, especially important in TNM stage III and IV patients. For stage III and IV patients with low PIGF levels, aggressive treatment is still recommended.

Figure 3 Immunohistochemical stain showed a positive placenta growth factor (PIGF) (A) and vascular endothelial growth factor (VEGF) (B) reaction, mainly in tumour cells, while Flt-1 (C) protein was expressed in both tumour cells and endothelial cells. A–C, 200 x; stage III; arrows, tumour cells; arrowhead, endothelial cell.

Figure 4 (A) Protein levels of placenta growth factor (PIGF) were significantly higher in colorectal tumour tissues than in corresponding non-tumour tissues, measured by an ELISA method. (B) PIGF protein expression levels were significantly correlated with mRNA expression levels.
Immunochemical localisation studies showed that PlGF, VEGF, and their receptor, Flt-1, were all localised in tumour cells. We hypothesised that colon cancer cells secrete PlGF as well as VEGF and that both factors serve paracrine and autocrine functions in stimulating angiogenesis as well as cancer growth and metastasis.

Several clinical efforts are currently underway to evaluate the therapeutic potential of inhibitors of VEGF or VEGFR-2. In recent studies, the VEGF specific antibody bevacizumab prolonged survival of colorectal cancer patients and had a direct and rapid antivascular effect in human rectal cancer.29 However, VEGF and VEGFR-2 are not only involved in pathological angiogenesis but also in normal vessel growth and maintenance.29 Furthermore, VEGF has a direct effect on motor neurone survival,30 lung maturation,31 liver regeneration,32 control of blood pressure,33 and glomerular development.34 This raises the question of whether long term inhibition of VEGF may also affect these processes. PlGF, in contrast with VEGF, affects blood vessel formation only under pathological and not under physiological conditions,35 except during normal physiological angiogenesis in the placenta.36 37 Our results showed that PlGF, but not VEGF, was significantly upregulated in colorectal cancer and correlated with survival. Thus PlGF may be a safer therapeutic target for colorectal cancer.

In summary, we demonstrated that both PlGF and VEGF expression levels in tumour tissues are higher than in non-tumour tissues in patients with colorectal cancer. The ratio of PlGF levels in tumour tissues to PlGF levels in non-tumour tissues, but not the analogous ratio for VEGF, correlated with stage of colorectal cancer. We conclude that PlGF expression correlates with disease progression and survival status, and may be used as a prognostic indicator for colorectal cancer.

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Conflict of interest: None declared.

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REFERENCE

Liver failure after delivery

Clinical presentation
A 32 year old, gravida 2, para 2, was admitted at 36 weeks' gestation with upper abdominal pain. During her second pregnancy she frequently had epistaxis. At 39 weeks' gestation she delivered a healthy girl after an elective caesarean section which was complicated by a total blood loss of 1.5 litres. Progressive liver failure and shock led to her transport to our hospital one day post partum. She had no history of alcohol abuse, viral hepatitis, and was not receiving any medications.

Continuing abdominal bleeding was suspected and at laparotomy 5 litres of blood were found in the abdominal cavity from sites of recently lysed adhesions. On day 4 post partum, liver failure worsened with grade IV encephalopathy necessary to perform an orthotopic liver transplantation. The procedure was complicated by severe intraoperative bleeding (30 litres). A section of the explanted liver is illustrated in fig 1. Postoperative follow up was complicated by intra-abdominal infections and steroid refractory chronic rejection leading to failure of the graft. She was successfully retransplanted four months later and is currently doing well.

Question
What was the mechanism of liver failure in this patient?
See page 709 for answer
This case is submitted by:

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Figure 1
Placenta growth factor expression is correlated with survival of patients with colorectal cancer

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