ELSEVIER

European Journal of Cancer 41 (2005) 2819–2827

European Journal of Cancer

www.ejconline.com

Placenta growth factor is over-expressed and has prognostic value in human breast cancer

Christian Parr [a,*], Gareth Watkins [a], Mike Boulton [b], Jun Cai [b], Wen G. Jiang [a]

[a] *Metastasis and Angiogenesis Research Group, Department of Surgery, Wales College of Medicine, Cardiff University, Heath Park, Cardiff CF14 4XN, UK*

[b] *Cell and Molecular Biology Unit, School of Optometry and Vision Sciences, Redwood Building, King Edward VII Ave., Cardiff University, Cardiff, CF10 3NB, UK*

Received 11 March 2005; received in revised form 19 July 2005; accepted 28 July 2005
Available online 4 November 2005

Abstract

Placenta growth factor (PlGF) belongs to the vascular endothelial growth factor (VEGF) family, a group of angiogenic factors that are crucial for tumour angiogenesis. Very little is known about the significance of PlGF in human cancer. We hypothesise that PlGF may have a potent influence in breast cancer. This study examined PlGF levels in human breast cancer in relation to patient's clinical parameters. PlGF expression and distribution was examined quantitatively using real-time quantitative polymerase chain reaction (Q-PCR) on a cohort of human breast cancer tissue ($n = 119$) and background breast tissue ($n = 33$), qualitatively using reverse transcriptase polymerase chain reaction (RT-PCR) on a range of cell lines, and immunohistochemically on patient samples. All these techniques revealed that PlGF expression was dramatically increased ($P = 0.028$) in breast cancer tissues compared with normal breast tissue. We demonstrate that PlGF displays prognostic value through analysis of patient survival status (6-year follow-up), as elevated levels of PlGF were significantly associated ($P = 0.017$) with recurrence, metastasis and patient mortality. Our study has shown that PlGF is over-expressed in breast cancer tissues and correlates with patient prognosis, and is likely to play a major role in the pathogenesis of tumours.
© 2005 Elsevier Ltd. All rights reserved.

Keywords: Placenta growth factor; Vascular endothelial growth factor; Vascular endothelial growth factor receptor-1; Angiogenesis; Breast cancer; Prognosis; Real-time quantitative polymerase chain reaction; Immunohistochemistry

1. Introduction

Angiogenesis is an essential component of tumour development [1] and is tightly regulated by a network of pro-angiogenic and anti-angiogenic factors [2]. The tumour itself is known to produce many of the proteins that drive the angiogenic process. Of the known angiogenic factors, vascular endothelial growth factor (VEGF) has been established as a potent inducer of tumour angiogenesis that can activate both endothelial cell proliferation and migration [3,4]. VEGF has been shown to exert a pro-angiogenic influence in a variety of human tumours [5–8], and has been shown to act as an independent prognostic indicator for breast cancer patients [9,10]. Placenta growth factor (PlGF) belongs to the VEGF family and was originally identified from a term placenta cDNA library as a factor related to VEGF, with the ability to promote endothelial cell proliferation [11].

Presently, the biological role and signalling mechanisms mediating the cellular actions of PlGF remain poorly understood. VEGF is able to interact and induce signalling events through both the VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1) receptors, whereas PlGF only

* Corresponding author. Tel.: +44 29 2074 8394; fax: +44 29 2076 1623.
E-mail address: parrc@cf.ac.uk (C. Parr).

0959-8049/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2005.07.022

possesses the ability to bind to VEGFR-1 [12]. However, a recent study demonstrated that activation of VEGFR-1, by PlGF, also led to the intermolecular transphosphorylation of VEGFR-2 [13]. Importantly, PlGF-mediated responses via VEGFR-1 differed from VEGF-driven events, as PlGF uniquely stimulated the phosphorylation of specific VEGFR-1 tyrosine residues and expression of distinct downstream target genes [13]. Carmeliet and colleagues [14] and Luttun and colleagues [15] highlight the important role played by PlGF and VEGFR-1 in inducing the angiogenic switch in pathological conditions.

PlGF is not produced by the majority of normal human tissues [12]. However, PlGF can be found expressed in vascular endothelium, umbilical vein endothelial cells, trophoblasts, the eye, uterine natural killer cells and dentine matrix [16–21]. Studies on cancer cell lines and tumour tissues so far indicate that PlGF expression was upregulated in human gastric adenocarcinoma, renal cell cancer, melanoma, cervical squamous cell carcinoma and meningiomas, whereas PlGF was found to be downregulated in thyroid carcinoma and cervical adenocarcinoma [22–29].

To clarify the role of PlGF in human cancer, we examined the expression of PlGF, through real-time quantitative polymerase chain reaction (Q-PCR) and immunohistochemical studies, in a cohort of human breast cancer specimens. The degree to which PlGF was expressed in these breast tissues may correlate with clinical parameters and the prognosis of patients with breast cancer.

2. Materials and methods

2.1. Cell lines and culture

All cell lines used in this study were obtained from the European Collection for Animal Cell Culture (ECACC, Porton Down, Salisbury, United Kingdom (UK)) apart from the human prostate cancer cell lines that were purchased from the American Type Culture Collection (ATCC, Rockville, Maryland, United States of America (USA)).

This study used human prostate cancer cells (DU-145, PC-3, CA-HPV-10), human breast cancer cells (MDA-MB-157, MDA-MB-231, MDA-MB-435S, MDA-MB-436, MDA-MB-453, MCF7, BT474, BT549, ZR-75-1), human colorectal cancer cells (HRT-18, HT-115), human pancreatic cancer cells (MIA PACA-2), human bladder cancer cells (EJ-138, T-24), human melanoma cells (G-361), human lung carcinoma cells (A-549), human hepatocellular carcinoma cells (PLC-PRF-5), human fibroblast cells (MRC-5, human fibroblasts), a human epithelial cell line (ECV-304) and a human endothelial cell line (HECV).

Cells were routinely cultured with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum, penicillin and streptomycin (Gibco BRC, Paisley, UK).

2.2. Human breast specimens

A total of 152 breast samples were obtained from breast cancer patients (33 background normal breast tissue and 119 breast cancer tissue). These tissues were collected immediately after mastectomies, and snap-frozen in liquid nitrogen. Background normal mammary tissues were removed from the same patients. The pathologist verified normal background and cancer specimens, and it was confirmed that the background samples were free from tumour deposits. For patient clinical data see Table 1.

2.3. Preparation of total cellular RNA

Total cellular RNA was isolated from the homogenised breast samples and human cell lines using the AB Gene Total RNA Isolation Reagent (Advanced Biotechnologies Ltd, Epsom, Surrey, UK). The concentration of RNA was determined through spectrophotometric measurement (WPA UV 1101, Biotech Photometer, Cambridge, UK).

Table 1
Breast cancer patient clinical data details

Clinical data	Grouping	Sample number
Tissue sample	Background	33
	Tumour	119
Nottingham prognostic index	1	67
	2	37
	3	15
Tumour grade	1	23
	2	41
	3	55
Tumour node metastasis	1	68
	2	39
	3	8
	4	4
Survival status	Good prognosis	89
	Poor prognosis	30
Histological sub-types	Ductal	93
	Lobular	13
	Muscinous	2
	Medullary	2
	Tubular	2
	Others	7
ER status	Negative	71
	Positive	48

2.4. Reverse transcription polymerase chain reaction (RT-PCR)

cDNA was prepared using 0.5 μg of the RNA sample and a reverse transcription kit (Sigma, Poole, Dorset, UK). The quality of DNA was verified using β-actin. β-actin forward and reverse primers were 5′-ATGATATCGCCGCGCTCGTC-3′ and 5′-CGCTCGGTGAGGATCTTCA-3′, respectively, and gave products approximately 0.58 kb. PlGF forward and reverse primers were 5′-GGAGCTGACGTTCTCTCAG -3′ and 5′-ACTGAACCTGACCGTACAGTTACCTCCGGGGAACAG-3′. PCR was performed in a GeneAmp PCR system 2400 thermocycler (Perkin–Elmer, Norwalk CT, USA). Conditions for PCR were 40 s at 94 °C, 60 s at 55 °C, 60 s at 72 °C (30 cycles). PCR products were loaded onto a 2% agarose gel and electrophoretically separated. The gel was then visualised under ultraviolet light following ethidium bromide staining.

2.5. Real-time quantitative polymerase chain reaction

The iCycler IQ system (BioRad, Camberley, UK) was employed [30,31], to quantify the level (shown as copies/μl from internal standard) of PlGF in the breast specimens. Breast cDNA samples were then examined for PlGF expression using the primers described above, along with a set of standards and negative controls. The Q-PCR technique utilised the Amplifluor system (Intergen Inc, UK) and real-time quantitative polymerase chain reaction (Q-PCR) master mix (ABgene, Surrey, UK), in conjunction with a universal probe (UniPrimerTM). Real-time Q-PCR conditions were 95 °C for 12 min, followed by 65 cycles at 95 °C for 15 s, 55 °C for 60 s and 72 °C for 20 s. The results of the test molecules were normalised against levels of β-actin, using a β-actin quantitation kit from Perkin–Elmers (Perkin–Elmers, Surrey, UK). The epithelial content within the tumours was taken into account by normalising PlGF against cytokeratin19. CK19 forward and reverse primers were 5′-CAGGTCCGAGGTTACTGAC-3′ and 5′-ACTGAACCTGACCGTACACACTTTCTGCCAGTGTGTCTTC-3′, respectively.

2.6. Immunohistochemical staining of breast specimens

Frozen sections of breast tumour and background tissue were cut at a thickness of 6 μm using a cryostat. The sections were mounted on super frost plus microscope slides, air-dried and then fixed in a mixture of 50% acetone and 50% methanol. The sections were then placed in Optimax wash buffer (Menerium, Oxford, UK) for 5–10 min to rehydrate. Sections were incubated for 20 min in a 0.6% bovine serum albumen (BSA) blocking solution and probed with PlGF antibody, and without primary antibody as a negative control. Antibody to the PlGF epitope mapped to the C-terminus of human PlGF, and was purchased from Santa-Cruz Biotechnologies Inc. (Santa-Cruz, CA, USA). Following extensive washings, sections were incubated for 30 min in the secondary biotinylated antibody (Multilink Swine anti-goat/mouse/rabbit immunoglobulin, Dako Inc., Glostrup, Denmark). Following washings, the Avidin Biotin Complex (Vector Laboratories, Peterborough, UK) was then applied to the sections, followed by extensive washing steps. Diamino-benzidine chromogen (Vector Labs, Peterborough, UK) was then added to the sections, and incubated in the dark for 5 min. Sections were then counter stained in Gill's haematoxylin and dehydrated in ascending grades of methanol before clearing in xylene and mounting under a cover slip (staining was independently assessed by the authors).

2.7. Wounding/migration assay

The migration of transformed human endothelial cells (HECV) cells across a wounded surface was examined under different treatment combinations, and has been described in a previous study [32]. Cells at a density of 50 000/well were seeded into a 24-well plate and allowed to reach confluence. The layer of cells was then scraped with a fine gauge needle to create a wound of approximately 200 μm. Treatments consisted of recombinant human PlGF (30 ng/ml) (R&D systems, Abingdon, UK), or a combination of recombinant PlGF and anti-VEGFR-1 (120 ng/ml) (Santa Cruz, CA, USA). For details of treatment concentrations and ratios see previous studies [33]. The movement of cells to close the wound was recorded and analysed as described previously using a time-lapsed video system [32]. After the addition of a treatment, the cells motile qualities were monitored and recorded on video for 100 min. Wound closure/cell migration was evaluated with motion analysis software (Optimus 6) and results were exported to a spreadsheet (Excel) for further evaluation and interpretation.

2.8. Growth assay

HECV were seeded in a 96-well plate at a density of 7000 cells/well, and allowed to adhere overnight. Following this incubation period, the treatments of PlGF (30 ng/ml) and a combination of PlGF and anti-VEGFR-1 (120 ng/ml) were added and incubated at 37 °C for 72 h. A solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added to the cells (200 μg/well) and incubated for 4 h at 37 °C, then the cells were lysed with Triton (10%) and the intensity of the colour released was determined by a plate reader (Titertek Multiskan, Eflab, Finland). The number of cells was shown as absorbance units.

2.9. Statistical analysis

The results were assessed using non-paired (two-sided) Student's *t*-test. PlGF transcript values obtained in the study are given as mean copy number ±SD. A *P*-value <0.05 was defined as statistically significant.

3. Results

3.1. Screening of human cell lines for PlGF

A variety of 24 human normal and cancer cell lines were examined for PlGF expression through RT-PCR (Fig. 1 and Table 2). PlGF was expressed in the large majority of the human cancer cell lines examined. However, only 4 of the 9 breast cancer cell lines examined (numbers 1–9 in Fig. 1) revealed any detectable level of PlGF transcripts. These breast cancer cell lines displayed weak to moderate levels of PlGF. Of the remaining human cancer cell lines, PlGF was found to be strongly expressed in the prostate (numbers 10–12), colorectal (numbers 13 and 14), bladder (numbers 18 and 19) and melanoma (number 21). Human fibroblasts (numbers 15 and 16) revealed little or no detectable levels of PlGF. The normal epithelial and endothelial cell lines expressed a moderate to low level of PlGF.

Table 2
Summary of qualitative PlGF expression

No.	Cell line	Origin	PlGF
1	MDA-MB-157	Breast cancer	[a]
2	MDA-MB-231	Breast cancer	[b]
3	MDA-MB-436	Breast cancer	[a]
4	MDA-MB-453	Breast cancer	–
5	MDA-MB-43S5	Breast cancer	[b]
6	BT474	Breast cancer	–
7	BT549	Breast cancer	–
8	MCF 7	Breast cancer	–
9	ZR-75-1	Breast cancer	–
10	PC-3	Prostate cancer	[c]
11	DU-145	Prostate cancer	[c]
12	CA-HPV-10	Prostate cancer	[b]
13	HT-115	Colorectal cancer	[b]
14	HRT-18	Colorectal cancer	[b]
15	MRC5	Fibroblast	–
16	Human fibroblast	Fibroblast	[a]
17	MIA-PACA-2	Pancreatic cancer	[b]
18	EJ-138	Bladder cancer	[b]
19	T-24	Bladder cancer	[c]
20	A-549	Lung cancer	[b]
21	G-361	Melanoma	[c]
22	PLC-PRF-5	Liver cancer	[b]
23	ECV-304	Epithelial	[b]
24	HECV	Endothelial	[a]

–, not detected.
[a] Weak expression.
[b] Moderate expression.
[c] Strong expression.

3.2. Expression of PlGF in paired breast specimens (normal versus cancer)

We also performed conventional RT-PCR on a panel of paired breast tissues (*n*, normal; *t*, tumour). Both normal breast tissue and breast cancer tissue were found to express PlGF mRNA, albeit at minimal levels in normal breast specimens. Overall, PlGF mRNA was expressed to a higher degree in the breast cancer samples when compared with normal breast tissue (Fig. 2).

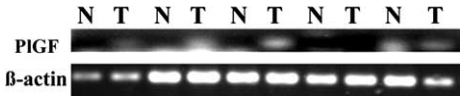

Fig. 2. Reverse transcriptase polymerase chain reaction (RT-PCR) on a panel of paired breast tissues (*n*, normal; *t*, tumour). PlGF expression was found to be expressed to a higher degree in the tumour specimens compared with the paired background breast tissue specimen. PlGF expression in normal breast tissue was minimal.

3.3. Immunohistochemical staining of human breast specimens

PlGF immunostaining was observed in the human breast tissue sections. We reveal that the PlGF protein level was dramatically elevated in the breast tumour specimens compared with the normal breast tissue. The patients' tumour tissues displayed a more intense and widespread level of staining in the breast cancer cells compared with the very weak staining of epithelial cells in the normal breast tissue (Fig. 3). These results confirm our RT-PCR and Q-PCR data in showing that breast cancer tissues have significantly enhanced PlGF levels.

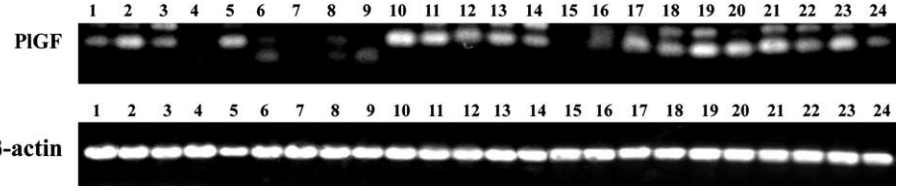

Fig. 1. Reverse transcriptase polymerase chain reaction (RT-PCR) expression of PlGF in 24 human cell lines expression. PlGF was expressed by a large majority of the human cancer cell lines examined. *Note:* additional larger sized bands were sequenced and found to be non-specific.

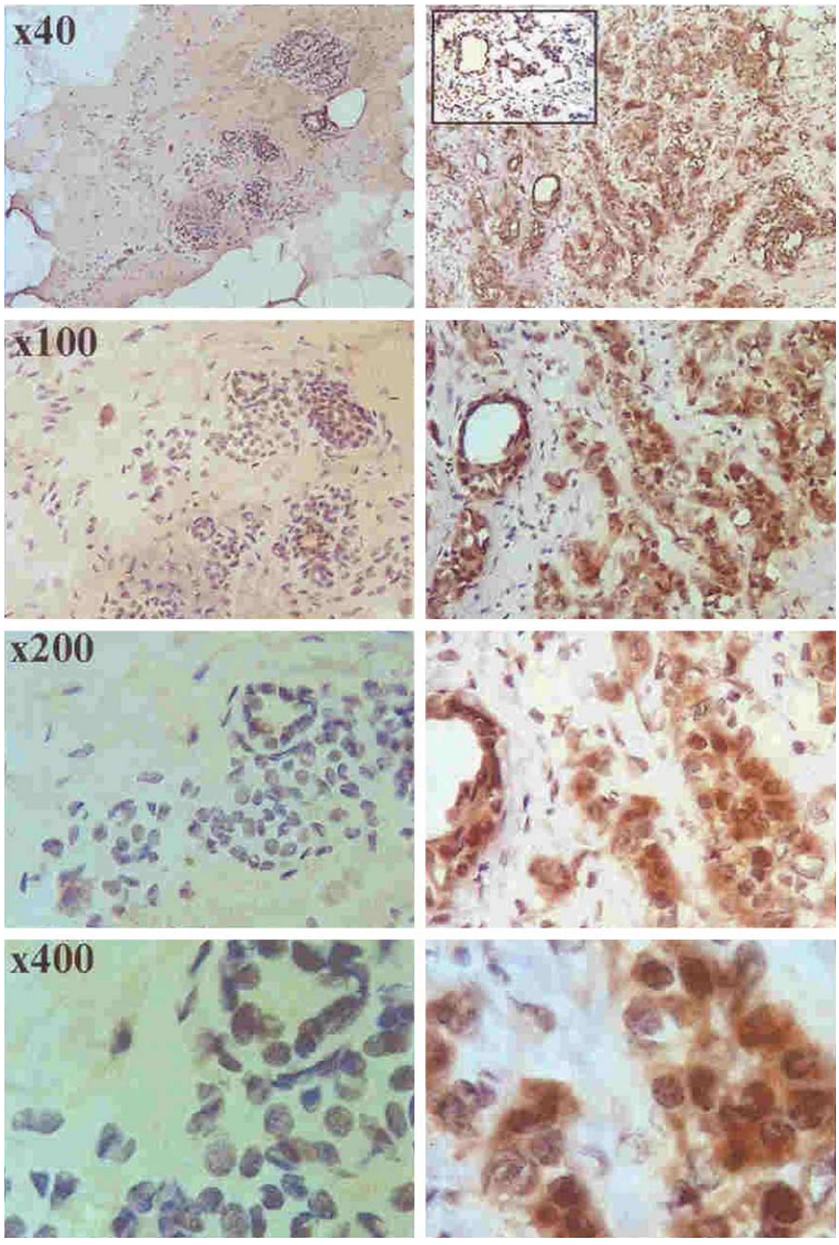

Fig. 3. Immunohistochemical staining of human breast specimens. Normal background breast tissue (left panels) *versus* breast cancer tissue (right panels) from breast cancer patients. Breast cancer tissues were also stained in the absence of a PlGF primary antibody to act as a negative control (insert in top right plate). These sections demonstrate that the PlGF is expressed at far higher levels in the breast tumour specimens compared with the normal breast tissue. The breast tumour specimens displayed a high and widespread level of PlGF staining that was far more intense in the breast carcinoma cells, compared with the low/absent levels of PlGF in the epithelial cells and fibroblasts of normal background breast tissue.

3.4. Quantification of PlGF expression in human breast tissues

We quantified the PlGF transcript expression in the breast specimens (tumour $n = 119$, background $n = 33$) using real-time Q-PCR (all Q-PCR values are displayed as mean transcript copies). We show that the PlGF expression value in tumours was 0.665 ± 0.35, compared with 0.03 ± 0.017 in the normal tissue. Therefore, our results reveal that the breast cancer tissues express significantly higher levels of PlGF ($P = 0.028$), compared with the low levels observed in the normal background breast tissues (Fig. 4(A)).

3.5. PlGF expression in relation to prognosis

We examined the PlGF level between the Nottingham Prognostic Index (NPI) groups of breast cancer patients (NPI-1, $n = 67$; NPI-2, $n = 37$; NPI-3, $n = 15$). The NPI-1 group represented patients with a good prognosis and

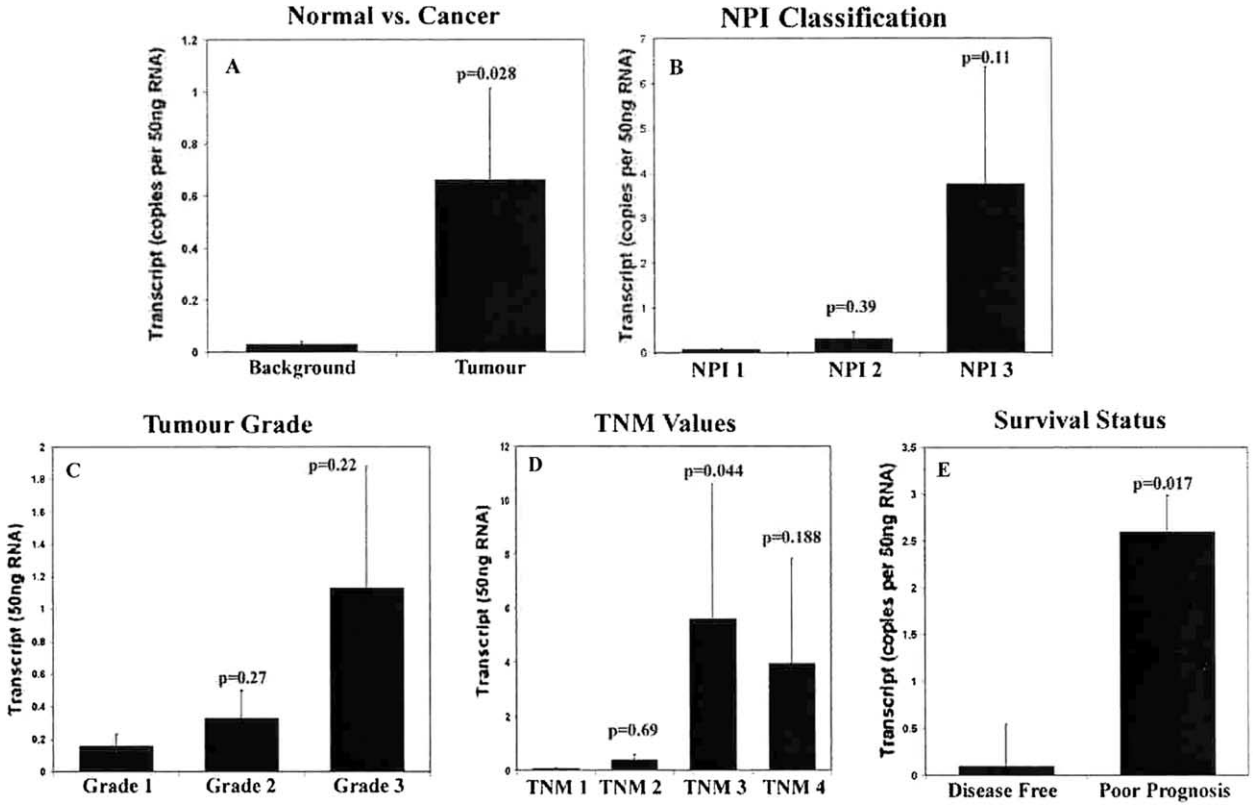

Fig. 4. Quantitative-PCR analysis of PlGF expression in human breast cancer tissues (A–E). (A) PlGF expression is significantly elevated in human breast cancer tissues compared with normal background breast tissue ($P = 0.028$). (B) Patients with some degree of node involvement have a higher level of PlGF expressed in the tumours, compared with the tumour of patients without any node involvement, although, these values did not reach significance (C) Levels of PlGF appeared to correlate with poor differentiation. The lowest levels of PlGF were observed in the well-differentiated grade 1 tumours, although these values did not reach statistical significance. (D) Comparison of PlGF expression between the TNM classification groups revealed that PlGF was expressed to significantly higher levels ($P = 0.044$) in patients with an overall poor outlook (TNM 3) compared with those with good outlook (TNM 1). (E) Patients who were alive and well with no recurrence were allocated to the good prognosis group, whereas, those who had recurrence, metastasis to distant areas or had died as a result of breast cancer were allocated to the poor prognosis group. Patients with a poor prognosis had dramatically elevated levels of PlGF ($P = 0.017$).

an NPI index <3.4, the NPI-2 group contained patients with a moderate prognosis and an NPI index of 3.4–5.4, whereas the NPI-3 patients had a poor prognosis with an NPI index of >5.4. An increased degree of lymph node involvement was associated with increasing levels of PlGF (Fig. 4(B)). The patients assigned to the NPI-1 group had a PlGF transcript value of 0.085 ± 0.028, compared with the NPI-2 group with 0.312 ± 0.171 and NPI-3 with 3.76 ± 2.68. A Kruskal–Wallis test has shown that there is a significant association between increased levels of PlGF transcript and high NPI status ($P = 0.022$). Overall, patients with node involvement had raised levels of PlGF compared with those without any degree of node involvement. However, this correlation between PlGF and prognosis did not reach statistical significance in these instances ($P = 0.13$).

3.6. PlGF and breast tumour grade

PlGF values in the moderate grade 2 (0.33 ± 0.171, $n = 41$) and poorly differentiated and grade 3 (1.13 ± 0.75, $n = 55$) tumours were elevated compared with well-differentiated grade-1 tumours (0.16 ± 0.02, $n = 23$). This trend linking PlGF to a poor patient outlook did not reach statistical significance with the current sample number (grade 1 *versus* grade 2, $P = 0.27$; grade 1 *versus* grade 3, $P = 0.22$) (Fig. 4(C)). Additionally, we examined PlGF expression within the different tumour histological subtypes, although there was no apparent difference between the lobular, ductal or other tumour types. However, Kaplan–Meier survival analysis identified PlGF as a disease-free survival factor in oestrogen receptor (ER) negative tumours ($P = 0.013$).

3.7. Tumour-node-metastasis classification of patients

PlGF expression correlated with patient outlook through tumour-node-metastasis (TNM) grouping (TNM 1, $n = 68$; TNM 2, $n = 39$; TNM 3, $n = 8$; TNM 4, $n = 4$). Levels of PlGF were found to be significantly elevated in tumours of patients who had an overall poor outlook (TNM 3, 5.57 ± 5.47; TNM 4,

3.96 ± 3.91), compared with those patients with a relatively good prognosis (TNM 1, 0.084 ± 0.025; TNM 2, 0.41 ± 0.19). We reveal that PlGF was expressed to significantly higher degree ($P = 0.044$) in patients with an overall poor outlook (TNM 3) compared with those with a good outlook (TNM 1) (Fig. 4(D)).

3.8. PlGF expression and survival status

We assessed the survival status of the breast cancer patients in association with PlGF levels, with an average of a 6-year follow-up period (Fig. 4(E)). Patients were divided into two groups, those who remained disease-free were assigned to the good prognosis group ($n = 89$), whereas, the patients who had recurrence, metastasis to a distant site or had died as a result of breast cancer were allocated to the poor prognosis group ($n = 30$). The quantity of PlGF from each tumour specimen was assessed and we reveal that the patients with a poor prognosis had dramatically elevated levels of PlGF ($P = 0.017$). Our results show that the good prognosis group had low levels of PlGF (0.099 ± 0.042) compared with the statistically higher levels observed in the poor prognosis group (2.6 ± 1.51).

3.9. PlGF did not significantly enhance endothelial cell migration

The cell motility assay utilised time-lapsed video monitoring to provide quantitative data on PlGF's ability to enhance HECV cell migration. The mean distance moved for the cells over 100 min was determined for each treatment. The addition of PlGF slightly increased the degree of endothelial cell migration; however, these results did not reach significance. Therefore, no significant differences in cell motility were observed with recombinant PlGF treatment (Fig. 5).

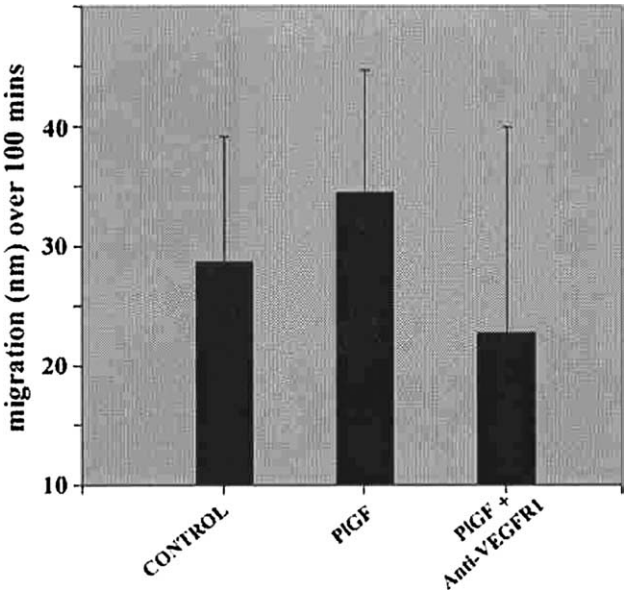

Fig. 5. Endothelial motility assay. This assay assessed the motile properties of HUVEC cells in the presence anti-VEGFR-1 (120 ng/ml) and/or PlGF (30 ng/ml). No significant differences in endothelial cell motility were observed in the presence of PlGF.

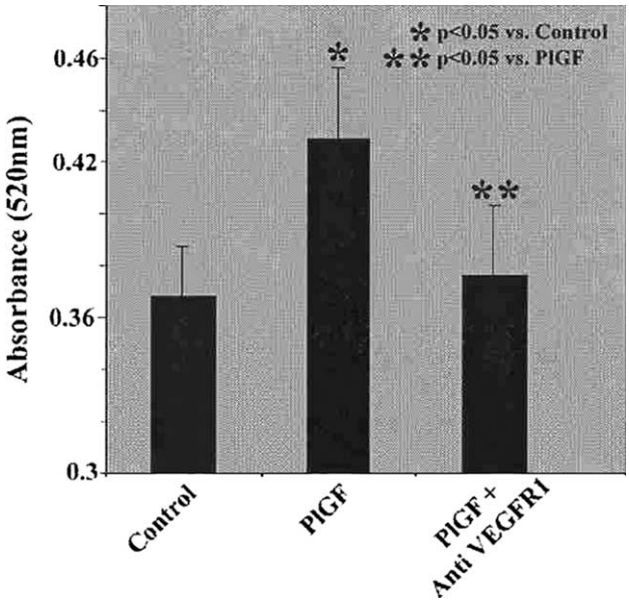

Fig. 6. Endothelial proliferation assay. Over a 72-h incubation period, there was a significant increase in the rate of proliferation ($P = 0.036$) in the HUVEC cells treated with recombinant PlGF (30 ng/ml) compared with the control HUVEC cells. This enhanced proliferation was significantly suppressed ($P = 0.027$) through the addition of an antibody against VEGFR-1 (120 ng/ml).

3.10. PlGF increased human endothelial cell proliferation

The proliferation assay examined the effect of PlGF on the proliferation rate of HECV cells (Fig. 6). The results showed that, over a 72-h period, there was a significant increase in the rate of proliferation in the endothelial cells cultured in PlGF compared with the control ($P = 0.036$). PlGF exerts its influence through its interaction with the VEGFR-1 receptor, however, when PlGF was added in combination with an antibody to VEGFR-1, the influence of PlGF was significantly inhibited ($P = 0.027$). We confirm that PlGF plays a role in human endothelial cell proliferation, via VEGFR-1 signalling pathways.

4. Discussion

PlGF plays a putative role in tumour angiogenesis. Studies report that PlGF stimulates formation of mature, leakage-resistant vessels in a manner comparable in efficiency to VEGF [34,35]. However, it was recently discovered that PlGF switches on its own genetic

program of angiogenic genes, separate to that of VEGF [13]. Recent studies in mice revealed that loss of PlGF impaired tumour angiogenesis, while an over-expression enhanced vascularisation and vessel permeability [35,36]. PlGF, and its receptor VEGFR-1 (Flt-1), while being minimally expressed in quiescent vessels, are strongly upregulated in a variety of pathological conditions [37]. Therefore, PlGF does appear to enhance tumour angiogenesis, although there are contrasting views whether this factor influences physiological angiogenesis. Our study is the first to quantitate PlGF expression in human breast tissue samples from breast cancer patients. This study also assessed PlGF levels in relation to patient clinical parameters.

PlGF is not expressed in the majority of normal adult human tissues, and we confirm that PlGF was absent or at very low levels in the normal breast tissue from breast cancer patients. Screening a panel of 24 human cells lines revealed that PlGF was expressed in a large variety of human cancer cell lines, including breast, colorectal, bladder and prostate. This study demonstrates that PlGF was strongly expressed in the tumour tissue of the breast cancer patient specimens, and that a high degree of expression was indicative of a poor prognosis for the patient. We confirmed that PlGF was dramatically upregulated in the patients' breast cancer tissue compared with the minimal levels observed within normal breast tissues via immunohistochemical staining, and both qualitative and quantitative PCR. Interestingly, a recent study also demonstrates that PlGF was over-expressed in nearly 90% of gastric cancers compared with non-tumour mucosa, and this expression correlated with patient clinical outcome, whereas VEGF did not [22]. Therefore, PlGF demonstrates strong prognostic potential. We also assessed the function PlGF in a series of *in vitro* assays and report that addition of recombinant PlGF enhanced endothelial cell proliferation through its interaction with VEGFR-1 (Flt-1); however, PlGF did not significantly enhance endothelial cell migration or tubule formation in a 3-D culture system. Lack of significant results may be due to the fact that the endothelial cells themselves produce abundant levels of PlGF in culture, which reduces their sensitivity to exogenous PlGF.

Our study demonstrates that PlGF expression correlates with breast cancer patient prognosis, as high levels of PlGF were significantly associated with a higher degree of lymph node involvement, poorly differentiated tumours and an overall poor outlook for the patient. Therefore, we show that PlGF may exercise its role only at specific fragments of the cancer progression pathway, such as the later stages of the disease, and that PlGF may also act in tumour specific manner. Following radio-immunotherapy, surviving tumour cells upregulate the expression of specific angiogenic factors depending on the type of tumour, and PlGF was shown to increase microvessel density but decrease the number of mature vessels [38,39]. Therefore, it has been suggested that PlGF may play a role in the rapid restoration of a tumour blood supply after treatment, thus may enhance the possibly of recurrence. Tumour angiogenesis is a complex process; therefore the reported responses from PlGF/VEGR-1 signalling will make PlGF a desirable potential therapeutic target. We conclude that PlGF is over-expressed in human breast cancer tissues and displays prognostic value in breast cancer patients.

Conflict of interest

None declared.

Acknowledgement

We thank the Breast Cancer Campaign for supporting this work.

References

1. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000, **100**, 57–70.
2. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996, **86**, 353–364.
3. Ferrara N. VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer* 2002, **2**, 795–803.
4. Dvorak HF. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. *J Clin Oncol* 2002, **20**, 4368–4380.
5. Plate KH, Breier G, Weich HA, *et al*. Vascular endothelial growth factor is a potential tumor angiogenesis factor in human gliomas *in vivo*. *Nature* 1992, **359**, 845–848.
6. Berse B, Brown LF, Van de Water L, *et al*. Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. *Mol Biol Cell* 1992, **3**, 211–220.
7. Zhang HT, Craft P, Scott PA, *et al*. Enhancement of tumor growth and vascular density by transfection of vascular endothelial cell growth factor into MCF-7 human breast carcinoma cell. *J Natl Cancer Inst* 1995, **87**, 213–219.
8. Maeda K, Chung YS, Ogawa Y, *et al*. Prognostic value of vascular endothelial growth factor expression in gastric carcinoma. *Cancer* 1996, **77**, 858–863.
9. Linderholm B, Tavelin B, Grankvist K, *et al*. Vascular endothelial growth factor is of high prognostic value in node negative breast carcinoma. *J Clin Oncol* 1998, **16**, 3121–3128.
10. Gasparani G, Toi M, Gion M. Prognostic significance of vascular endothelial growth factor protein in node-negative breast carcinoma. *J Natl Cancer Inst* 1997, **89**, 139–147.
11. Maglione D, Guerriero V, Viglietto G, *et al*. Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. *Proc Natl Acad Sci USA* 1991, **88**, 9267–9271.
12. Cao Y, Weidong RJ, Pang Q, *et al*. Placenta growth factor: identification and characterization of a novel isoform generated by RNA alternative splicing. *Biochem Biophys Res Commun* 1997, **235**, 493–498.

13. Autiero M, Waltenberger J, Communi D, *et al*. Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat Med* 2003, **9**, 936–943.
14. Carmeliet P, Moons L, Luttun A, *et al*. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med* 2001, **7**, 575–583.
15. Luttun A, Autiero M, Tjwa M, *et al*. Genetic dissection of tumor angiogenesis: are PlGF and VEGFR-1 novel anti-cancer targets? *Biochim Biophys Acta* 2004, **1654**, 79–94.
16. Hauser S, Weich HA. A heparin-binding form of placenta growth factor (PlGF-2) is expressed in human umbilical vein endothelial cells and in placenta. *Growth Factors* 1993, **9**, 259–268.
17. Vuorela P, Hatva E, Lymboussaki A. Expression of vascular endothelial growth factor and placenta growth factor in human placenta. *Biol Reprod* 1997, **56**, 489–494.
18. Rakic JM, Lambert V, Devy L, *et al*. Placental growth factor, a member of the VEGF family, contributes to the development of choroidal neovascularization. *Ophthalmol Vis Sci* 2003, **44**, 3186–3193.
19. Khaliq A, Foreman D, Ahmed A, *et al*. Increased expression of placenta growth factor in proliferative diabetic retinopathy. *Lab Invest* 1998, **78**, 109–116.
20. Li XF, Charnock-Jones DS, Zhang E, *et al*. Angiogenic growth factor messenger ribonucleic acids in uterine natural killer cells. *J Clin Endocrinol Metab* 2001, **86**, 1823–1834.
21. Roberts-Clark DJ, Smith AJ. Angiogenic growth factors in human dentine matrix. *Arch Oral Biol* 2000, **45**, 1013–1016.
22. Chen CN, Hsieh FJ, Cheng YM, *et al*. The significance of placenta growth factor in angiogenesis and clinical outcome of human gastric cancer. *Cancer Lett* 2004, **213**, 73–82.
23. Matsumoto K, Suzuki K, Koike H, *et al*. Prognostic significance of plasma placental growth factor levels in renal cell cancer: an association with clinical characteristics and vascular endothelial growth factor levels. *Anticancer Res* 2003, **23**, 4953–4958.
24. Donnini S, Machein MR, Plate KH, *et al*. Expression and localization of placenta growth factor and PlGF receptors in human meningiomas. *J Pathol* 1999, **189**, 66–71.
25. Lacal PM, Failla CM, Pagani E, *et al*. Human melanoma cells secrete and respond to placenta growth factor and vascular endothelial growth factor. *J Invest Dermatol* 2000, **115**, 1000–1007.
26. Nomura M, Yamagishi S, Harada S, *et al*. Placenta growth factor (PlGF) mRNA expression in brain tumors. *J Neurooncol* 1998, **4**, 123–130.
27. Takahashi A, Sasaki H, Kim SJ, *et al*. Markedly increased amounts of messenger RNAs for vascular endothelial growth factor and placenta growth factor in renal cell carcinoma associated with angiogenesis. *Cancer Res* 1994, **54**, 4233–4237.
28. Kodama J, Seki N, Tokumo K, *et al*. Placenta growth factor is abundantly expressed in human cervical squamous cell carcinoma. *Eur J Gynaecol Oncol* 1997, **18**, 508–510.
29. Viglietto G, Maglione D, Rambaldi M, *et al*. Upregulation of vascular endothelial growth factor (VEGF) and downregulation of placenta growth factor (PlGF) associated with malignancy in human thyroid tumors and cell lines. *Oncogene* 1995, **11**, 1569–1579.
30. Parr C, Watkins G, Mansel RE, *et al*. The hepatocyte growth factor regulatory factors and human breast cancer. *Clin Cancer Res* 2004, **10**, 202–211.
31. Jiang WG, Douglas-Jones A, Mansel RE. Level of expression of PPAR-gamma and its co-activator (PPAR-GCA) in human breast cancer. *Int J Cancer* 2003, **106**, 752–757.
32. Jiang WG, Hiscox S, Parr C, *et al*. Antagonistic effects of NK4, a novel HGF variant, on the *in vitro* angiogenesis of human vascular endothelial cells. *Clin Cancer Res* 1999, **5**, 3695–3703.
33. Cai J, Ahmad S, Jiang WG, *et al*. Activation of vascular endothelial growth factor receptor-1 sustains angiogenesis and Bcl-2 expression via the phosphatidylinositol 3-kinase pathway in endothelial cells. *Diabetes* 2003, **52**, 2959–2968.
34. Luttun A, Tjwa M, Moons L, *et al*. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med* 2002, **8**, 831–840.
35. Carmeliet P. VEGF gene therapy: stimulating angiogenesis or angioma-genesis. *Nat Med* 2001, **6**, 1102–1103.
36. Odorisio T, Schietroma C, Zaccaria ML, *et al*. Mice overexpressing placenta growth factor exhibit increased vascularization and vessel permeability. *J Cell Sci* 2002, **115**, 2559–2567.
37. Luttun A, Autiero M, Tjwa M, *et al*. Genetic dissection of tumor angiogenesis: are PlGF and VEGFR-1 novel anti-cancer targets. *Biochim Biophys Acta* 2004, **1654**, 79–94.
38. Taylor AP, Osorio L, Craig R, *et al*. Tumor-specific regulation of angiogenic growth factors and their receptors during recovery from cytotoxic therapy. *Clin Cancer Res* 2002, **8**, 1213–1222.
39. Taylor AP, Rodriguez M, Adams K, *et al*. Altered tumor vessel maturation and proliferation in placenta growth factor-producing tumors: potential relationship to post-therapy tumor angiogenesis and recurrence. *Int J Cancer* 2003, **105**, 158–164.