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PGF Isoforms, PLGF-1 and PGF-2, in Colorectal Cancer and the Prognostic Significance

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Abstract. *Aim: This study examined the expression of PLGF-1 and PGF-2 in human colorectal cancer and how changes in expression may be linked to prognosis of the disease. Materials and Methods: Expression of PLGF transcripts in primary colorectal tumours (n=94) and background tissue (n=80) were analysed by Q-PCR. Immunohistochemistry was carried out on a number of matched background and tumour sections. Results: There was a progressive increase in Dukes A to C (430±134 versus 674±2680) and with TNM 1 to 3 as compared to background tissues (479±152 versus 777±327 p=0.032). Interestingly, there was an increase in PLGF-1 expression in patients with poor outcome compared with patients remaining disease-free. PGF-2 expression was significantly elevated in tumours compared to normal (4558±240 versus 859±67.3 p<0.0001). Moreover, expression was increased in patients with poor prognosis compared to those remaining disease free (4585±285 versus 3909±446). Conclusion: There is increased expression of the PLGF isoforms in human colorectal cancer.*

Angiogenesis, is a key component of the metastatic pathway. In the physiological condition, there is a balance between pro-angiogenic and anti-angiogenic factors which can be unbalanced in certain pathological conditions such as cancer, atherosclerosis, and diabetic retinopathy (1). The survival and growth of colorectal tumour and thus metastases are dependent on the balance of the growth factors implicated in tumour angiogenesis. Vascular endothelial growth factor (VEGF) appears to be one of the most specific and potent regulators of angiogenesis, which regulates endothelial proliferation, permeability, and survival and is therefore amongst the pro-angiogenic factorts implicated in tumour angiogenesis. VEGF is a mitogen for vascular endothelial cell derived from arteries, veins, and lymphatics (2-4). Different VEGF family member appears to have a specific function: VEGF-C and VEGF-D are principally lymphangiogenic factors (5). In addition, to the proliferative and anti-apoptotic action on endothelial cell, VEGF directly promotes proliferation and/or migration of VEGF receptor-expressing tumour cells and they are also involved in mobilization of endothelial and hemotopoietic stem cells (HSCs) from the bone marrow and their recruitment at the tumour location where they supply to tumour angiogenesis (6). High levels of VEGF expression and important vessels counts correlate with recurrence and metastasis of colorectal cancer, suggesting that VEGF expression could be a marker of colorectal cancer prognosis (7).

Placenta growth factor, PGF, is a member of the VEGF family which is highly expressed in trophoblast, the major cellular source in the placenta (8). It is also found expressed in vascular endothelium, umbilical vein endothelial cells, the eye, uterine natural killer cells and dentine matrix (9). It is a homodimeric glyprotein that displays significant homology with VEGF, sharing 53% of similarity in its overall amino acid residues. Human PGF gene has been mapped to chromosome 14q24. PGF-coding sequence is encoded by seven exons spanning an 800-kb long DNA interval (10). As a result of splicing the primary PGF transcript, PGF has at least three isoforms, PLGF-1, PGF-2 (also known as PLGF-2 in the following published studies by (8, 10-14) and PGF-3 (also known as PLGF-3 in the following published studies by (8, 10, 13). However, only one study has described a further isoform, PLGF-4 in human trophoblast and endothelial cells (8).

PGF mediate their effects through VEGFR-1. PGF-2 has a 21-amino acid heparin binding domain and is also able to bind both of the known PGF receptors: neuropilin-1 (Nrp-1) and FLT-1 while PLGF-1 and PGF-3, both missing the heparin binding domain, bind only FLT-1 (14).

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Several studies have documentated that VEGF and PGF wield similar biological functions in disturbing the growth of vascular endothelial cells (11). There are clear evidences that PGF promotes motility and invasiveness, two important features related to the epithelial to mesenchymal transformation that characterizes metastasis in tumours (14).

Results available from *in vitro* studies on the angiogenesis role of PGF are contradictory; some studies have revealed that PGF binding to VEGFR-1 (FLT-1) failed to produce any growth and angiogenesis in endothelial cell (EC), while others show that PGF/VEGFR-1 signalling promotes EC viability and angiogenesis. In placenta, it seems to be clear that PGF has a direct effect on EC, inducing its own signalling and by magnifying the angiogenensis promoted by VEGF.

In addition to endothelial cells, PGF has a chemotactic effect on monocytes. It has been demonstrated that after injection of PGF protein and adenovirus mediated PGF gene transfer there is a higher accumulation of macrophage (10).

PGF also acts on VEGFR-1 (FLT-1) expressing smooth muscle cells and pericytes promoting vessel maturation (6).

Colorectal cancer is the third most common form of cancer and the second leading cause of cancer-related death in the Western world. Colorectal cancer causes 655,000 deaths worldwide per year, including about 16,000 in the UK, where it is the second most common site (after lung) to cause cancer death according to the World Health organization in 2006. Recent statistics suggest an estimated 135,762 new cases of colorectal cancer with 52,180 deaths in 2007 (15).

To clarify the role of PLGF-1 and PGF-2 in colorectal tumour cancer and its prognostic significance, we examined the expression of both isoforms and how changes in expression of these may be linked to prognosis of the disease, through reverse transcription polymerase chain reaction (RT-PCR), real-time quantitative polymerase chain reaction (Q-PCR), and Immunohistochemical studies, in a cohort of human colorectal cancer specimens.

Materials and Methods

Human colorectal specimen. A total of 174 colorectal samples were obtained from colorectal cancer patient (80 background normal colorectal tissue and 94 colorectal cancer tissue), with consent of the patients and ethical committee. These tissues were collected immediately after colorectomies, and snap-frozen in liquid nitrogen. The pathologist verified normal background and cancer specimens, and it was confirmed that the background samples were free from tumour deposit.

Polymerase chain reaction (PCR). Human Colorectal cancer tissue was screened for endogenous expression of PLGF-1 and PGF-2. Conventional PCR was performed using cDNA together with PCR master mix using respective primers, PLGF-1 and PGF-2 forward and reverse primers were 5'-ATGCCGGTCATGAGGCTGTT CCCTTGCTT-3' and 5'-TTACCTCCGGGGAACAGCATCGCCG CAC-3', two products being distinguished by their charactistic size differences. The reaction conditions were: 94˚C for 5 min, 94˚C for 15 s, 61˚C for 30 s, 72˚C for 1 min and the final extension phase at 72˚C for 10 min for 42 cycles. The PCR products were separated on a 1.4% agarose gel and electrophoretically separated. The gel was then stained with ethidium bromide prior to examine under ultraviolet light and photographs taken.

Real-time quantitative polymerase chain reaction (Q-PCR). The Q-PCR systems used were the Amplifluor™ Uniprimer™ system (Intergen Company Oxford, UK) and Thermo-Start® (ABgene, Epsom, Surrey, UK). Specific primers were designed by the authors using Beacon Designers software (Biosoft, Palo Alto, California, USA) and manufacturated by Invitrogen (Invitrogen Life Technologies, Paisley, Scotland, UK). Using the Icycler IQ system (Bio-Rad, Hemel Hemptead, UK), which incorporates a gradient thermocycler and a 96-channel optical unit. Q-PCR was carried out using the specific primer pair, Uniprimer probe (FAM tagged, at one tenth of the primer concentration) and with the following conditions: enzyme activation at 95˚C for 12 min, 1 cycle, followed by 60 cycles of denaturing: 95˚C for 15s; annealing: 55˚C for 40s; extension: 72˚C for 25s. Using purified plasmids as internal standard, the level of each molecule cDNA (copies/50 ng RNA) in the colorectal samples was calculated. Q-PCR for β-actin was also performed on the same samples, to correct for any residual differences in the initial level of RNA in the specimens (in addition to spectrophotometry). The products were verified on agarose gel.

Primer pairs for Q-PCR were as follows: PLGF-1 forward and reverse primers (underlined sequence was the region that is complementary to the Uniprimer probe) were 5'-GTTCTCT CAGCACGTTCG-3' and 5'-ACTGAACCTGACCGTACACAT CGCCGCACCTTTC-3' and PGF-2 forward and reverse primers were 5'-GGAGCTGACGTTCTCTCAG-3' and 5'-ACTGAAC CTGACCGTACAGTTACCTCCGGGGAACAG-3'.

Immunohistochemical staining of colorectal specimens. This was based on methods we previously described (16-18). Frozen sections of colorectal tumour and background tissue were cut at a thickness of 6 μm using a cryostat. The sections were mounted on super frost 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 16% horse serum blocking solution and probed with PLGF-1 and PGF-2 antibody, and without primary antibody as a negative control to verify the binding specificity. Primary antibodies were purchased from Santa-Cruz Biotechnologies Inc. (Santa-Cruz, CA, USA). Following extensive washings, sections were incubated for 30 min in the secondary biotinylated antibody and following washings (ABC Standard kit, Vector Laboratories, Peterborough, UK). Diamino-benzine chromogen (Vector Laboratories, 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.

Immunohistochemical photographs of normal and tumour sections (N=20) were assessed as previously described (17).

Statistical analysis. Statistical analyses were carried out using paired samples Student's *t*-test (mean±SD) and by the non-parametric Mann-Whitney test (IQR) where appropriate. A *p*-value 0.05 was defined as statistically significant. Statistic tests were performed using the software SPSS 10.0 (SPSS Inc., Chicago, USA).

Results

Expression of PLGF-1 and PGF-2 in colorectal cancer tissue. PLGF-1 and PGF-2 transcripts were detected in colorectal cancer tissue and were seen as two discrete products, 447 bp and 512 bp in size, respectively.

Immunohistochemical staining of colorectal specimens. Colorectal cancer tissue stained positively for PLGF-1 and PGF-2. We reveal that the PLGF-1 and PGF-2 protein level was elevated in the colorectal tumour specimens as showed in Figure 1 (C and D for PLGF-1, G and H for PGF2) compared with the normal colorectal tissue as shown in Figure 1 (A and B for PlGF1, and, E and F for PGF2).

Quantification of PLGF-1 and PGF-2 in human colorectal tissues. We quantified the PLGF-1 and PGF-2 transcript expression in the colorectal specimens (tumour n=94, background n=80) using real-time Q-PCR (all values are displayed as mean transcript copies). We show that the PLGF-1 transcript value in normal colorectal tissue was 38.6±12.7, compared with 641±162 in the colorectal cancer tissue as shown in Figure 2A. These results reveal that PLGF-1 expression was significantly higher in colorectal cancer tissue than in normal colorectal tissue and this reached a level of significance (p=0.004).

When looking at PGF-2, expression level in cancer tissue was 859±67, compared with normal colorectal tissue 4558±240. Levels of PGF-2 were significantly higher in cancer tissue, this result reached again a level of significance (p<0.0001).

PLGF-1 and PGF-2 and TNM. Colorectal cancer patients were divided into four groups according to the most commonly used staging system, the TNM (for tumours/nodes/metastases).Our study show that PLGF-1 is expressed a higher level in advanced TNM staging. When comparing the levels of expression of TNM 1 and TNM 3 we can see a higher expression in TNM 3, (479±152) for TNM 1 *vs*. (777±327) for TNM 3, although this did not reach significance as shown in Figure 2B.

PGF-2 presents a similar pattern, a higher level of expression in TNM 4 when compared to TNM 1, (4528±487) for TNM 1 *vs*. (4532±1047) for TNM 4, this did not reach significance.

PLGF-1 and PGF-2 and Duke's classification. Prognostic indicator Duke's system was associated with significant changes in levels of PLGF-1, showing a higher level of expression in Duke's C when compared with Duke's A (674±134 *vs*. 430±134). However, when looking at PGF-2 our study did not show any significant changes between the different Duke's classifications as shown in Figure 2C.

PLGF-1 and PGF-2 and nodal status. We assigned 31 patients with node-positive and 39 patients with node negative tumours. PLGF-1 and PGF-2 in the node-positive were elevated compared with node-negative patients (696±276 *vs*. 510±152) for PLGF-1 and (4436±355 *vs*. 4186±227) for PGF-2) as shown in Figure 3.

PLGF-1 and PGF-2 and patient outcome. We assessed the survival status of the colorectal cancer patient in association with PLGF-1 and PGF-2 levels. Patients were divided into two groups, those who remained disease free were assigned to the good prognosis group (n=21), whereas, the patients who had recurrence, metastasis to a distant site or had died as a result of colorectal cancer were allocated to the poor prognosis group (n=49).

The quantity of PLGF-1 and PGF-2 from each tumour specimen was assessed. We reveal that the patients with a poor outcome had elevated levels of PLGF-1 (median values Disease free and Poor outcome 196 and 266 respectively), (–205.5, 122.9, 95.2% confidence interval) p=0.3835, although this did not reach a level of significance as shown in Figure 4. When looking at PGF-2 our study indicates that increased levels of PGF-2 are associated with poor outcome group (median values Disease free and Poor outcome 3550 and 4210 respectively), (–1689.3, 310, 95% confidence interval) p=0.2068, although this did not reach a level of significance.

Overall, there is an increase in PLGF-1 and PGF-2 in patients with a poor outcome compared with those remaining free.

Discussion

The current study evaluate some of these factors in relation with PLGF-1 and PGF-2 and investigated the expression of PLGF-1 and PGF-2, at the protein level and the messenger RNA level and whether it has a significant value in the prognosis of the colorectal cancer patients.

Real-time quantitative Q-PCR is a sensitive, reliable, reproductive and rapid method that allows broad range of gene transcript to be studied in a very small amount of biologic material. The current study has used this method to determine the levels of PLGF-1 and PGF-2 gene transcript. To our best knowledge this study is the first reporting that both isoforms were detected in all colorectal cancer tissues and the matched normal tissues.

We demonstrated that PLGF-1 and PGF-2 expression levels were significantly higher in tumour tissues compared with non-tumour tissues. These results correlate with the IHC analysis showed both PLGF-1 and PGF-2 were expressed mainly in tumour cells.

We confirm that PLGF-1 and PGF-2 mRNA levels were elevated when looking at clinical outcome for these patients. Levels of PLGF-1 and PGF-2 were higher in tumour tissues

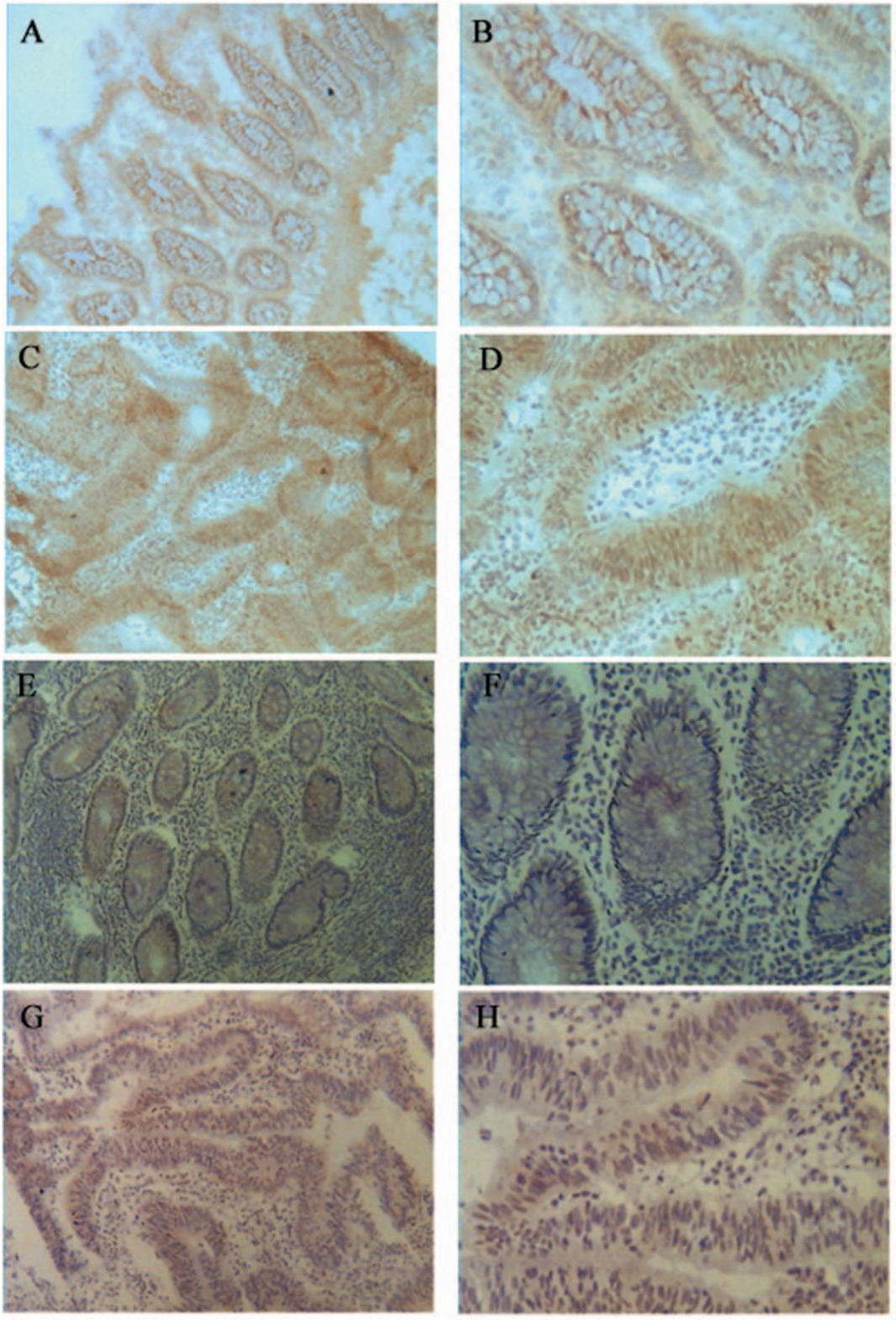

Figure 1. *Immunohistochemical staining for PLGF-1 and PGF-2 of human colorectal specimens. A and B showed the negative staining in normal background colorectal tissue for PLGF-1. C and D showed strong cytoplasmic staining of PLGF-1 in human colorectal cancer tissue. Similarly, E and F showed the negative staining in normal background colorectal tissue for PGF-2, while G and H showed cytoplasmatic staining for PGF-2 in human cancer tissue.*

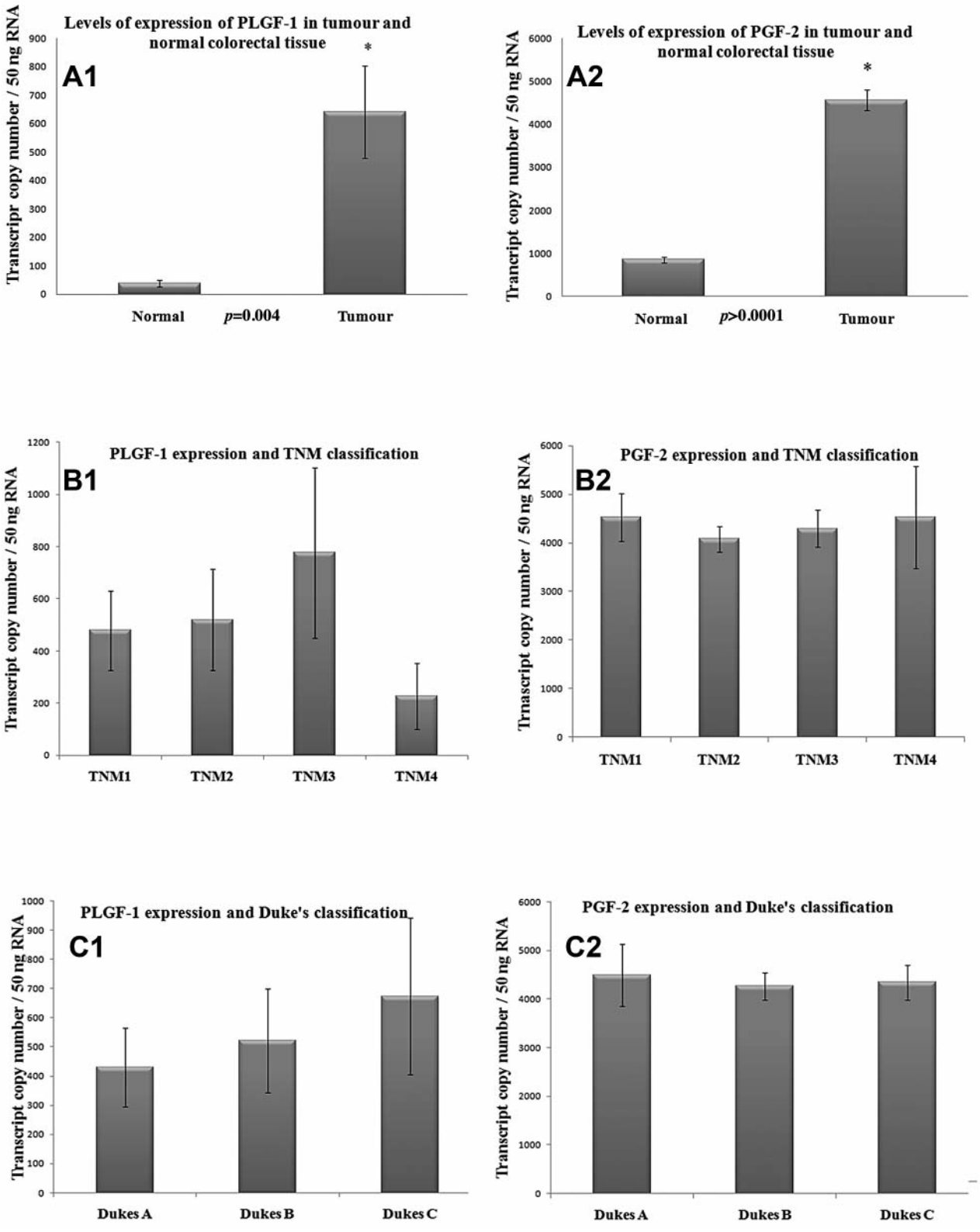

Figure 2. *Levels of transcript of PLGF-1 (left) and PGF-2 (right). A: tumour samples in comparison to background. B: levels of transcript of PLGF-1 and PGF-2 expression in correlation with the TNM staging system. C: Levels of PLGF-1 and PGF-2 transcript in correlation with the Duke's classification.*

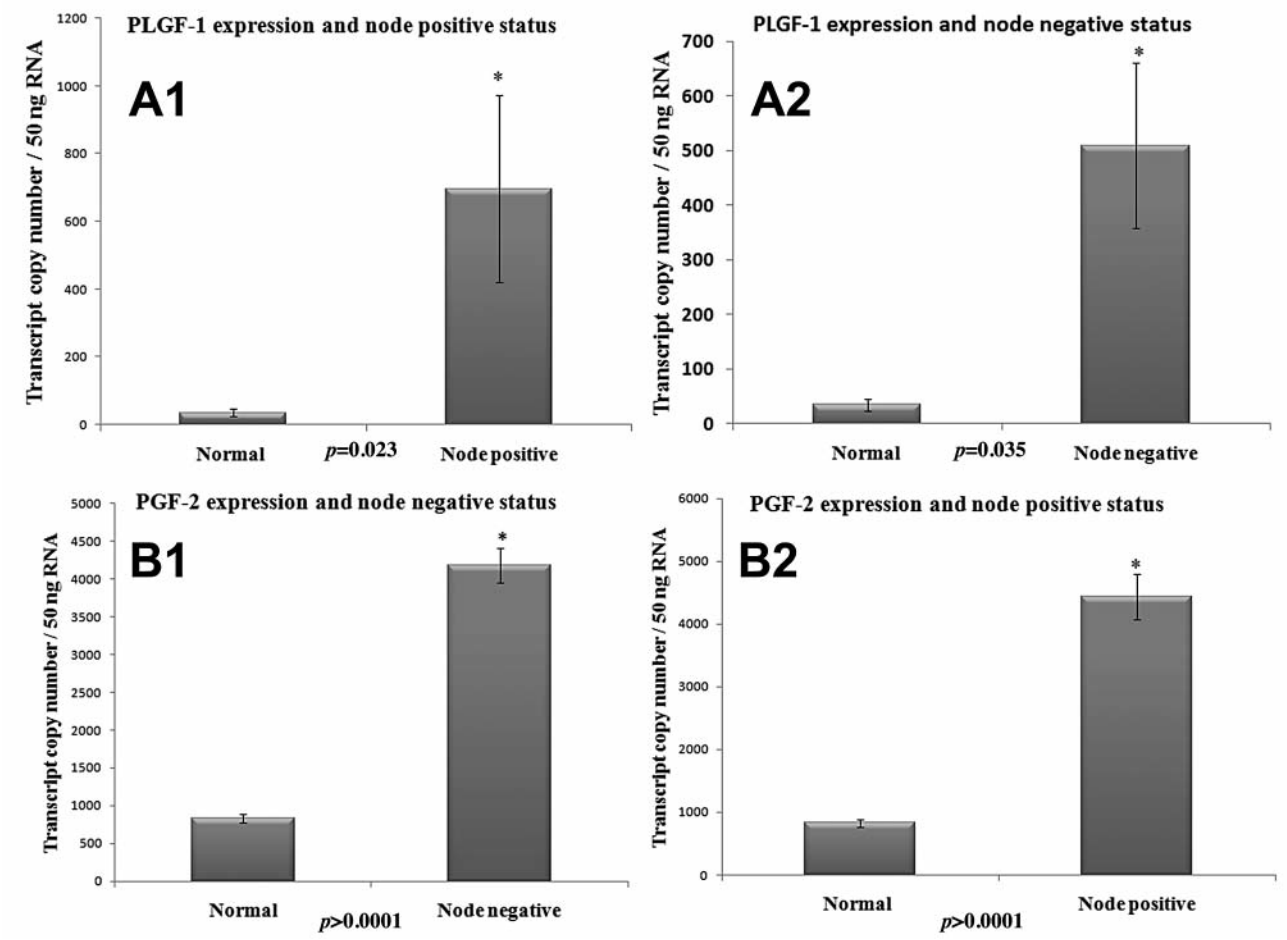

Figure 3. *Levels of transcript of PLGF-1 and PGF-2. A: PLGF-1 expression in node positive (A1) and node negative (A2) tumours in comparison with normal tissues. B: PGF-2 expression in node positive (B1) and node negative (B2) tumours in comparison with normal tissues.*

from patients who were categorised as the poor outcome group compared with those who were categorised as the disease free group. This high degree of expression was indicative for a poor prognosis for patient. Therefore, both isoforms demonstrate strong prognostic potential as shown in Figure 4.

The prognosis of colorectal cancer is affected by a number of factors which can be classified into those related to the tumour stage (Duke's classification and TNM system), clinical variables, pathological features, and oncogenic, molecular and immunologic variables. In the past years it has been a slow move from using the Duke's and Astler-Coller staging systems to using the TNM classification, a more precise description of the primary tumour, and the number of lymph involved (19).

When looking at the tumour stage PLGF-1 and PGF-2 appear to have a high level of expression in the earliest stages, I and II respectively. We can demonstrate from these results that PLGF-1 and PGF-2 are powerful predictors for a poor prognosis in patients based on the results of clinical outcome we have explained before.

Duke's system classification, first proposed by Dr Cuthbert E. Duke in 1932, has four classifications although we focused on the first three, which are Duke's A (tumour confined within the muscle layer), Duke's B (tumour has spread through the muscle layer but does not involve any lymph nodes) and Duke's C (any tumour involving the lymph nodes). Our results for PLGF-1 showed a high expression in Duke's C while PGF-2 does not show any significant difference among the three classifications. Thus, PLGF-1 correlates with a poor prognosis for patient described before.

The other method of classification is the TNM system which is much more precise than the Duke's classification. It compartmentalizes carcinomas according to the depth of invasion of the primary tumour, the absence or presence of

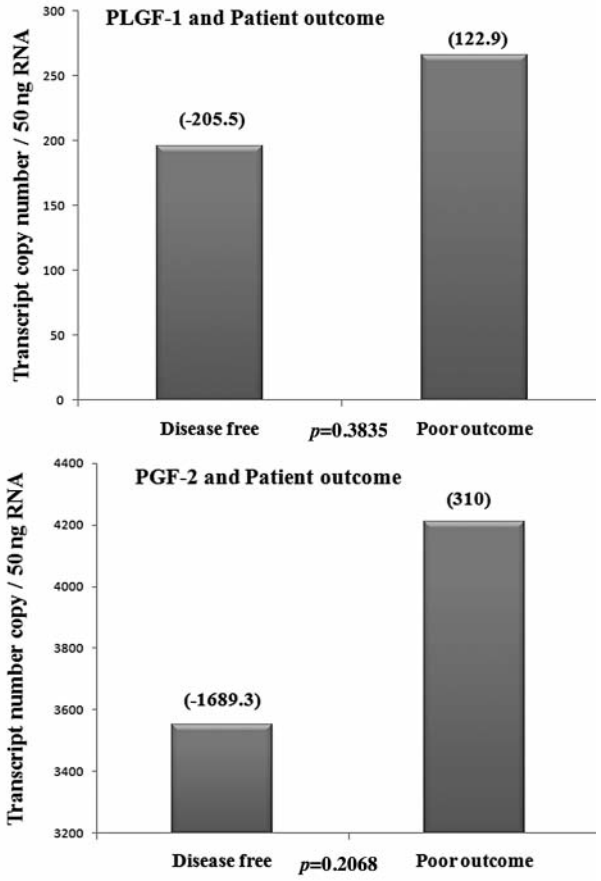

Figure 4. *Comparison of transcript levels of PLGF-1 and PGF-2 in correlation with clinical outcome.*

regional lymph node metastases, and the absence or presence of distant metastases. Tumours are grouped under stages I through IV. High levels of PLGF-1 were found in TNM 3 while PGF-2 presents a high level in TNM 4, demonstrating again the association of high levels of both isoforms with a poor prognosis in patient.

Lymph node status remains one of the most important prognostic factors in the prognosis of colorectal cancer. Node negative patients are usually not treated with adjuvant chemotherapy outside a clinical trial because of the lack of definitive evidence of survival benefit. Patients with node positive, on the other hand, should be treated with adjuvant chemotherapy because of the potential reduction of mortality up to 33% (20).

Our results for PLGF-1 and PGF-2 levels are dramatically elevated in both, node positive tumours and node negative tumours, thus, PLGF-1 and PGF-2 might be useful markers to select a correct moment for chemotherapy: after surgery (adjuvant), before surgery (neo-adjuvant) or primary therapy (palliative).

We can conclude that PLGF-1 and PGF-2 expression correlates with the disease progression and survival status, consistent with the results from Wei SC, Tsao PN, Yu SC *et al*. (7) although these results are irrespective of isoform.

These results suggest that both PLGF-1 and PGF-2 may play roles in colorectal cancer cancer formation and tumour progression, consistent with the hypothesis of Carmelit P, Moons L, Luttun A *et al*. (21) that PLGF exert synergistic effect on VEGF driven angiogenesis under pathological conditions.

In summary, this study has shown that PLGF-1 and PGF-2 expression of the angiogenic factor, PGF, are increased in colorectal tumour tissues than in normal colorectal tissue, and displays prognostic value in colorectal cancer patients. This indicates that blocking/targeting PLGF-1 and PGF-2 expression may have a promising therapeutic future in colorectal cancer. Although, these isoforms require further study to elucidate their role in colorectal cancer.

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