Chapter 7

Angiogenic markers Endoglin and Vascular Endothelial Growth Factor in Gastroenteropancreatic Neuroendocrine Tumours

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Abstract

Gastroenteropancreatic neuroendocrine tumours (GEP-NETs) are uncommon, heterogeneous neoplastic lesions. Angiogenesis, the process of new blood vessel formation, is required for tumour growth, progression and the development of metastases. This process is induced by several growth factors, including vascular endothelial growth factor (VEGF), and transforming growth factor beta 1 (TGF-β1). Endoglin is a co-receptor for TGF-β1 and a marker for angiogenic endothelial cells. The aim of the present study was to evaluate the expression and potential prognostic role of VEGF and endoglin in GEP-NETs.

Microvessel density (MVD) in GEP-NETs was evaluated using endoglin and CD31 immunohistochemistry. In addition, tissue levels of endoglin and VEGF were determined in homogenates by ELISA.

Endoglin was highly expressed on tumour endothelial cells. CD31 microvessel density in GEP-NETs was significantly higher compared to endoglin MVD. Two to four-fold higher tissue levels of endoglin and VEGF were seen in tumours compared to associated normal tissue. This increased endoglin tissue expression in tumours was significantly related to tumour size, presence of metastases and a more advanced tumour stage, whereas expression of VEGF was not.

Based on these findings, we suggest endoglin to be a potential marker to detect present and to predict future metastases. Assessment of endoglin tumour levels provides information on tumour aggressiveness which might be useful in the post-resection therapeutic approach of patients with GEP-NETs.

Introduction

Gastroenteropancreatic neuroendocrine tumours (GEP-NETs), including gastrointestinal carcinoids and pancreatic neuroendocrine tumours (PNETs), comprise a very heterogeneous group of neoplasia, with respect to tumour biology, histocytopathology and prognosis[1]. Despite a slow-growing nature, they are primarily malignant[2]. Angiogenesis, the formation of new blood vessels from the existing vascular bed, is a crucial process in tumour progression. When tumours reach a size of approximately 1 or 2 mm, they become dependent on neovascularisation, not only to provide them with nutrients and oxygen, but also as an exit route for metabolic waste products, further growth of the primary tumour, and eventually, metastatic spread[3]. One of the key factors in angiogenesis is vascular endothelial growth factor (VEGF) which has numerous effects on endothelial cells (ECs), including induction of migration and differentiation[4]. Several studies have addressed the prognostic implications of VEGF in patients with GEP-NETs, and trials investigating the action of the anti-VEGF antibody bevacizumab in patients with GEP-NETs are ongoing[5,6].

Another important growth factor, with a pivotal role in angiogenesis is transforming growth factor beta 1 (TGF-β1), a multifunctional cytokine that is involved in numerous physiological and pathological processes[7]. Endoglin (CD105) is a co-receptor for TGF-β1. As a result of its principal expression on ECs of newly formed blood vessels, several studies have suggested that endoglin is a specific marker of neovascularisation in various cancer types[8-10]. In pancreatic carcinomas, high endoglin microvessel density (MVD) has been found to be related to shorter survival and therefore, is suggested to be a prognostic marker[11]. In colorectal cancer, the vessel count by positive endoglin staining is able to identify patients at high risk of metastases[12].

In the present study, we assessed the tissue expression and levels of two key players in the process of angiogenesis, namely endoglin and VEGF, to assess their potential clinical implications in patients with GEP-NETs.

Materials and Methods

Patients

After surgical removal, tumour tissues were collected at the Department of Gastroenterology, Leiden University Medical Centre (LUMC), Leiden, and either frozen at -80◦C and/or embedded in paraffin for immunohistochemical staining. Sixty-eight homogenates (27 tumour samples and 41 normal samples) of 27 patients were available for the determination of tissue levels of endoglin. For the measurement of VEGF levels, one tumour sample was exhausted; therefore, the total number of tumour samples comprises 26. For CD31 and endoglin immunostaining, 50 and 49 samples, respectively, of 39 patients, were available. For most patients, but not all, both homogenates and paraffin slides were available. In total, 41 patients with GEP-NETs were included. GEP-NETs comprised pancreatic neuroendocrine tumours (PNETs) and gastrointestinal neuroendocrine tumours, which were also referred to as 'carcinoids'.

Clinicopathological information was obtained by evaluation of patients' medical files and pathology reports, when available. According to the classification of the World Health Organization for GEP-NETs, tumours were categorized into well-differentiated neuroendocrine tumour (NET), well-differentiated neuroendocrine carcinoma (NEC), or poorly differentiated NEC[13]. From some patients, the WHO classification was not assessable due to the lack of specified classification. This study was performed according to the guidelines of the Medical Ethics Committee of the LUMC in compliance with the Helsinki Declaration.

Immunohistochemistry

Immunohistochemistry was performed as follows. Tissues were fixed in formalin, embedded in paraffin and cut into 5 µm sections. After deparaffinisation and rehydration, endogenous peroxidases were blocked in methanol containing 0.3% H_20_2 (Merck, Darmstadt, Germany). Antigen retrieval was performed by boiling in 0.01M citrate buffer pH 6.0 for 10 minutes. Slides were incubated overnight at room temperature (RT) with primary antibodies: biotinylated goat anti-human endoglin (1:200, R&D Systems Europe, Abingdon, UK), or mouse monoclonal anti-

CD31 (1:400, Dako, Glostrup, Denmark) diluted in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA), as described previously[14]. Immunodetection was performed with a biotinylated goat anti-mouse antibody (for CD31) and horseradish peroxidase (HRP)-streptavidin complex (both Dako) for 30 minutes at RT. Staining was visualized using 0.05% 3,3'-diaminobenzidine (DAB, Sigma, Darmstadt, Germany) containing 0.0038% H_20_2. Colon carcinomas were used as positive controls. Negative controls were included by omitting the primary antibodies. Representative photomicrographs were taken with an Olympus BX-51TF microscope equipped with a DP23-3-5 camera.

The endoglin and CD31 MVD in the tumour-bearing area were quantified by computerized analysis. Four representative tumour areas for either endoglin or CD31 were selected and photographed at a 100x magnification. Images were binarized and the extent of staining was quantified using ImageJ 1.43u (National Institutes of Health, Bethesda, MD, U.S.A.). Finally, the average MVD out of four photographs was taken. The microvessel quantification was performed blinded, that is, without knowledge of patients or tumour characteristics, and expressed as the number of pixels per field x 1,000.

Quantitative human endoglin and VEGF determinations in tissue samples

Tissues were homogenized and protein concentrations were determined according to Lowry *et al.*[14,15]. Endoglin levels were determined in tissue homogenates, using a commercially available quantitative immunoassay (ELISA) for human endoglin, performed according to the manufacturer's instructions (R&D Systems), as described before[14]. VEGF tissue levels were determined using a commercially available duoset (R&D Systems) as described before[16]. Endoglin and VEGF levels were expressed as ng/mg and pg/mg protein, respectively.

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences version 16 (SPSS) and GraphPad Prism version 5. Unpaired *t* test and one-way ANOVA were used to compare mean levels of endoglin and VEGF between

various data sets. Orthogonal regression analysis and Pearson's correlation (*r*) were used to explore the relationship between two variables. Survival curves were plotted using the method of Kaplan and Meier. Results are reported as mean ± S.E. A p-value of <0.05 was considered statistically significant.

Results

Overall, 41 patients with NETs were included (Table 1) of which the majority were female. Most patients (28/41) had a solitary primary tumour, while 13/41 patient had multiple primaries. Primary tumours of 23/41 patients were localized in the pancreas, 5/41 in the duodenum, 10/41 in the small bowel , 1/41 in the appendix, 1/41 in the sigmoid, and in one patient, the exact primary tumour location was unknown. Functional tumours were mainly insulinomas (42.1%) and gastrinomas (52.6%). Tumour size was significantly different between the groups, *P*=0.01, with a smaller tumour size for functional PNETs. Metastases were seen in the majority of patients, with an almost equal distribution of lymph node or liver location. Angioinvasion was present in only 18.3% of the tumours.

Endoglin and VEGF tissue levels were measured in 27 tumour samples from 18 patients with GEP-NETs. Endoglin and VEGF levels were significantly increased in tumours compared to (associated) normal tissues (Table 2). However, among the various types of GEP-NETs, both endoglin and VEGF levels were comparable. Interestingly, metastatic tumours showed significantly higher endoglin levels compared to those in primary lesions. VEGF levels were also increased in metastases, although not significantly. Furthermore, well-differentiated NECs showed significantly higher endoglin levels compared to well-differentiated NETs. Again, this difference in VEGF levels was not statistically significant, although levels in well-differentiated NECs were also increased. Of particular interest, we observed that primary tumour tissues of patients who had developed (lymph node or liver) metastases displayed significantly higher endoglin levels than from those without metastases. Neither endoglin nor VEGF levels were (significantly) related to other clinicopathological parameters including patients' age, sex, the

hormonal status (i.e., functional or non-functional) of the PNETs, or the presence of angioinvasion.

Table 1. Patient and tumour characteristics			
Patients (n=41)		**Tumours (n=60)**	
Age	**Years**	**Primary or metastatic**	**n (%)**
Mean ± s.d.	47 ± 14	Primary	45 (75.0%)
Range	20 - 77	Metastasis	15 (25.0%)
Sex	**n (%)**	**Angioinvasion**	**n (%)**
Male	17 (41.5%)	Present	11 (18.3%)
Female	24 (58.5%)	Absent	49 (81.7%)
Tumour type	**n (%)**	**Tumour size**	**Mean ± s.d. (cm)**
Carcinoid	12 (29.3%)	Carcinoids	3.4 ± 2.7
Functional PNET	19 (46.3%)	Functional PNETs	1.9 ± 1.7
Non-functional PNET	10 (24.4%)	Non-functional PNETs	3.6 ± 2.4
Tumour grade	**n (%)**		
Well-differentiated NET	13 (31.7%)		
Well-differentiated NEC	26 (63.4%)		
Poorly differentiated NEC	1 (2.4%)		
Unknown	1 (2.4%)		
Metastases	**n (%)**		
Present	26 (63.4%)		
Lymph node only	9 (34.6%)		
Liver only	7 (26.9%)		
Both	10 (38.5%)		
Absent	15 (36.6%)		

Table 1. Patient and tumour characteristics.

Endoglin tissue levels, but not tissue levels of VEGF, were found to increase with tumour size (Figure 1). Finally, endoglin tumour levels showed no significant correlation with VEGF tumour levels (r=0.11 with P=0.59).

Figure 1. Orthogonal linear regression analysis of tumour size and endoglin levels

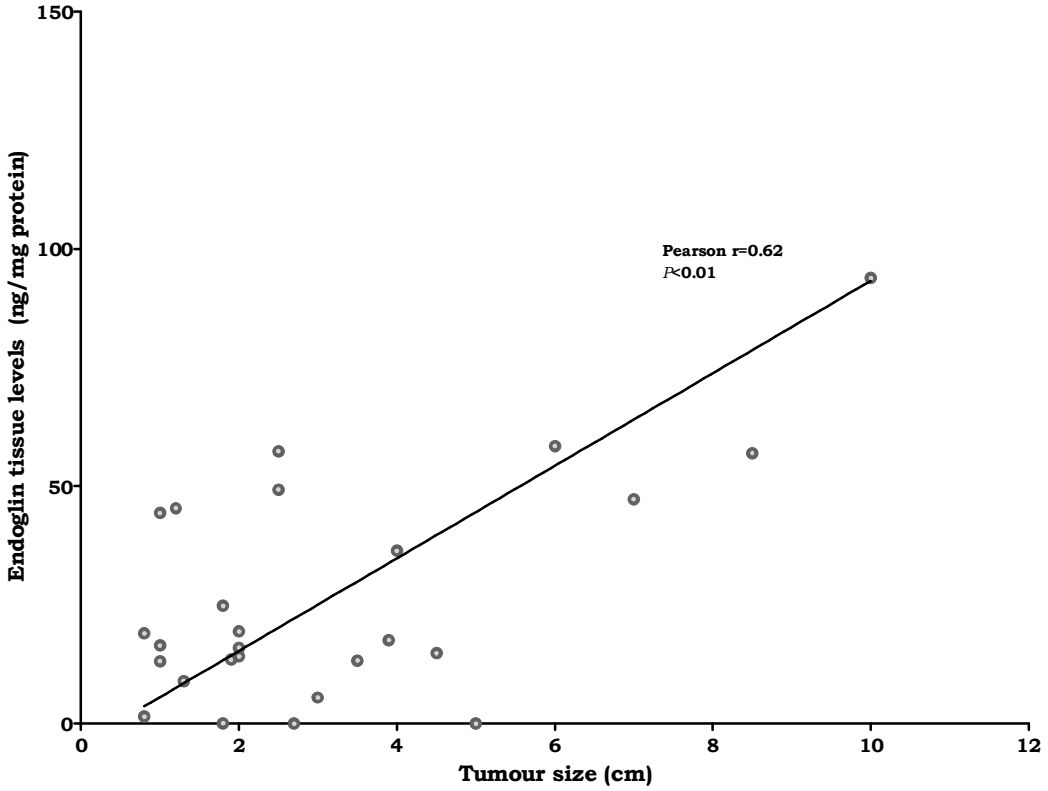

Figure 1. Orthogonal regression analysis of endoglin tissue levels and tumour size (n=26). Increasing endoglin levels in tumours are significantly correlated with a greater tumour size, r=0.62 with p<0.01.

The immunohistochemical expression of endoglin and CD31 was analyzed in 39 patients with GEP-NETs. All tumours showed expression for CD31 and endoglin on intratumour vascular ECs. Endoglin expression was mainly observed on ECs of small tumour-associated blood vessels, while its expression in normal, non-tumourous tissue was weak or negative, in contrast to CD31 staining (Figure 2).

Figure 2. Immunostaining of gastroenteropancreatic neuroendocrine tumours

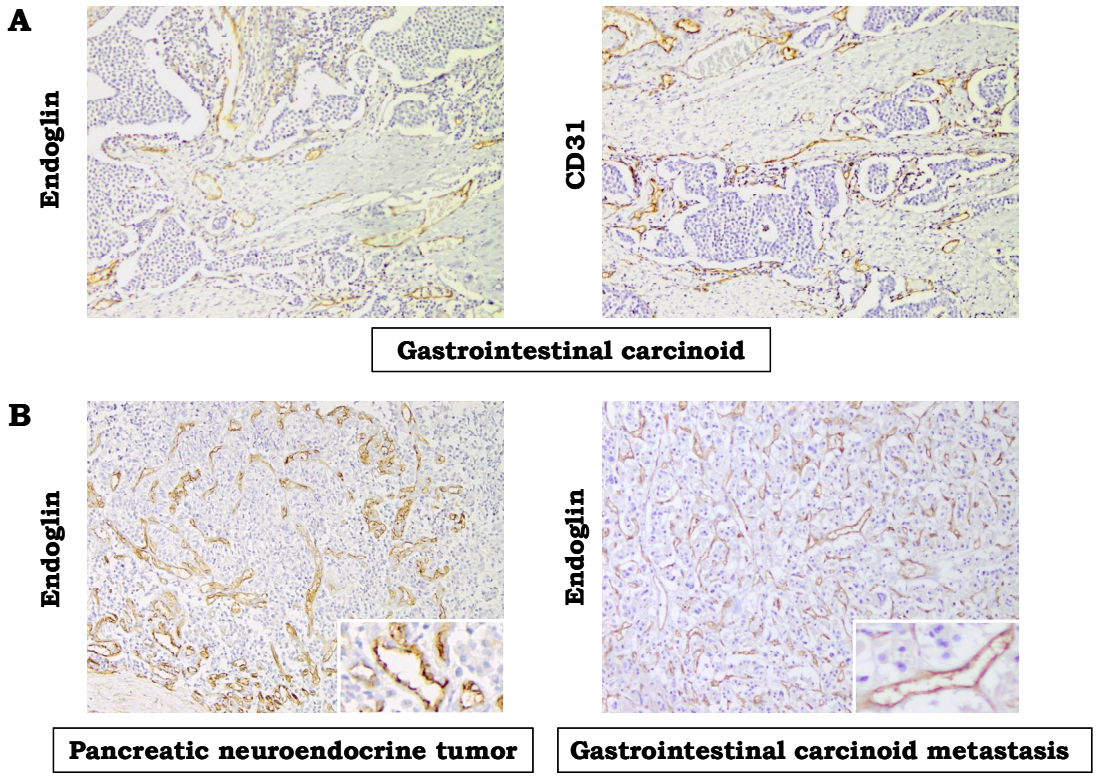

Figure 2. Immunostaining of endoglin and CD31 on peritumoural and intratumoural vessels in GEP-NETs. A) Endoglin staining is limited to angiogenic vessels, whereas CD31 stains both old and new blood vessels in tumour tissue. Magnification 100x. B) Representative endoglin staining in a pancreatic neuroendocrine tumour and a gastrointestinal carcinoid metastasis (mesenterium of small bowel). Magnification 100x. Inserts show a higher magnification at 200x.

The CD31 MVD was found to be significantly higher than the endoglin MVD in 73% of the tumour samples, $P<0.01$. No significant differences in endoglin and CD31 MVD were observed between carcinoids and PNETs (Table 3). Furthermore, both endoglin and CD31 MVD were not significantly related to clinicopathological parameters such as patients' age, sex, tumour size, functionality, and angioinvasion.

Table 2. Mean endoglin and VEGF levels in GEP-NETs in relation to clinicopathological parameters

	Endoglin (ng/mg)				VEGF (pg/mg)			
	n	Mean	S.E.	*P*	*n*	Mean	S.E.	*P*
Tissues								
Normals	38	12.1	2.0	**<0.01**	38	75.0	9.5	**<0.01**
Tumours	27	26.8	4.5		26	316.8	46.0	
Tumour type - tumours								
Carcinoid	8	35.3	11.4	0.37	8	354.9	72.0	0.67
Functional PNET	14	25.4	4.7		13	274.4	46.7	
Non-functional PNET	5	16.8	8.7		5	366.2	186.8	
Origin								
Primary tumours	19	18.8	3.9	**<0.01**	18	293.2	52.0	0.45
Metastastic tumours	8	45.7	9.0		8	369.9	95.8	
WHO classification								
Well-differentiated NETs	6	7.6	5.2	**0.02***	6	200.2	52.8	0.21*
Well-differentiated NECs	20	32.9	4.0		19	328.5	60.2	
Poorly-differentiated NECs	1	19.0	ND		1	795.0	ND	
Primary tumours: Metastases								
Present	12	24.8	5.2	**0.04**	11	339.5	76.4	0.28
Absent	7	8.5	3.5		7	220.6	54.8	

Table 2. Mean values of endoglin and VEGF levels in GEP-NETs in relation to major clinicopathological parameters. Bold p-values are considered statistically significant.
*Result of unpaired t-test to compare well-differentiated NETs with well-differentiated NECs.

Table 3. MVD scores in GEP-NETs in relation to clinicopathological parameters								
	MVD-endoglin				**MVD-CD31**			
	n	**Mean***	**S.E.***	***P***	***n***	**Mean***	**S.E.***	***P***
Tumour type - tumours								
Carcinoid	11	55	107	0.30	13	123	23	0.75
Functional PNET	24	65	8		23	106	18	
Non-functional PNET	14	85	18		14	100	17	
Origin								
Primary tumours	36	66	8	0.58	37	111	13	0.69
Metastatic tumours	13	75	15		13	101	24	
WHO classification								
Well-differentiated NETs	13	69	18	*0.93***	13	76	12	*0.08***
Well-differentiated NECs	33	67	7		34	121	15	
Poorly-differentiated NECs	1	212	x		1	82	x	
Primary tumours: Metastases								
Present	19	66	9	0.96	20	138	18	**0.05**
Absent	17	67	14		17	88	15	

Table 3. MVD determined by endoglin and CD31 in GEP-NETs in relation to clinicopathological parameters. Bold p-values are considered statistically significant. *Values x 1,000 pixels per area. ** Result of unpaired *t* test to compare well-differentiated NETs with well-differentiated NECs.

Endoglin and CD31 MVD were significantly correlated with endoglin tumour levels; r=0.64 with P<0.01 (Figure 3) and r=0.58 with P<0.01, respectively. VEGF tumour levels were not correlated with endoglin MVD (r=0.28 with P=0.25), but were borderline significantly correlated with CD31 MVD, r=0.43 with P=0.07.

Figure 3. Correlation between MVD and tissue levels of endoglin in tumours

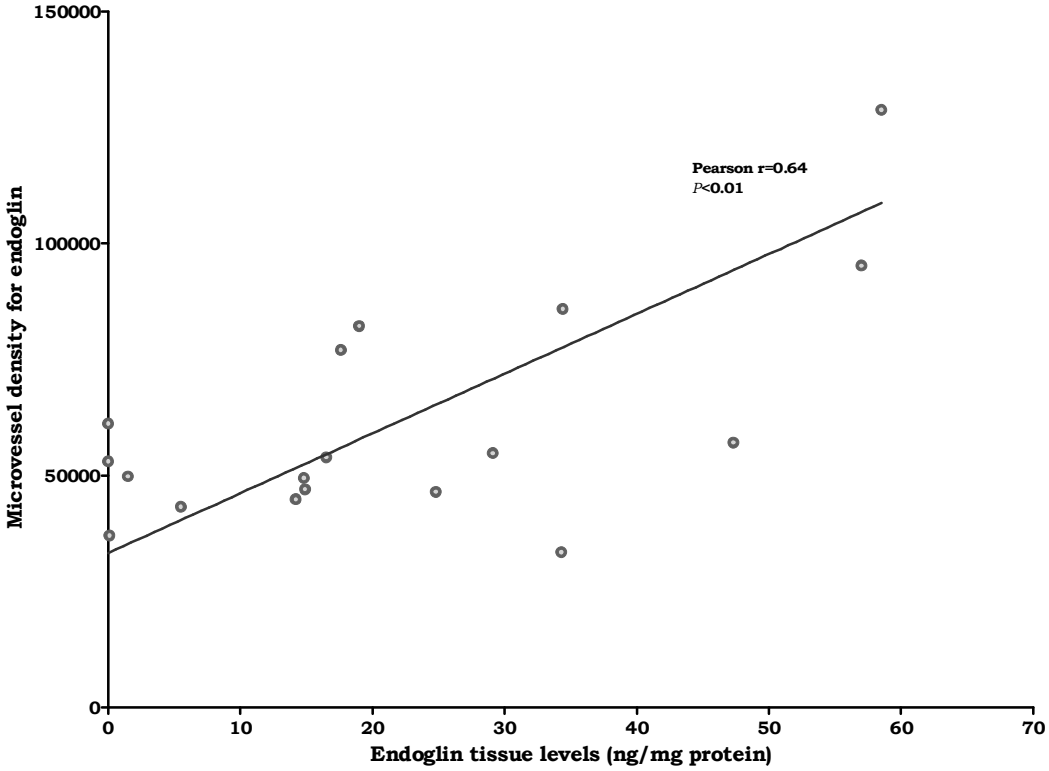

Figure 3. Correlation analysis of the endoglin MVD and endoglin tissue levels in tumours (n=17). For one patient in whom endoglin tissue levels were assessed, no paraffin slides for MVD determination was available. Endoglin MVD is significantly correlated with tumour levels of endoglin, r=0.64 with p<0.01.

To evaluate the prognostic potential of endoglin and VEGF tissue levels, Kaplan Meier survival analysis was performed (Figure 4) by dividing the patients into two groups (i.e. low versus high) using the mean value of endoglin and VEGF tumour levels (Table 2). Both endoglin and VEGF tissue levels were not significantly related to patient survival. Furthermore, patients were divided into two groups based on the MVD of endoglin and CD31. Both parameters were not significantly correlated with overall survival of these patients.

Figure 4a. Survival analysis on tissue levels

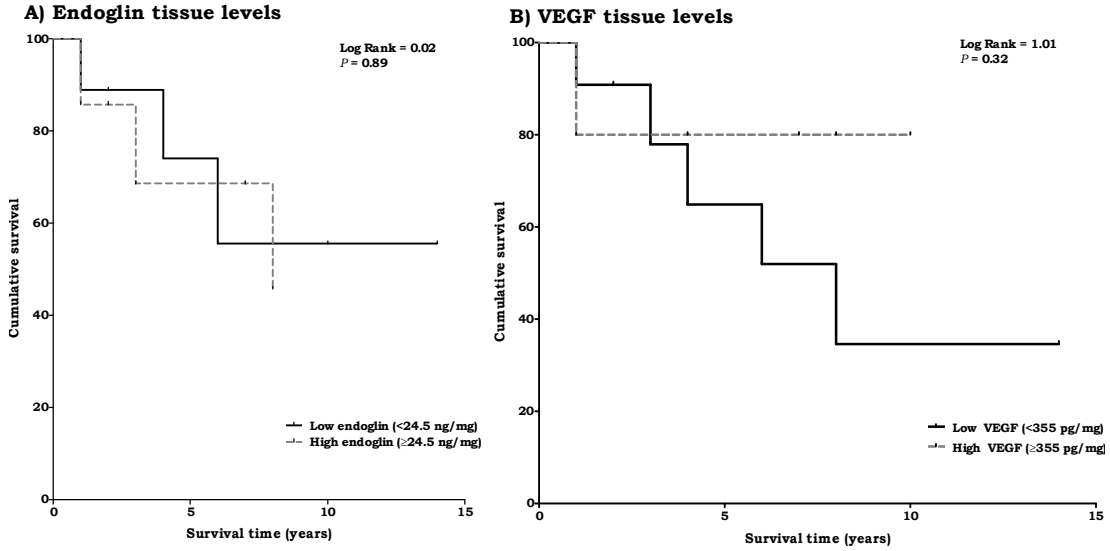

Figure 4b. Survival analysis on MVD

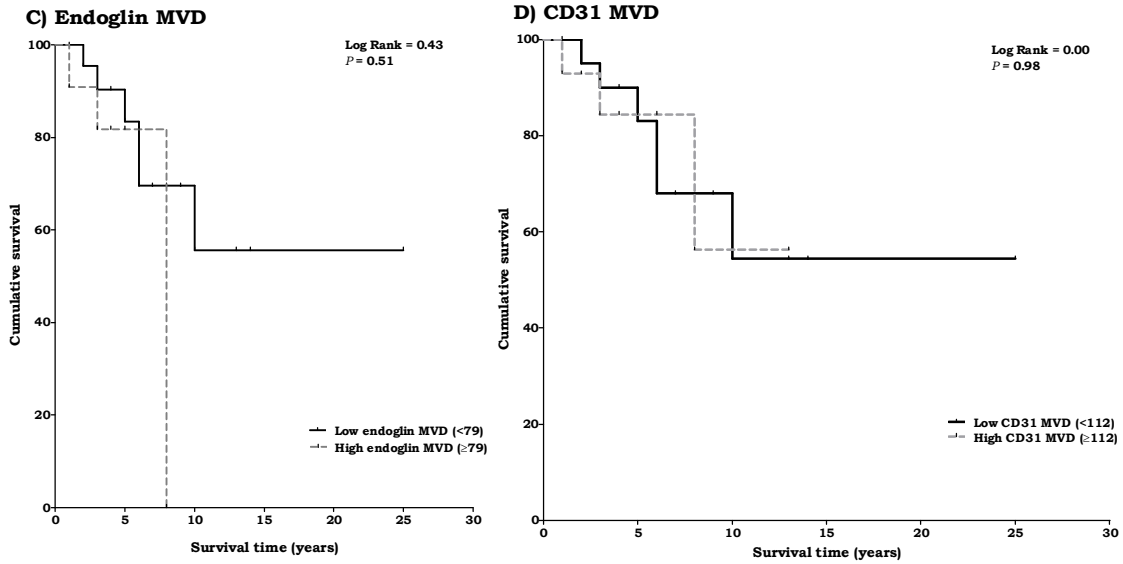

Figure 4. Kaplan Meier survival analysis for endoglin tumour levels (a), VEGF tumour levels (b), endoglin MVD (c) and CD31 MVD (d). Patients were divided into two groups based on mean tumour levels (a,b) or mean MVD-scores (c,d). None of the parameters showed a significant relation with survival of the patients.

Discussion

In this study, we observed that the expression of the angiogenic cell marker endoglin was related to tumour size, aggressiveness and metastatic potential in patients with GEP-NETs, whereas expression of another key player in angiogenesis, namely VEGF, was not.

In general, GEP-NETs are highly vascularised. In recent years it has become clear that angiogenesis has important effects on tumour progression in several cancers,

and the therapeutic role of angiogenesis inhibitors in the treatment of cancers is increasing[17,18]. In this study, we investigated whether endoglin and VEGF were related to any clinicopathological characteristics of GEP-NETs and evaluated their potential prognostic implications.

By immunohistochemistry, we observed high endoglin expression on vascular ECs in tumour tissues of GEP-NETs. In contrast to CD31, immunopositivity of endoglin was mainly observed on newly formed blood vessels, which indicates that endoglin is more representative of tumour neovascularisation than the pan-endothelial marker CD31.

Furthermore, we found that endoglin tissue levels were significantly higher in tumours compared to normal tissues. Interestingly, we observed that an increased endoglin expression was indicative of metastatic disease. Endoglin levels were higher in metastases compared to primary tumours, and primary tumours with metastases showed higher endoglin levels compared to tumours without metastases. Additionally, endoglin levels were increased in well-differentiated NECs compared to well-differentiated NETs, and higher endoglin levels were related to larger tumour size in patients with GEP-NETs. In several cancers, the extent of tumour angiogenesis was shown to be reflective of their potency to become invasive and form metastases[19,20]. Our data indicate that tissue endoglin may serve as a potential assessment marker for the tumour aggressiveness (i.e., NEC versus NET) and the presence of metastases following tumour resection. In the context of anti-cancer therapy, anti-endoglin treatment might provide a new effective anti-angiogenic strategy for GEP-NETs, but more research is needed. However, several promising *in vivo* and *in vitro* studies using anti-endoglin antibodies for anti-cancer treatment have recently been published[21].

In the present study, we did not evaluate the immunohistochemical expression of VEGF. High immunoexpression of VEGF on GEP-NETs has already been shown by others, but opposing results regarding the prognostic role of VEGF in these tumours have been reported; Takahashi *et al.* found no correlation of VEGF-A immunoexpression with growth of blood vessels, haematogenous spread or tumour growth in pancreatic endocrine tumours. In contrast, Zhang *et al.* have

revealed that strong expression of VEGF was associated with increased angiogenesis and poor prognosis in patients with GEP-NETs [22,23]. However, we determined tissue VEGF expression in GEP-NETs and found that VEGF tissue levels showed a similar pattern to endoglin, but were not significantly related to any clinicopathological parameter. Therefore, we assume that, although VEGF is most likely to be involved in the process of neoplastic blood vessel formation in GEP-NETs, this key mediator of angiogenesis is not the appropriate prognostic marker in these tumours. In contrast, our data suggest that endoglin can function as a predictive marker for the development of metastases in GEP-NETs. Endoglin is a co-receptor for TGF-β1. Among the various members of the TGF-β family, TGF-β1 is mostly involved in cancer, and has been shown to stimulate angiogenesis[24]. Endoglin is an important modulator of the TGF-β response, particularly in tumour pathogenesis[25]. In another study by our group, strongly increased tissue levels of endoglin were observed in colorectal cancers, whereas premalignant lesions displayed endoglin levels comparable to those in normal tissues, which supports the pivotal role of endoglin in tumour progression[14].

The fact that neither endoglin nor VEGF levels were associated with patient survival might be due to the relatively good prognosis of the patients. Gastrointestinal carcinoids show a 5-year survival rate of about 70%, whereas PNETs have a reported 5-year survival rate ranging from 25 to 100%, even in the case of (unresectable) liver metastases[26,27]. In our study cohort, 10/18 patients in whom endoglin or VEGF levels were determined were still alive at the end of the study (median survival 8 years), which makes it unlikely to use one of these parameters as a predictor of outcome or survival marker. However, our data support a role for endoglin in identifying patients with GEP-NETs at risk for metastasis.

It is worth reiterating that the current study involved a relatively small number of patients. Nevertheless, GEP-NETs are a rare disease with a low incidence, which leads to general scarcity of patients and samples. However, we believe that the significant differences observed here are representative and illustrate the differential expression pattern of endoglin and VEGF among GEP-NETs.

In conclusion, we suggest that endoglin is a potential marker to predict present and future metastases, which might help to optimize the therapeutic approach in patients with GEP-NETs.

References

1. Barakat MT, Meeran K, Bloom SR. Neuroendocrine tumours. *Endocr Relat Cancer* 2004;11:1-18.
2. Poncet G, Villaume K, Walter T, Pourreyron C, Theillaumas A, Lépinasse F, Hervieu V, Cordier-Bussat M, Scoazec JY, Roche C. Angiogenesis and tumor progression in neuroendocrine digestive tumors. *J Surg Res* 2009;154:68-77.
3. Folkman J. Tumor angiogenesis. *Adv Cancer Res* 1974; 19: 331-358.
4. Veikkola T, Alitalo K. VEGFs, receptors and angiogenesis. *Semin Cancer biol* 1999;9:211-220.
5. Strosberg JR, Kvols LK. A review of the current clinical trials for gastroenteropancreatic neuroendocrine tumours. *Expert Opin Investig Drugs* 2007;16:219-224.
6. Yao JC, Phan A, Hoff PM, Chen HX, Charnsangavej C, Yeung SC, Hess K, Ng C, Abbruzzese JL, Ajani JA. Targeting vascular endothelial growth factor in advanced carcinoid tumour: a random assignment phase II study of depot octreotide with bevacizumab and pegylated interferon alpha-2b. *J Clin Oncol* 2008;26:1316-1323.
7. Hawinkels LJ, Verspaget HW, van Duijn W, van der Zon JM, Zuidwijk K, Kubben FJ, Verheijen JH, Hommes DW, Lamers CB, Sier CF. Tissue level, activation and cellular localisation of TGF-β1 and association with survival in gastric cancer patients. *Br J Cancer* 2007;97:398-404.
8. Li C, Guo B, Bernabeu C, Kumar S. Angiogenesis in breast cancer: the role of transforming growth factor beta and CD105. *Microsc Res Tech* 2001;52:437–449.
9. Fonsatti E, Altomonte M, Nicotra MR, Natali PG, Maio M. Endoglin (CD105): a powerful therapeutic target on tumour-associated angiogenetic blood vessels. *Oncogene* 2003;22:6557–6563.
10. Zijlmans HJ, Fleuren GJ, Hazelbag S, Sier CF, Dreef EJ, Kenter GG, Gorter A. Expression of endoglin (CD105) in cervical cancer. *Br J Cancer* 2009;100:1617-1626.
11. Yoshitomi H, Kobayashi S, Ohtsuka M, Kimura F, Shimizu H, Yoshidome H, Miyazaki M. Specific expression of endoglin (CD105) in endothelial cells of intratumoral blood and lymphatic vessels in pancreatic cancer. *Pancreas* 2008;37:275-281.
12. Romani AA, Borghetti AF, Del Rio P, Sianesi M, Soliani P. The risk of developing metastatic disease in colorectal cancer is related to CD105-positive vessel count. *J Surg Oncol* 2006;93:446-455.
13. Rindi G, Klöppel G. Endocrine tumors of the gut and pancreas tumor biology and classification. *Neuroendocrinology* 2004;80:12-15.
14. Hawinkels LJ, Kuiper P, Wiercinska E, Verspaget HW, Liu Z, Pardali E, Sier CF, ten Dijke P. Matrix Metalloproteinase-14 (MT1-MMP)-mediated endoglin shedding inhibits tumor angiogenesis. *Cancer Res* 2010;70:4141-4150.

15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265–275.
16. Hawinkels LJ, Zuidwijk K, Verspaget HW, de Jonge-Muller ES, van Duijn W, Ferreira V, Fontijn RD, David G, Hommes DW, Lamers CB, Sier CF. VEGF release by MMP-9 mediated heparan sulphate cleavage induces colorectal cancer angiogenesis. *Eur J Cancer* 2008;44:1904-1913.
17. Quesada AR, Muñoz-Chápuli R, Medina MA. Anti-angiogenic drugs: from bench to clinical trials. *Med Res Rev* 2006;26:483-530.
18. Nussenbaum F, Herman IM. Tumor angiogenesis: insights and innovations. *J Oncol* 2010; Epub 2010 Apr 26.
19. Weidner N, Carroll PR, Flax J, Blumenfeld W, Folkman J. Tumour angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am J Pathol* 1993;143:401-409.
20. Weidner N, Semple JP, Welch WR, Folkman J. Tumour angiogenesis and metastasis – correlation in invasive breast carcinoma. *N Engl J Med* 1991;324:1-8.
21. Fonsatti E, Nicolay HJ, Altomonte M, Covre A, Maio M. Targeting cancer vasculature via endoglin/CD105: a novel antibody-based diagnostic and therapeutic strategy in solid tumours. *Cardiovasc Res* 2010;86:12-19.
22. Zhang J, Jia Z, Li Q, Wang L, Rashid A, Zhu Z, Evans DB, Vauthey JN, Xie K, Yao JC. Elevated expression of vascular endothelial growth factor correlates with increased angiogenesis and decreased progression-free survival among patients with low-grade neuroendocrine tumours. *Cancer* 2007;109:1478-1486.
23. Takahashi Y, Akishima-Fukasawa Y, Kobayashi N, Sano T, Kosuge T, Nimura Y, Kanai Y, Hiraoka N. Prognostic value of tumor architecture, tumor-associated vascular characteristics, and expression of angiogenic molecules in pancreatic endocrine tumors. *Clin Cancer Res* 2007;13:187-196.
24. Bierie B, Moses HL. Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. *Nat Rev Cancer* 2006;6:506-520.
25. Bernabeu C, Lopez-Novoa JM, Quintanilla M. The emerging role of TGF-β superfamily coreceptors in cancer. *Biochim Biophys Acta* 2009;1792:954-973.
26. Modlin IM, Oberg K, Chung DC, Jensen RT, de Herder WW, Thakker RV, Caplin M, Delle Fave G, Kaltsas GA, Krenning EP, Moss SF, Nilsson O, Rindi G, Salazar R, Ruszniewski P, Sundin A. Gastroenteropancreatic neuroendocrine tumours. *Lancet Oncol* 2008;9:61-72.
27. Metz DC, Jensen RT. Gastrointestinal neuroendocrine tumors: pancreatic endocrine tumors. *Gastroenterology* 2008;135:1469-1492.