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Angiogenesis: A prognostic determinant in pancreatic cancer?

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ABSTRACT

Angiogenesis has been associated with disease progression in many solid tumours, however the statement that tumours need angiogenesis to grow, invade and metastasise seems no longer applicable to all tumours or to all tumour subtypes. Prognostic studies in pancreatic cancer are conflicting. In fact, pancreatic cancer has been suggested an example of a tumour in which angiogenesis is less essential for tumour progression.

The aim of the present study was therefore to measure angiogenesis in two anatomically closely related however prognostically different types of pancreatic cancer, pancreatic head and periampullary cancer, and investigate its relation with outcome.

Vessels were stained by CD31 on original paraffin embedded tissue from 206 patients with microscopic radical resection (R0) of pancreatic head ($n = 98$) or periampullary cancer ($n = 108$). Angiogenesis was quantified by microvessel density (MVD) and measured by computerised image analysis of three randomly selected fields and investigated for associations with recurrence free survival (RFS), cancer specific survival (CSS), overall survival (OS) and conventional prognostic factors.

MVD was heterogeneous both between and within tumours. A higher MVD was observed in periampullary cancers compared with pancreatic head cancers ($p < .01$). Furthermore, MVD was associated with lymph node involvement in pancreatic head ($p = .014$), but not in periampullary cancer ($p = .55$). Interestingly, MVD was not associated with RFS, CSS or with OS.

In conclusion, angiogenesis is higher in periampullary cancer and although associated with nodal involvement in pancreatic head cancer, pancreatic cancer prognosis seems indeed angiogenesis independent.

1. Introduction

Pancreatic cancer has a highly invasive and metastatic potential. Up to 80% of patients present with locally advanced disease or distant metastasis, precluding them from resection. And even following curative resection, recurrent disease remains a major problem, almost half of the patients relapse within the first year.[1] Several attempts have been made to im-

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prove outcome by adjuvant treatment regimens, however with disappointing results.[2]

Disease progression through to the formation of metastasis is a highly selective multi-step process, dependant on both tumour characteristics and environmental factors. Angiogenesis plays an important role in tumour growth and progression by supplying necessary oxygen, growth factors and nutrients, as well as by facilitating the dissemination of tumour cells.[3–7] In an attempt to translate observations from experimental models to clinical practice, quantification of tumour vessels has been performed and correlations with clinicopathological factors and outcome have been investigated. Irrespective of the method used, the amount of tumour microvessels was associated with recurrent disease and poor survival in several primary tumours, including melanoma,[8] breast,[9–19] lung cancer,[20–22] prostate,[23] bladder,[24] ovary,[25] colorectal carcinoma,[26] hepatocellular carcinoma[27] and also in their metastatic counterparts.[28] However, not all studies identified a negative relation with outcome,[29–37] a study on colorectal cancer[38] and one on node negative breast cancer[39] even suggested a beneficial effect of a higher microvessel density on prognosis. Quantification of tumour microvessels has also been performed in some relatively small sized studies on pancreatic cancer, however again results are conflicting.[40–48]

Not all tumours need angiogenesis to grow, invade and metastasise. Some tumours apply alternative mechanisms such as co-option, mosaicism, vasculogenesis or intussesceptive vascular growth, to obtain blood vessels.[49] Furthermore, the role of angiogenesis as a prognostic marker cannot be generalised to all tumour types. Pancreatic cancer was opted an example of a tumour type that is less dependent on angiogenesis for tumour progression. Even within a certain type of cancer, subtypes are not necessarily equally dependant on angiogenesis for their growth and progression. Gastric cancer is an example in which the process of growth and metastasis of one subtype, diffuse type gastric cancer, is less angiogenesis dependent than the other, intestinal type gastric cancer.[50,51]

The aim of the present study was therefore to quantify tumour angiogenesis in a large cohort of two anatomically closely related however prognostically distinct types of pancreatic cancer, pancreatic head and periampullary cancer and elucidate whether angiogenesis is a prognostic marker in either of these types of pancreatic cancer.

2. Patients and methods

2.1. Patient population

Retrospectively 231 patients treated for pancreatic adenocarcinoma with curative intent at Erasmus Medical Center between 1987 and 2008, who had no microscopically residual tumour (R0), were identified. Tumours were classified by location, having its origin either in the pancreatic head or periampullary region, the latter group comprising of tumours originating in the Ampulla of Vater or the distal common bile duct. Tumour samples originating before the new 2002 UICC TNM classification were re-evaluated according to these new criteria.

Representative tumour areas were encircled on original haematoxylin/eosin slides by a GI pathologist with special expertise in pancreatic pathology and staining was performed on corresponding formalin fixed, paraffin embedded tissue.

During the above-mentioned period two randomised control studies were ongoing in our center. Between September 1987 and April 1995, 17 patients were randomised to the treatment arm of the European Organisation for Research and Treatment of Cancer (EORTC) 40891 trial, receiving two courses of 5-FU as a continuous infusion (max 1500 mg/day) followed by radiotherapy (20 Gy). From June 2000 up to its closure in March 2007, 32 patients were randomised to the treatment arm of a trial combining intra-arterial chemotherapy and radiotherapy. Patients received six cycles of intra-arterial mitoxantrone (10 mg/m^2), folinic acid (170 mg/m^2/day), 5-FU (600 mg/m^2/day) and cisplatinum (60 mg/m^2), the first cycle followed by radiotherapy (54 Gy). These trials and the results have been described in detail elsewhere.[52–54]

At the time of the present report, the median follow-up duration was 19 months (range 0–192 months). Recurrence free survival (RFS) was defined as the time from date of surgery to the date of first proof of disease recurrence (locally, distant or both) or to death without relapse. Overall survival (OS) was computed as the number of months from resection to death of any cause as registered by the social security death index (SSDI). For cancer specific survival (CSS) patients that died without recurrent disease were excluded. Patients who died in hospital following procedure related complications were excluded from analysis with respect to survival as their death was unrelated to tumour biology and would have introduced a confounding influence on survival analysis.

2.2. CD31 expression by immunohistochemistry

Immunohistochemistry was performed according to the protocol used in clinical practice at our institution and was optimised for CD31.

Briefly, 4 μM sections were deparaffinised in xylene and rehydrated through decreasing ethanol series ending in distilled water. Antigen retrieval was performed by microwave heating (20 min preheating followed by 20 min of cooking) in Tris–ethylene diamine tetra-acetic acid (EDTA) buffer pH 9.0. Endogenous peroxidase activity was quenched using 0.3% hydrogen peroxide (H_2O_2) in PBS for 20 min. Sections were incubated overnight at 4 °C with a ready to use mouse monoclonal antibody to CD31 (JC70A, Dako Netherlands B.V., Heverlee, Belgium). Followed by incubation with the secondary antibody (Dako REAL Envision HRP Rabbit/Mouse) for 30 min at room temperature, immunostaining was developed by immersion in diaminobenzidine. Slides were washed extensively between each of the above steps. Nuclei were counterstained with Harris Haematoxylin, followed by dehydration, fixation and finally covered using Leica multistainer and robotic coverslipper (ST5020 and CV 5030, Leica Microsystems B.V., Rijswijk, Netherlands). Positive and negative controls were included in each run.

2.3. Microvessel density (MVD) analysis

Tumour areas were examined with a Leica DM-RXA microscope. Three random ×160 fields (×16 objective, ×10 ocular)

were captured using a Sony 3CCD DXC 950 camera connected to a computer. Images were analysed using UTHSCSH Image Tool. Briefly, colours were separated and the 24bits binary blue image was transferred to grayscale followed by setting an automatic threshold that clearly identified the CD31 positive endothelial cells. Fixed light intensity was used throughout the analysis. MVD was recorded from each of the three high-power fields and either the highest count or the average was used for analysis. MVD was calculated as the number of CD31 positive pixels per picture.

2.4. *Statistical analysis*

Statistical analysis was performed using SPSS version 15.0 for Windows. Differences in MVD between groups were compared with Student's *t*-test or ANOVA when appropriate. The correlation between MVD and established prognostic factors was carried out using linear regression analysis. In these analyses of MVD, logarithmically transformed values (base 10) were used for an approximate normal distribution.

The distributions of RFS, CSS and OS were estimated using Kaplan–Meier curves. In one model the median MVD was used as potential prognostic cut-off; in another the 25th, 50th and 75th percentile were evaluated. Univariate associations were tested using Log-rank test. Cox proportional hazards regression model was used to test whether outcome measures were independent of other established prognostic factors, such as tumour extension (*T*-status), mode of differentiation and nodal status. All *p* values reported are two sided and values $\leqslant .05$ were considered statistically significant.

3. Results

3.1. *Patient population*

Tissue blocks were available from 222 out of 231 patients. Sixteen slides could not be scored due to poor quality. As a result immunostaining was correlated with established prognostic factors for 206 cases. Ten patients died during postoperative stay and were thus excluded from survival analysis. The age of the patients ranged from 36 to 87 years (median 65 years) and the study population included slightly more males than females (113 versus 83). Ninety-one tumours originated in the head of the pancreas whereas 105 patients had periampullary cancer.

3.2. *Microvessel density (MVD)*

ANOVA showed large variations between tumours (SD = 0.24) and between the different random spots within a tumour (SD = 0.22). Therefore the average values per tumour were calculated and used for further analysis. The average MVD from the three pictures analysed, ranged from 0.3 to 12.4 percent/patient (mean 3.1; median 2.7). We also calculated the highest MVD measured among the three spots. The highest MVD ranged from 0.5 to 13.6 percent/patient (mean 4.4; median 3.8).

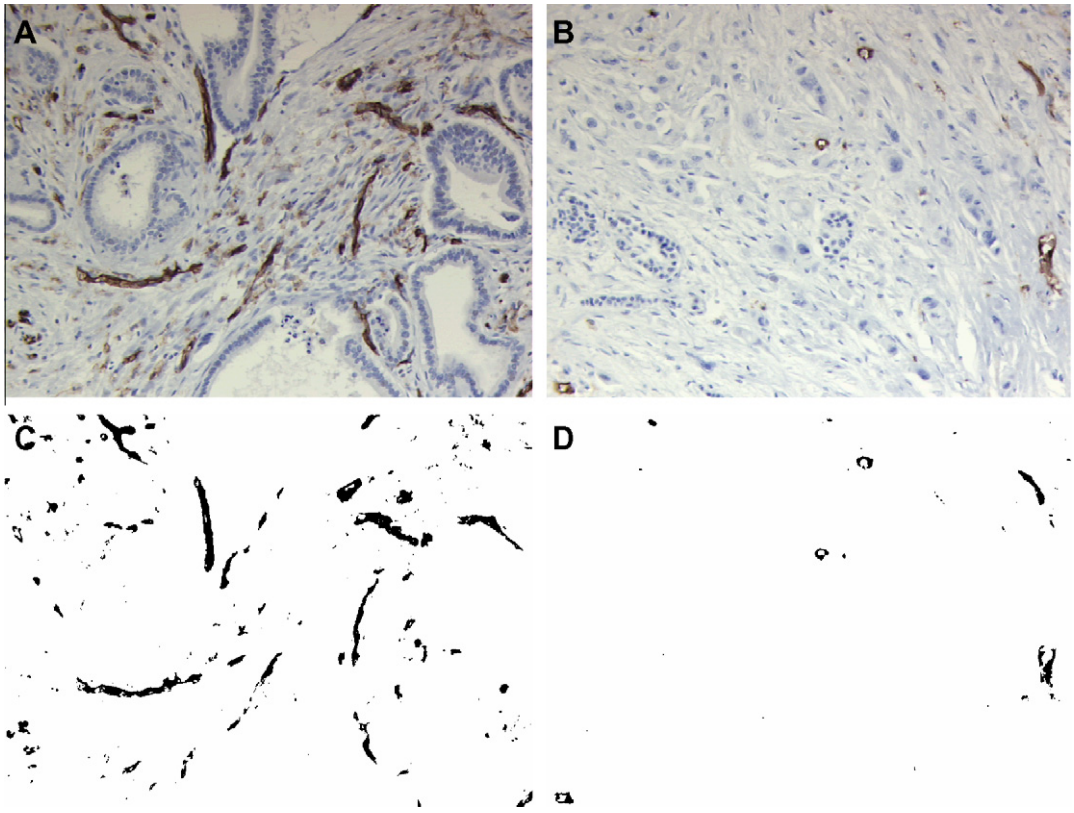

Fig. 1 – Two examples of pancreatic cancer tissue immunohistochemically stained with CD31 antibody (A and B) (16× magnification) and their binary overlays (C and D) showing a tumour with respectively high (A) and low microvessel density (MVD) (B).

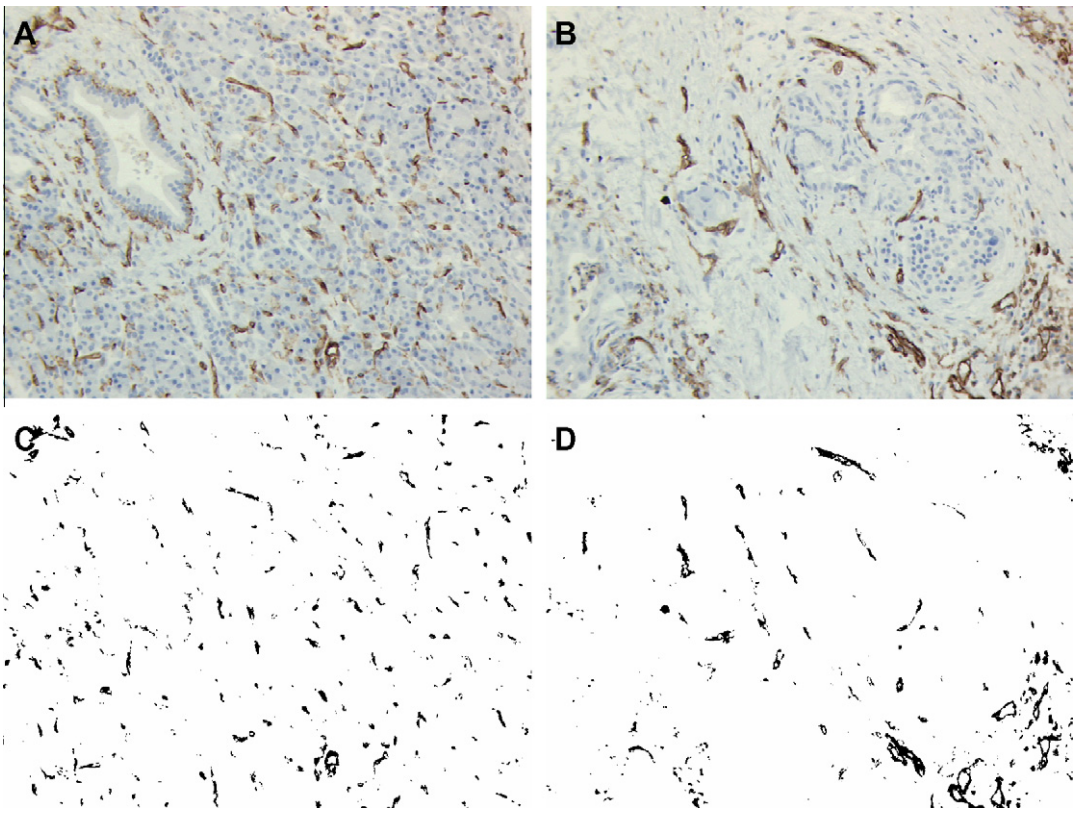

Fig. 2 – Two examples of normal pancreas tissue immunohistochemically stained with CD31 antibody (A and B) and their binary overlays (C and D) (16× magnification) suggesting homogeneous vessel distribution.

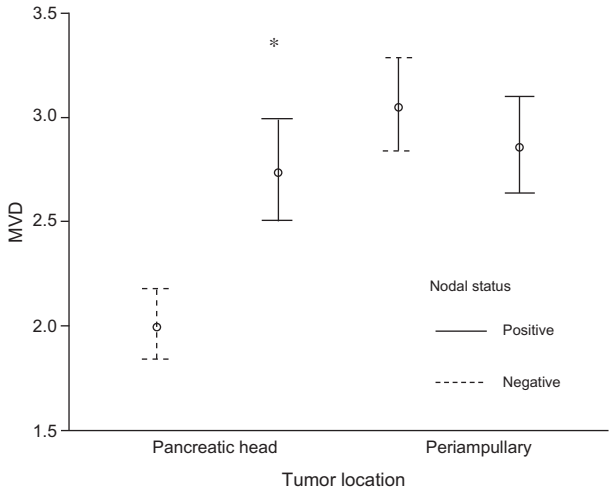

Fig. 3 – Microvessel density (MVD) (±1 SE) in node positive (——) and node negative (- - - -) tumours for respectively pancreatic head and periampullary cancer. In patients with nodal involvement tumours showed a higher proportion of vessels than those with clean lymph nodes. However this was only true for pancreatic head cancer (*$p = .014$).

Both the average and the highest MVD were not normally distributed. In Fig. 1, two examples are given of such a random field. The first represents a patient with an average MVD of 3.6, the second a patient with an average MVD of 0.9.

Fig. 2 represents the vessel distribution in two spots of normal pancreas demonstrating rather homogeneous vessel distribution.

3.3. *Pathologic correlations*

The average MVD of pancreatic head tumours was less than MVD of tumours originating in the periampullary region (respectively 2.4 and 2.9 percent; $p < .01$). Furthermore, nodal status correlated with the average MVD in pancreatic head tumours ($p = .014$), whereas this was not the case in perampullary tumours ($p = .55$). In pancreatic head tumours with lymph node involvement a larger proportion of the tumour was occupied by vessels (Fig 3). There was no correlation with *T*-status or with mode of differentiation. This was true for both pancreatic head and periampullary cancer.

When the highest instead of the average MVD was used for analysis, findings were similar to analysis using the average MVD. However, in addition to nodal status, mode of differentiation also correlated with vessel area ($p = .047$). Generally, moderately and poorly differentiated pancreatic head cancers were more vascularised than well-differentiated pancreatic head cancers.

3.4. *Clinical correlations*

All conventional prognostic factors proved highly significant in predicting RFS, CSS and OS in univariate analysis. In multivariate analysis however, tumour extension (*T* status) lost

its prognostic value for all outcome measures. The type of pancreatic cancer, i.e. pancreatic head or periampullary cancer, proved to be the strongest independent predictor of outcome (data not shown).

To test whether the relation between MVD and the outcome measures differed between the two types of pancreatic cancer, we tested for effect modification ('interaction') by tumour type in the Cox-models. This test showed that the type of pancreatic cancer did not determine the effect of MVD on outcome (all $p > .19$ for RFS, CSS and OS). The same was done for the highest MVD measured, with similar conclusions. Therefore, for survival analysis, both tumours were treated as one group, however for direct interpretation purposes Kaplan–Meier curves are presented for the two tumour types separately.

MVD was divided into quartiles and the four groups were tested for trend regarding relation with outcomes. No trend for an association of MVD with RFS ($p = .77$), CSS ($p = .79$) or OS ($p = .84$) was observed (Figs. 4–6). In fact none of the four groups showed a significant difference with any of the other groups following pair wise comparison.

Following multivariate analysis, only tumour type, nodal status and differentiation proved to be independent prognostic factors for RFS, CSS and OS. MVD provided no additional prognostic information (Table 1). Furthermore, the effect of MVD on outcome did not significantly depend on N-category either (all $p > .21$ for RFS, CSS and OS).

All analyses were repeated with the highest MVD instead of the average MVD with similar conclusions (data not shown).

4. Discussion

This is the largest study to date, quantifying tumour angiogenesis and evaluating its effect on the prognosis of pancreatic cancer.

The pan-endothelial marker CD31 was used to stain tumour vessels and the proportion of vessel to tumour area (MVD) was measured using an automated system to quantify tumour angiogenesis. MVD appeared to be heterogeneous between, but also within tumours, while vessel density in normal pancreatic tissue appeared more homogeneous.

Furthermore, a higher MVD was observed in periampullary cancer as compared to pancreatic head cancer. Microvessel recruitment is the result of the angiogenic potential of the tumour cell itself and its interaction with the surrounding extracellular matrix. The angiogenic potential of a tumour cell is likely a reflection of its origin. Although both pancreatic head and periampullary cancer have a close anatomic relation, they have different stem cell origins. This difference in origin could explain the different molecular characteristics and behaviour of both tumours, reflected by the currently observed difference in MVD but also the previously observed differences in expression rates of c-erb,[55] p53, Ki-67,[56] SMAD 4[57] and HMGA1 and the effect on prognosis of both tumours.[58]

No association of MVD with other prognostic factors could be identified in periampullary cancer, whereas in pancreatic head cancer, a higher MVD coincided with lymph node involvement. Interestingly, Khan and co-workers observed

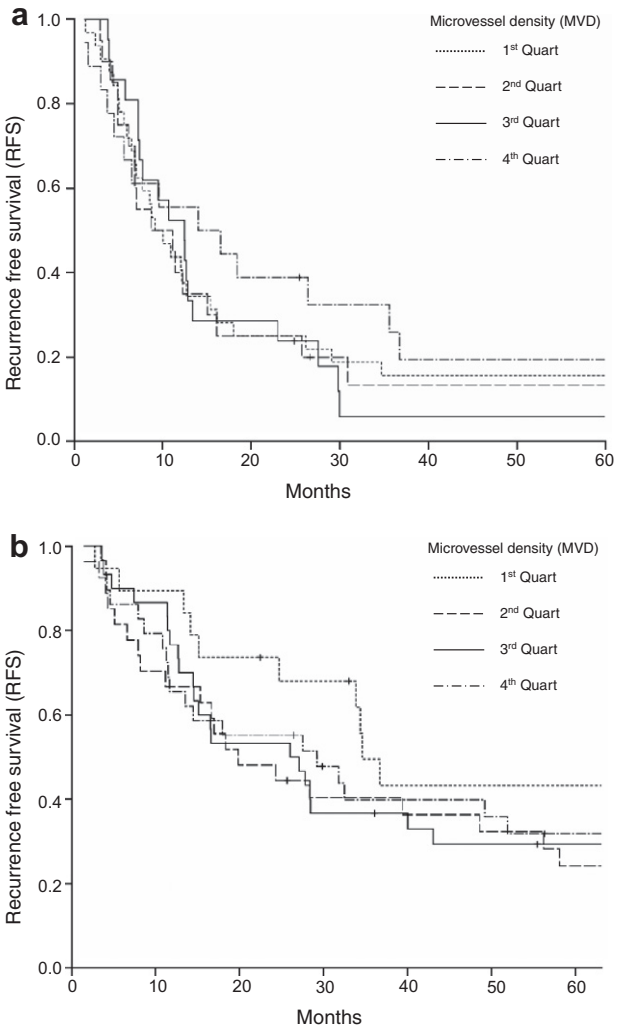

Fig. 4 – Recurrence free survival (RFS) of patients treated for adenocarcinoma of respectively the pancreatic head (a) and periampullary (b) region. Microvessel density (MVD) did not have an impact on RFS of pancreatic cancer ($p = .77$).

the exact opposite: an association with nodal involvement in ampullary cancer instead of pancreatic cancer.[45] When the highest MVD measured was used for analysis, MVD was associated with both nodal status and differentiation. This is in line with another study in pancreatic cancer in which the vascular surface density and the number of vessels per mm^2 stroma correlated with poor histological differentiation.[44] The same investigators also found a relationship between MVD and tumour size and in yet another study an association with stage was observed.[41] In other tumours associations with tumour size,[11,12,14,18,27,35,36] *T* status,[17] differentiation[9,11–13,17,23,35,36] or lymph node status[9,11,12,18,36] were observed in some studies but not in others.[15,24–26,31–34,38]

Following correlation with established prognostic factors, the association of MVD with survival was assessed. Although we intended to evaluate tumour angiogenesis as a prognostic factor in pancreatic head and periampullary cancer separately, no interaction was identified between the type of pancreatic cancer and outcome. A separate analysis was

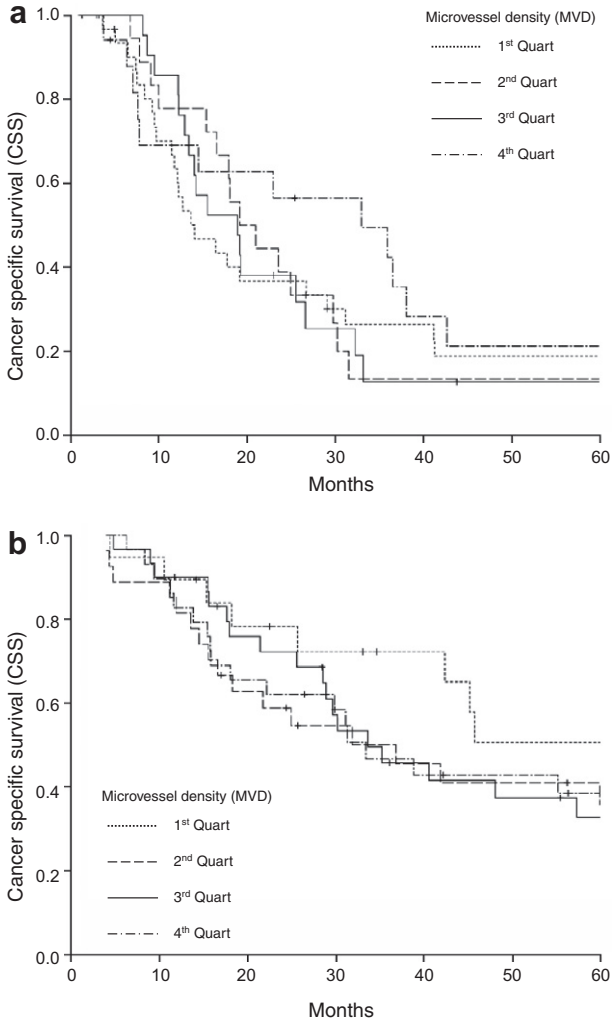

Fig. 5 – Cancer specific survival (CSS) of patients treated for adenocarcinoma of respectively the pancreatic head (a) and periampullary (b) region. Microvessel density (MVD) did not have an impact on CSS of pancreatic cancer ($p = .79$).

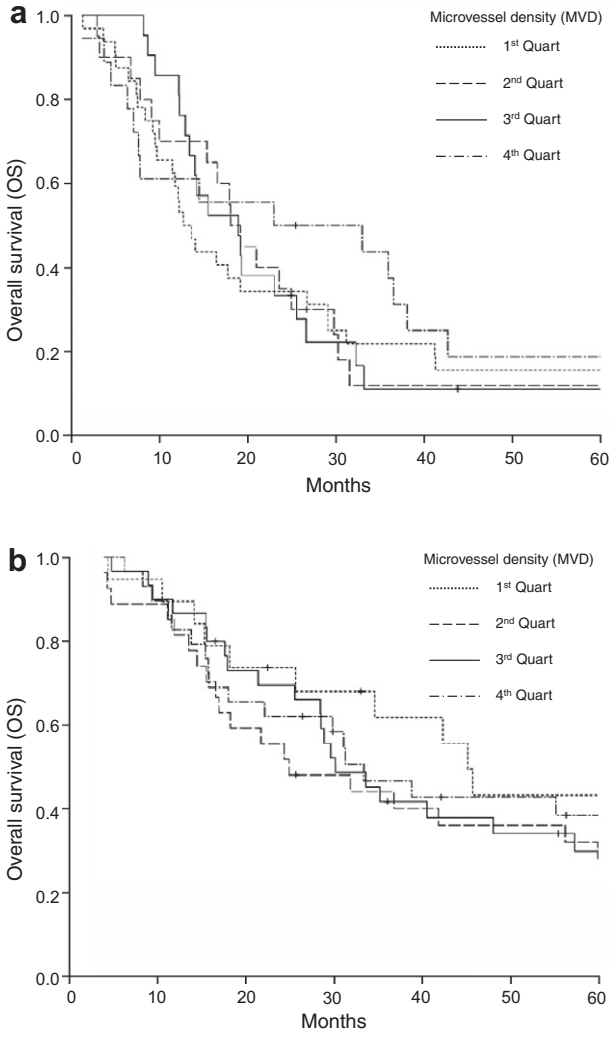

Fig. 6 – Overall survival (OS) of patients treated for adenocarcinoma of respectively the pancreatic head (a) and periampullary (b) region. Microvessel density (MVD) did not have an impact on OS of pancreatic cancer ($p = .84$).

therefore not justified. Consequently we analysed both types as one group. A possible reason for the lack of an interaction between tumour type and outcome could be the small difference between the actual MVD, 2.4 and 2.9 percent for pancreatic head and periampullary cancer, respectively, classifying both as relative hypoxic tumours.

In our cohort of patients with pancreatic cancer, MVD did not provide additional prognostic information to conventional prognostic factors such as nodal involvement and degree of differentiation. This is in line with some reports on the prognostic role of MVD in pancreatic cancer,[40,41,46] however in sharp contrast to others.[41–45,47,48]

Possible reasons for these inconsistent results are not limited to the difference in study population, but may be due to poor standardisation of the methodology. The assessment of MVD is generally a tedious process with poor reproducibility. First, as originally proposed by Weidner and colleagues, the most vascularised area (the so called 'hot-spot') needs to be selected. Then, each separate microvessel needs to be counted. Both selection processes are highly subjective and consequently likely associated with inter-observer variation. Furthermore, exclusively counting vessels neglects the vessel size aspect, and might underestimate the metastatic potential of a tumour as hypothesised by Nagakawa et al. who showed that liver metastasis occurred more often when tumour invasion was present in the middle sized and the large vessels compared to the smaller sized ones.[46] An observation already made by Liotta and co-workers in the early seventies. They found a linear relationship between the number of vessels >30 μm and the number of tumour cell- and cell clump washout following perfusion of a xenografted fibrosarcoma.[3] However, the disadvantage of using vessel size to quantify MVD is again the subjective bias and interobserver variability associated with counting individual vessels.

To reduce the subjective bias and interobserver variability associated with selecting a hot-spot and counting individual microvessels, in the current study areas were selected randomly and analysed by automated computerised image anal-

Table 1 – Multivariate analysis of conventional prognostic factors and microvessel density (MVD) on outcome in pancreatic cancer.

Factor	N	Recurrence free survival (RFS)				Cancer specific survival (CSS)				Overall survival (OS)			
		%5 year	HR	95% (CI)	p	%5 year	HR	95% CI	p	%5 year	HR	95% CI	p
Tumour type					<.001				<.001				<.001
Pancreatic head[a]	91	14				17				15			
Periampullary	105	31	0.49	0.35–0.68		38	0.49	0.34–0.71		34	0.49	0.35–0.68	
Tumour extension					.09				.13				.08
T 1/2[a]	50	44				48				45			
T 3/4	142	15	1.43	0.95–2.15		21	1.44	0.90–2.29		18	1.45	0.96–2.20	
Nodal involvement					<.001				<.001				.001
No[a]	92	37				42				38			
Yes	104	10	1.89	1.35–2.67		16	2.07	1.41–3.03		13	1.77	1.26–2.50	
Differentiation					.003				.002				.008
Well[a]	32	43				51				46			
Moderately	129	21	1.49	0.94–2.37	.09	26	1.66	0.98–2.84	.06	22	1.61	1.01–2.57	.047
Poorly	34	12	2.55	1.47–4.41	.001	16	2.89	1.57–5.33	.001	15	2.42	1.39–4.22	.002
MVD			1.00[b]	0.83–1.19	.96		0.93[b]	0.77–1.13	.48		0.95[b]	0.79–1.13	.54

HR: hazard ratio; %5 year: % 5 year survival by univariate analysis; 95% CI: 95% confidence interval.
[a] Reference category.
[b] Effect of doubling MVD.

ysis. Conveniently, the pixel/area aspect of the analysis performed does not completely neglect the vessel size aspect discussed above since a large vessel contains more pixels than a small vessel. Finally, the average of the randomly selected areas was taken to correct for the heterogeneous nature of tumours.

Another reason for the differences observed between studies could be the antibody or agent used to detect the vessels. Antigen specificity is a major problem when staining endothelial cells. In case of CD34, lymphatic vessels, perivascular stromal cells as well as other stromal elements can be stained. The disadvantage of factor VIII is that it is absent on part of the capillary endothelium in tumour tissue. Disadvantages associated with staining for CD31, are the co-staining of inflammatory cells and frequent antigen loss due to fixatives that contain acetic acid. However inflammatory cells are easy to distinguish from endothelial cells and microwave antigen retrieval effectively abolishes the problem of antigen loss.[59] Furthermore, JC-70, the antibody used in the current study, has the advantage over factor VIII of being present also on immature blood vessels. Consequently, counts using this marker are 30% higher than those using factor VIII.[16] In 1996 an international consensus was developed by the experts in the field of MVD assessment. In this consensus CD31, the agent used in the current study, was chosen as the agent of choice for assessment of MVD on paraffin sections.

The lack of an association with prognosis could be explained in several ways. First, pancreatic cancer is known to be a hypoxic tumour.[60–63] Pancreatic cancer is characterised by an extensive extracellular matrix deposition, called desmoplasia, creating increased intratumoural pressure and tumour-vessel distances, resulting in a hypoxic environment.[64] Quite some research has been done in the last decennium on the role of pancreatic stellate cells in this extracellular matrix deposition. Recently one of the expert groups suggested that this hypoxic and fibrotic environment of pancreatic cancer might be due to both fibrogenic effects of pancreatic stellate cells and anti-angiogenic effects of cancer cells.[65] Evidence has been presented that hypoxia might act as a physiological selective agent against apoptosis-competent cells in tumours, thus promoting the clonal expansion of cells that acquire mutations in their apoptotic programs.[66]

Secondly, angiogenesis is but one step in the multi-step process of tumour progression and metastasis formation. It might be necessary but not sufficient to produce metastasis.

In conclusion, the present study shows that periampullary cancer is more vascularised than pancreatic head cancer and although angiogenesis as quantified by microvessel density correlates with nodal MVD head cancer and thus gives some information on the malignant potential, pancreatic cancer seems indeed an example of a tumour in which prognosis is not dependent on angiogenesis.

This would also explain the poor response to anti-VEGF treatment observed in pancreatic cancer.[67,68]

Conflict of interest statement

None declared.

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REFERENCES

1. Boeck S, Ankerst DP, Heinemann V. The role of adjuvant chemotherapy for patients with resected pancreatic cancer: systematic review of randomized controlled trials and meta-analysis. *Oncology* 2007;**72**(5–6):314–21.
2. Merchant N, Berlin J. Past and future of pancreas cancer: are we ready to move forward together? *J Clin Oncol* 2008;**26**(21):3478–80.
3. Liotta LA, Kleinerman J, Saidel GM. Quantitative relationships of intravascular tumor cells, tumor vessels, and pulmonary metastases following tumor implantation. *Cancer Res* 1974;**34**(5):997–1004.
4. Folkman J. What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 1990;**82**(1):4–6.
5. Folkman J. How is blood vessel growth regulated in normal and neoplastic tissue? G.H.A. Clowes memorial award lecture. *Cancer Res* 1986;**46**(2):467–73.
6. Fidler IJ. Critical factors in the biology of human cancer metastasis: twenty-eighth G.H.A. Clowes memorial award lecture. *Cancer Res* 1990;**50**(19):6130–8.
7. Liotta LA, Steeg PS, Stetler-Stevenson WG. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* 1991;**64**(2):327–36.
8. Srivastava A, Laidler P, Davies RP, Horgan K, Hughes LE. The prognostic significance of tumor vascularity in intermediate-thickness (0.76–4.0 mm thick) skin melanoma. A quantitative histologic study. *Am J Pathol* 1988;**133**(2):419–23.
9. El-Gohary YM, Metwally G, Saad RS, et al. Prognostic significance of intratumoral and peritumoral lymphatic density and blood vessel density in invasive breast carcinomas. *Am J Clin Pathol* 2008;**129**(4):578–86.
10. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis–correlation in invasive breast carcinoma. *N Engl J Med* 1991;**324**(1):1–8.
11. Weidner N, Folkman J, Pozza F, et al. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. *J Natl Cancer Inst* 1992;**84**(24):1875–87.
12. Horak ER, Leek R, Klenk N, et al. Angiogenesis, assessed by platelet/endothelial cell adhesion molecule antibodies, as indicator of node metastases and survival in breast cancer. *Lancet* 1992;**340**(8828):1120–4.
13. Gasparini G, Weidner N, Bevilacqua P, et al. Tumor microvessel density, p53 expression, tumor size, and peritumoral lymphatic vessel invasion are relevant prognostic markers in node-negative breast carcinoma. *J Clin Oncol* 1994;**12**(3):454–66.
14. Nieto Y, Woods J, Nawaz F, et al. Prognostic analysis of tumour angiogenesis, determined by microvessel density and expression of vascular endothelial growth factor, in high-risk primary breast cancer patients treated with high-dose chemotherapy. *Br J Cancer* 2007;**97**(3):391–7.
15. Acenero MJ, Gonzalez JF, Gallego MG, Ballesteros PA. Vascular enumeration as a significant prognosticator for invasive breast carcinoma. *J Clin Oncol* 1998;**16**(5):1684–8.
16. Uzzan B, Nicolas P, Cucherat M, Perret GY. Microvessel density as a prognostic factor in women with breast cancer: a systematic review of the literature and meta-analysis. *Cancer Res* 2004;**64**(9):2941–55.
17. Dhakal HP, Bassarova A, Naume B, et al. Breast carcinoma vascularity: a comparison of manual microvessel count and Chalkley count. *Histol Histopathol* 2009;**24**(8):1049–59.
18. Sullivan CA, Ghosh S, Ocal IT, et al. Microvessel area using automated image analysis is reproducible and is associated with prognosis in breast cancer. *Hum Pathol* 2009;**40**(2):156–65.
19. Gasparini G, Barbareschi M, Boracchi P, et al. 67-kDa laminin-receptor expression adds prognostic information to intra-tumoral microvessel density in node-negative breast cancer. *Int J Cancer* 1995;**60**(5):604–10.
20. Macchiarini P, Fontanini G, Hardin MJ, Squartini F, Angeletti CA. Relation of neovascularisation to metastasis of non-small-cell lung cancer. *Lancet* 1992;**340**(8812):145–6.
21. Fontanini G, Lucchi M, Vignati S, et al. Angiogenesis as a prognostic indicator of survival in non-small-cell lung carcinoma: a prospective study. *J Natl Cancer Inst* 1997;**89**(12):881–6.
22. Meert AP, Paesmans M, Martin B, et al. The role of microvessel density on the survival of patients with lung cancer: a systematic review of the literature with meta-analysis. *Br J Cancer* 2002;**87**(7):694–701.
23. Weidner N, Carroll PR, Flax J, Blumenfeld W, Folkman J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am J Pathol* 1993;**143**(2):401–9.
24. Bochner BH, Cote RJ, Weidner N, et al. Angiogenesis in bladder cancer: relationship between microvessel density and tumor prognosis. *J Natl Cancer Inst* 1995;**87**(21):1603–12.
25. Hollingsworth HC, Kohn EC, Steinberg SM, Rothenberg ML, Merino MJ. Tumor angiogenesis in advanced stage ovarian carcinoma. *Am J Pathol* 1995;**147**(1):33–41.
26. Vermeulen PB, Van den Eynden GG, Huget P, et al. Prospective study of intratumoral microvessel density, p53 expression and survival in colorectal cancer. *Br J Cancer* 1999;**79**(2): 316–22.
27. Poon RT, Ng IO, Lau C, et al. Tumor microvessel density as a predictor of recurrence after resection of hepatocellular carcinoma: a prospective study. *J Clin Oncol* 2002;**20**(7): 1775–1785.
28. Rajaganeshan R, Prasad R, Guillou PJ, et al. The influence of invasive growth pattern and microvessel density on prognosis in colorectal cancer and colorectal liver metastases. *Br J Cancer* 2007;**96**(7):1112–7.
29. Kato T, Steers G, Campo L, et al. Prognostic significance of microvessel density and other variables in Japanese and British patients with primary invasive breast cancer. *Br J Cancer* 2007;**97**(9):1277–86.
30. Carnochan P, Briggs JC, Westbury G, Davies AJ. The vascularity of cutaneous melanoma: a quantitative histological study of lesions 0.85–1.25 mm in thickness. *Br J Cancer* 1991;**64**(1):102–7.
31. Hall NR, Fish DE, Hunt N, et al. Is the relationship between angiogenesis and metastasis in breast cancer real? *Surg Oncol* 1992;**1**(3):223–9.
32. Axelsson K, Ljung BM, Moore 2nd DH, et al. Tumor angiogenesis as a prognostic assay for invasive ductal breast carcinoma. *J Natl Cancer Inst* 1995;**87**(13):997–1008.
33. Goulding H, Abdul Rashid NF, Robertson JF, et al. Assessment of angiogenesis in breast carcinoma: an important factor in prognosis? *Hum Pathol* 1995;**26**(11):1196–200.
34. Costello P, McCann A, Carney DN, Dervan PA. Prognostic significance of microvessel density in lymph node negative breast carcinoma. *Hum Pathol* 1995;**26**(11):1181–4.
35. Vincent-Salomon A, Carton M, Zafrani B, et al. Long term outcome of small size invasive breast carcinomas independent from angiogenesis in a series of 685 cases. *Cancer* 2001;**92**(2):249–56.
36. Mohammed RA, Ellis IO, Elsheikh S, Paish EC, Martin SG. Lymphatic and angiogenic characteristics in breast cancer: morphometric analysis and prognostic implications. *Breast Cancer Res Treat* 2009;**113**(2):261–73.
37. Pignataro L, Carboni N, Midolo V, et al. Clinical relevance of microvessel density in laryngeal squamous cell carcinomas. *Int J Cancer* 2001;**92**(5):666–70.

38. Lindmark G, Gerdin B, Sundberg C, et al. Prognostic significance of the microvascular count in colorectal cancer. *J Clin Oncol* 1996;**14**(2):461–6.
39. Medri L, Nanni O, Volpi A, et al. Tumor microvessel density and prognosis in node-negative breast cancer. *Int J Cancer* 2000;**89**(1):74–80.
40. Ellis LM, Takahashi Y, Fenoglio CJ, et al. Vessel counts and vascular endothelial growth factor expression in pancreatic adenocarcinoma. *Eur J Cancer* 1998;**34**(3):337–40.
41. Fujimoto K, Hosotani R, Wada M, et al. Expression of two angiogenic factors, vascular endothelial growth factor and platelet-derived endothelial cell growth factor in human pancreatic cancer, and its relationship to angiogenesis. *Eur J Cancer* 1998;**34**(9):1439–47.
42. Fujioka S, Yoshida K, Yanagisawa S, et al. Angiogenesis in pancreatic carcinoma: thymidine phosphorylase expression in stromal cells and intratumoral microvessel density as independent predictors of overall and relapse-free survival. *Cancer* 2001;**92**(7):1788–97.
43. Ikeda N, Adachi M, Taki T, et al. Prognostic significance of angiogenesis in human pancreatic cancer. *Br J Cancer* 1999;**79**(9–10):1553–63.
44. Karademir S, Sokmen S, Terzi C, et al. Tumor angiogenesis as a prognostic predictor in pancreatic cancer. *J Hepatobiliary Pancreat Surg* 2000;**7**(5):489–95.
45. Khan AW, Dhillon AP, Hutchins R, et al. Prognostic significance of intratumoural microvessel density (IMD) in resected pancreatic and ampullary cancers to standard histopathological variables and survival. *Eur J Surg Oncol* 2002;**28**(6):637–44.
46. Nagakawa Y, Aoki T, Kasuya K, Tsuchida A, Koyanagi Y. Histologic features of venous invasion, expression of vascular endothelial growth factor and matrix metalloproteinase-2 and matrix metalloproteinase-9, and the relation with liver metastasis in pancreatic cancer. *Pancreas* 2002;**24**(2):169–78.
47. Niedergethmann M, Hildenbrand R, Wostbrock B, et al. High expression of vascular endothelial growth factor predicts early recurrence and poor prognosis after curative resection for ductal adenocarcinoma of the pancreas. *Pancreas* 2002;**25**(2):122–9.
48. Takagi K, Takada T, Amano H. A high peripheral microvessel density count correlates with a poor prognosis in pancreatic cancer. *J Gastroenterol* 2005;**40**(4):402–8.
49. Vermeulen PB, Gasparini G, Fox SB, et al. Second international consensus on the methodology and criteria of evaluation of angiogenesis quantification in solid human tumours. *Eur J Cancer* 2002;**38**(12):1564–79.
50. Ellis LM, Walker RA, Gasparini G. Is determination of angiogenic activity in human tumours clinically useful? *Eur J Cancer* 1998;**34**(5):609–18.
51. Takahashi Y, Cleary KR, Mai M, et al. Significance of vessel count and vascular endothelial growth factor and its receptor (KDR) in intestinal-type gastric cancer. *Clin Cancer Res* 1996;**2**(10):1679–84.
52. Klinkenbijl JH, Jeekel J, Sahmoud T, et al. Adjuvant radiotherapy and 5-fluorouracil after curative resection of cancer of the pancreas and periampullary region: phase III trial of the EORTC gastrointestinal tract cancer cooperative group. *Ann Surg* 1999;**230**(6):776–82 [discussion 782-4].
53. Smeenk HG, van Eijck CH, Hop WC, et al. Long-term survival and metastatic pattern of pancreatic and periampullary cancer after adjuvant chemoradiation or observation: long-term results of EORTC trial 40891. *Ann Surg* 2007;**246**(5):734–40.
54. Morak MJ, van der Gaast A, Incrocci L, et al. Adjuvant intra-arterial chemotherapy and radiotherapy versus surgery alone in resectable pancreatic and periampullary cancer: a prospective randomized controlled trial. *Ann Surg* 2008;**248**(6):1031–41.
55. Vaidya P, Kawarada Y, Higashiguchi T, et al. Overexpression of different members of the type 1 growth factor receptor family and their association with cell proliferation in periampullary carcinoma. *J Pathol* 1996;**178**(2):140–5.
56. Takashima M, Ueki T, Nagai E, et al. Carcinoma of the ampulla of Vater associated with or without adenoma: a clinicopathologic analysis of 198 cases with reference to p53 and Ki-67 immunohistochemical expressions. *Mod Pathol* 2000;**13**(12):1300–7.
57. McCarthy DM, Hruban RH, Argani P, et al. Role of the DPC4 tumor suppressor gene in adenocarcinoma of the ampulla of Vater: analysis of 140 cases. *Mod Pathol* 2003;**16**(3):272–8.
58. Van der Zee JA, Ten Hagen TL, Hop WC, et al. Differential expression and prognostic value of HMGA1 in pancreatic head and periampullary cancer. *Eur J Cancer* 2010;**46**(18):3393–9.
59. Hasan J, Byers R, Jayson GC. Intra-tumoural microvessel density in human solid tumours. *Br J Cancer* 2002;**86**(10):1566–77.
60. Hosoki T. Dynamic CT of pancreatic tumors. *AJR Am J Roentgenol* 1983;**140**(5):959–65.
61. Koong AC, Mehta VK, Le QT, et al. Pancreatic tumors show high levels of hypoxia. *Int J Radiat Oncol Biol Phys* 2000;**48**(4):919–22.
62. Sofuni A, Iijima H, Moriyasu F, et al. Differential diagnosis of pancreatic tumors using ultrasound contrast imaging. *J Gastroenterol* 2005;**40**(5):518–25.
63. Sakamoto H, Kitano M, Suetomi Y, et al. Utility of contrast-enhanced endoscopic ultrasonography for diagnosis of small pancreatic carcinomas. *Ultrasound Med Biol* 2008;**34**(4):525–32.
64. Olive KP, Jacobetz MA, Davidson CJ, et al. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. *Science* 2009;**324**(5933):1457–61.
65. Erkan M, Reiser-Erkan C, Michalski CW, et al. Cancer-stellate cell interactions perpetuate the hypoxia-fibrosis cycle in pancreatic ductal adenocarcinoma. *Neoplasia* 2009;**11**(5):497–508.
66. Graeber TG, Osmanian C, Jacks T, et al. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature* 1996;**379**(6560):88–91.
67. Gemcitabine plus bevacizumab no better than gemcitabine alone in clinical study of 600 patients with pancreatic cancer. *Cancer Biol Ther* 2007;**6**(2):137.
68. Van Cutsem E, Vervenne WL, Bennouna J, et al. Phase III trial of bevacizumab in combination with gemcitabine and erlotinib in patients with metastatic pancreatic cancer. *J Clin Oncol* 2009;**27**(13):2231–7.