Histol Histopathol (2010) 25: 1239-1246

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Angiogenesis index CD105 (Endoglin)/CD31 (PECAM-1) as a predictive factor for invasion and proliferation in intraductal papillary mucinous neoplasm (IPMN) of the pancreas

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Summary. Background: Intraductal papillary-mucinous neoplasm (IPMN) of the pancreas is an increasingly diagnosed entity since its definition by the World Health Organization in 1996. It has a broad clinical spectrum ranging from benign to malignant tumors. Optimum treatment is controversial and a better understanding of the development of IPMN of the pancreas and identification of potential prognostic factors will help to address this. Angiogenesis plays an elementary role in the development of malignant tumors and may well also be important in the development of IPMN of the pancreas. Therefore we investigated endothelial cell marker CD31 (PECAM-1) and angiogenesis associated marker CD105 (Endoglin) by immunohistochemistry.

Methods: Thirty-two cases of surgically resected IPMN were chosen retrospectively and clinical data were obtained. Specimens were stained for proliferation marker (Ki-67), CD31 and CD105 by immunohistochemistry. A CD105/CD31 Angiogenesis ratio (AR) was established to determine the proliferating fraction of endothelial cells.

Results: The AR is significantly elevated in invasive IPMN of the pancreas (Mann-Whitney-U Test, $p<0.05$) and is associated with the Ki-67-labelling-index, demonstrating synergy between tumor-growth and neovascularisation. Invasive IPMN of the pancreas is associated with significantly lower recurrence-free and overall survival.

Conclusions: Neovascularisation plays an important role in the tumorigenesis of invasive IPMN of the pancreas, and therefore angiogenesis-associated molecules like CD105 and CD31 might be useful tools as prognostic markers. Furthermore, the results indicate a potential role for adjuvant anti-angiogenic therapies in selected patients with recurring and/or invasive IPMN of the pancreas.

Key words: IPMN of the pancreas, Neovascularisation, CD105 (Endoglin), CD31 (PECAM-1), Ki-67 index

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Introduction

Intraductal papillary-mucinous neoplasms (IPMN) of the pancreas were defined and classified by the World Health Organization (WHO) in 1996 (Klöppel et al., 1996). The term IPMN replaced the numerous different names for mucin-producing epithelial tumors of the pancreas which usually have a papillary architecture with associated dilation of the ducts.

Since then IPMN has been increasingly reported, and nowadays clinically 7% of pancreatic neoplasms and up to 16% of resected pancreatic neoplasms are characterized as IPMN's (Traverso et al., 1998; Falconi et al., 2001; Sohn et al., 2001; Balzano et al., 2005). Furthermore, it is diagnosed in nearly 50% of resected pancreatic cysts (Fernandez-del Castillo et al., 2003).

The WHO classification differentiates IPMN into benign adenomas, borderline tumors and carcinomas which may be invasive or non-invasive. Four subtypes of IPMN are described, the most common being the intestinal type, followed by the pancreaticobiliary, oncocytic and gastric types respectively (Furukawa et al., 2005).

According to the adenoma-carcinoma-sequence

normal ductal epithelium is replaced by columnar mucus producing cells with variable degrees of cellular atypia (Azar et al., 1996; Kosmahl et al., 2004; Salvia et al., 2004; Andrejevic-Blant et al., 2007).

Most of the patients have symptoms similar to chronic pancreatitis (D'Angelica et al., 2004). The treatment of choice is resection, which can be performed in 80-90% of the cases (Andrejevic-Blant et al., 2007). Radicality of resection is still under debate, ranging from limited resection to total oncologic pancreatectomy in cases of multiple IPMN's (Tanaka et al., 2006).

The main reason for the controversies surrounding the optimal treatment is the lack of non-invasive predictors of the clinical course of the disease. One well known predictor is the histopathological invasion of the tumor. 5-year survival rates of non-invasive IPMN range from 77-100%, whereas in contrast this is 26%-46% with invasive IPMN (Falconi et al., 2001; Chari et al., 2002). The grade of dysplasia seems to have no significant influence on survival (Falconi et al., 2001; Chari et al., 2002; Sohn et al., 2004; Wada et al., 2005), in contrast to factors such as lymph node involvement, serum bilirubin, tumor size and location (branch-duct versus main-duct), histology (tubular versus colloidal), angioinfiltration and the presence of blood vessels (Sohn et al., 2001, 2004; D'Angelica et al., 2004; Salvia et al., 2004). IPMN have a significantly better prognosis than ductal adenocarcinoma (Maire et al., 2002; Salvia et al., 2004; Sohn et al., 2004).

In various malignant entities angiogenesis correlates with patient survival, e.g. carcinomas of colon, prostate and cervix, ductal adenocarcinoma and neuroendocrine tumors of the pancreas (Weidner, 1995; Karademir et al., 2000; Duff et al., 2003; Marion-Audibert et al., 2003; Yoshitomi et al., 2008). To determine the microvascular density (MVD) a specific immunohistochemical staining of the micro-vessels (capillaries and small venules) is required.

Expression of endothelial markers such as CD31 (PECAM-1) and CD105 (Endoglin) has been shown to be of prognostic value for solid tumours such as carcinoma of pancreatic, breast and colorectal origin. CD105 particularly seems to be strongly associated with neovascularisation and therefore is postulated to have a higher prognostic impact than CD31 (Takahashi et al., 2001; Dales, Garcia, Andrac et al., 2004; Dallas et al., 2008; Yoshitomi et al., 2008).

The aim of this study was to determine the prognostic value of the angiogenesis-rate in IPMN of the pancreas and to characterize the influence of neovascularisation on the malignant potential of the tumors.

Materials and methods

Patients and Clinical data

The study was approved by the Ethics Committee of the Chamber of Physicians in Hamburg, Germany. Written informed consent was obtained from all patients for using the resected samples for research purposes.

Surgically resected IPMN and cases in which there was a suspicion of IPMN treated at the University Medical Center Hamburg-Eppendorf between 1997 and 2004 were chosen retrospectively.

Tissue-Samples were re-reviewed according to the WHO-classification by a single pathologist. Thirty-two cases were identified and classified according the WHO definition for IPMN. In addition, normal pancreas specimens were used for comparison.

Clinical data including sex, age at diagnosis, clinical symptoms, diagnoses, location and size of the tumor, macromorphologic type, resection margin, operation-method, recurrent, date and cause of death were obtained from a combination of clinical and pathological record reviews, reports of outside medical records and communication with patients and with their attending physicians. None of the patients received neoadjuvant treatment.

Survival data

Clinical follow-up data were obtained by reviewing the hospital records from the outpatient clinic, by direct communication with the attending physicians and from the cancer registry. Overall survival was calculated from the date of operation to the date of death or last follow-up.

Immunohistochemistry

Immunohistochemical staining was performed for 5 μm thick sections of formalin-fixed and paraffin-embedded tissues placed on pre-coated slides with 3-triethoxysilylpropylamin (Merck, Darmstadt, Germany). After deparaffinization with Rotihistole (Merck) and rehydration in ethanol and Tris-Buffer), tissue sections were pre-treated for 20 minutes with TRIS-Buffer at 95°C. After 15 minutes cooling down slides were incubated for 20 minutes with normal serum (rabbit immunoglobulin fraction, Code No X 0903, Dako, Glostrup, Denmark).

The primary antibodies used were a murine anti-human CD31 monoclonal antibody (IgG1, Clone JC70A), a murine anti-human CD105 monoclonal antibody (IgG Clone M3527 Dako) and a murine anti-human Ki-67 monoclonal antibody (IgG clone abMib-1, Dako). Anti-human CD105 and CD31 were each diluted at 1:50, and anti-human Ki-67 1:100 in TRIS-Buffer/BSA and slides were incubated for 30 minutes. For each sample one slide was incubated with irrelevant murine IgG2b (MOPC-141; Sigma, St. Louis, USA) as a negative control to determine the unspecific binding.

Secondary antibody (monoclonal rabbit anti mouse, Code No. Z 0259, Dako) was incubated for 30 minutes, as well as APAAP-antibody (APAAP mouse mono-

clonal, Code No. D 0651, Dako). Staining was performed with a New Fuchsine substrate for 30 minutes at room temperature. Except for the substrate all incubations were performed in a humidity chamber at 37°C. All washing steps were done with TRIS-Buffer. Counterstaining was performed with Haemalaun Mayer (Merck) for 30 seconds followed by Mayer's haematoxylin solution (Merck) for 7 minutes. Finally, slides were covered with coverslips with aqueous mounting medium (Aquatex; Merck).

Immunohistochemical analysis of the sections was performed by two independent investigators without knowledge of the patients' identity or clinical status. In discrepant cases, a pathologist reviewed the cases and a consensus was reached.

To determine the microvascular density (MVD), the microvessel count was assessed in the most vascularized areas of the lesions (in cases of invasive IPMN regardless of the location of invasion) (Dales et al., 2004) using a 40 objective (1.060 mm field diameter) with a Zeiss Axioplan microscope (Carl Zeiss International, Göttingen, Germany). Only small and large microvessels were taken into consideration, as previously described (Weidner et al., 1991). With the mean value of the vessel count in 20 different fields MVD per square millimeter was calculated.

Additionally, a Ki-67 labeling index was determined as marked cells/500 cells in each zone (a total of 1000 cells was counted).

Statistical analysis

SPSS for Windows (SPSS Inc., Chicago, IL USA) was used for statistical analysis. For each patient a CD105/CD31 ratio was calculated, dividing the MVD of CD105 by the MVD of CD31. Relationships between the immunostaining results of CD31, CD105, CD105/CD31 ratio, Ki-67 labeling index, WHO classification of IPMN and clinical data were calculated using a cross table and statistical analysis was performed with Fisher's-test. Therefore, the results of the immunohistochemical stainings and size of the tumors were each divided into a low and high level group (cut off levels: CD31: 150 vessels per square millimeter; CD105: 50 vessels per square millimeter; Ki-67 labeling index: 35% and CD105/CD31 ratio: 0.23; size: 2 cm). Group differences were calculated by the Mann-Whithney-U-Test. Survival curves were plotted using the Kaplan-Meier method and analyzed using the log-rank test. Correlation between CD105 and CD31 was examined using the Pearson correlation index.

P values less than 0.05 were considered statistically significant.

Results

Characteristics of the patients and pathologic findings

A total of 32 patients aged 36 to 84 years with the diagnosis intraductal papillary mucinous neoplasm of the pancreas (IPMN) were included in this study. 17 men (53%) and 15 women (47%) were treated surgically between 1997 and 2004.

Clinical symptoms were abdominal pain (47%), acute pancreatitis (25%), loss of weight (12%) and jaundice (6%).

Operation methods were pylorus preserving pancreaticoduodenectomy (13 patients (41%)), duodenum-preserving pancreatic head resection BEGER (11 patients (34%)), classical pancreaticoduodenectomy (5 patients (15%)), distal (2 patients (6%)) and total pancreatectomy (1 patient (3%))

Macromorphologic types and histopathologic findings are summarized in Table 1.

Median follow-up time of all patients included for survival analysis was 36 months. Of the 32 patients included retrospectively in our study, 9 (28%) experienced recurrence and 6 (25%) died as a result of tumor disease, 2 other patients died perioperatively because of surgical complications (6%).

Survival analysis by Kaplan-Meier method revealed a statistically significant better overall survival and recurrence free survival in patients with non-invasive IPMN (n=10) compared with invasive tumors (n=22) ($p<0.001$ by log rank test, Fig. 3).

Invasive IPMN were significantly larger than noninvasive (cut-off 2cm; $p>0.05$, Fisher's exact test).

Table 1. Comparison of high and low CD105/CD31 ratio with pathological characteristics of the 32 IPMN of the pancreas patients.

Variable		Endolgin/PECAM-1 ratio		
	n (%)	Low	High	p-value
Total	32 (100)	16 (50%)	16 (50%)	n.s.
Tumor localisation				
Head	29 (91)	15	14	
Body	1 (3)	1	0	
Tail	2 (6)	0	2	n.s.
Size				
<2 cm	14 (44)	7	7	
>2 cm	18 (56)	9	9	n.s.
Duct-type				
main	26 (81)	12	14	
small	6 (19)	3	3	n.s.
WHO-Classification				
Adenomas	4 (13)	4	0	
Borderline-tumors	15 (47)	9	6	
Carcinoma non-invasive	3 (9)	1	2	
Carcinoma invasive	10 (31)	2	8	<0.05
Recurrence				
no	9 (28)	12	11	
yes	23 (72)	4	5	
Ki-67 labeling index				
high (>35%)	18 (56)	12	6	
low (<35%)	14 (44)	4	10	<0.05

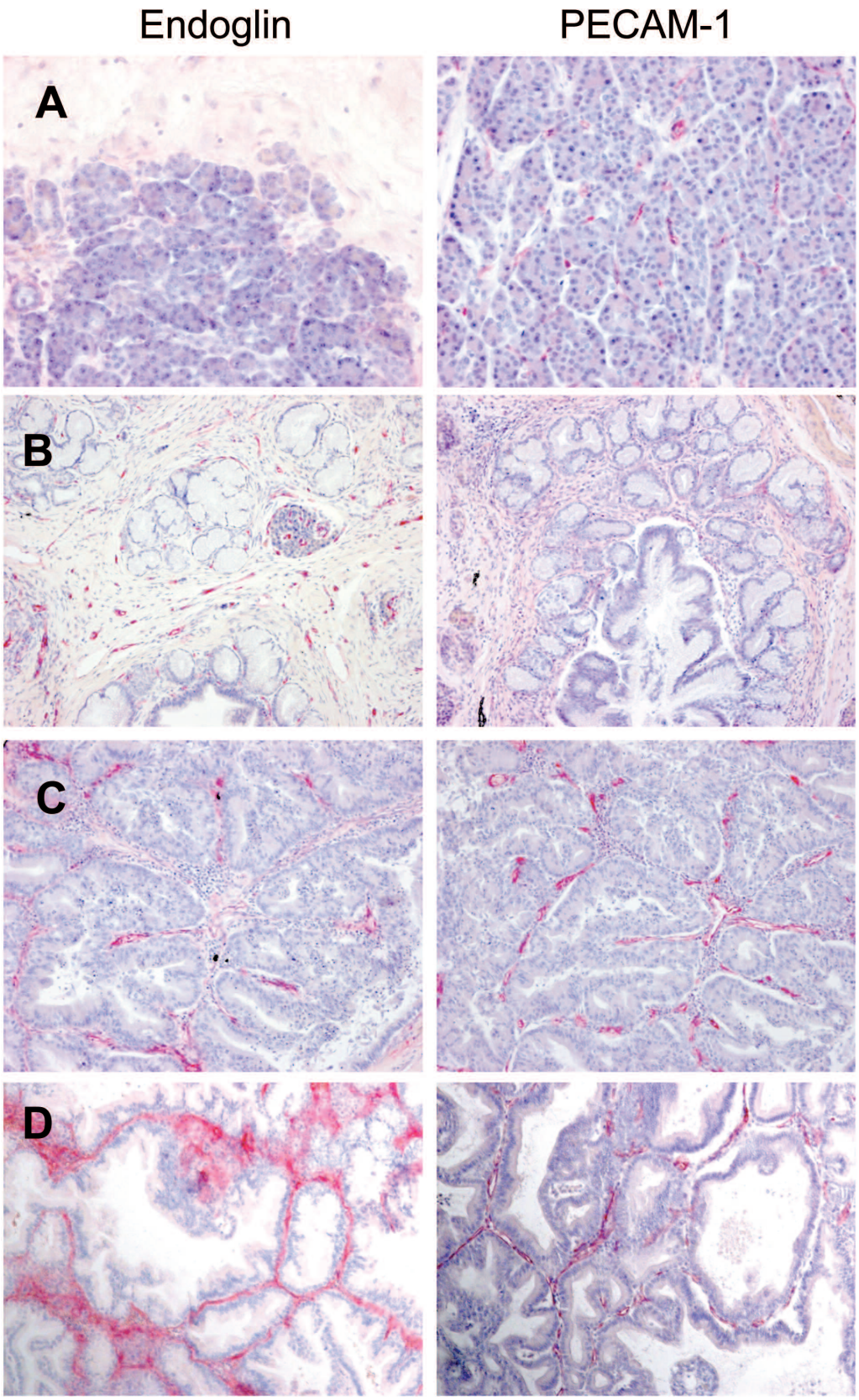

Fig. 1. CD105 (Endoglin) and CD31 (PECAM-1) immunohistochemical stainings of normal pancreatic tissue **(A)**, borderline IPMN **(B)**, lesions of non- invasive IPMN **(C)** and invasive IPMN **(D)**. Original magnification x 100.

The results of proliferation marker Ki-67 labeling index were divided into a high (>35%) and low level group (<35%). Stratified by invasiveness, Ki-67 labeling index showed no significant differences, but invasive tumors tended to have an increased Ki-67 labeling index (p=0.08, Mann-Whithney-U-Test, Fig. 2A). Tumors larger than two centimeters had a significantly higher level of Ki-67 labeling index than smaller tumors (p<0.05, Mann-Whithney-U-Test, Fig. 2A).

Ki-67 labeling index showed no significant association with sex, age at diagnosis, clinical symptoms, diagnosis, location of the tumor, macro-morphologic type, resection margin, recurrence and survival.

CD105 and CD31 immunohistochemistry

Anti-CD31 antibody stained ubiquitously the surface of endothelial cells in tumor as well as pancreatic healthy tissue, regardless of the size or kind of the vessels (Fig. 1). In contrast, anti-CD105 antibody failed to stain the vessels of healthy pancreatic tissue, but stained the endothelium of the peri- and intratumoral vessels, particularly capillary and small vessels with thin walls. Also, lymphatic vessels were positive for CD105-staininig. Vessels with larger diameter, with three layered walls were not stained with the anti-CD105 antibody.

The results of the MVD as assessed by the two different endothelium target molecules CD105 and CD31 showed a highly significant and solid Pearson correlation index (r=0.7; p<0.001).

To determine the proliferation fraction of the endothelium, a CD105/CD31 ratio was calculated. Invasive and non-invasive IPMN's showed significant differences in the CD105/PECAM ratio (p<0.05; Mann-Whithney-U-Test, Fig. 2B). Divided into a low and a high proliferation group (cut off 0.23) the CD105/PECAM ratio is significantly associated with invasion (Fisher's exact test p>0.05) and proliferation index Ki-67 (Fisher's exact test p>0.05).

The results of MVD determined by CD105 and CD31 immunohistochemistry showed no significant association with sex, age at diagnosis, clinical symptoms, diagnosis, location of the tumor, macro-morphologic type, resection margin or recurrence and survival.

We detected a significant difference in the CD31 MVD depending on the size of the tumor (p<0.05, Mann-Whithney-U-Test). In small tumors more CD31 stained vessels were detected than in large tumors.

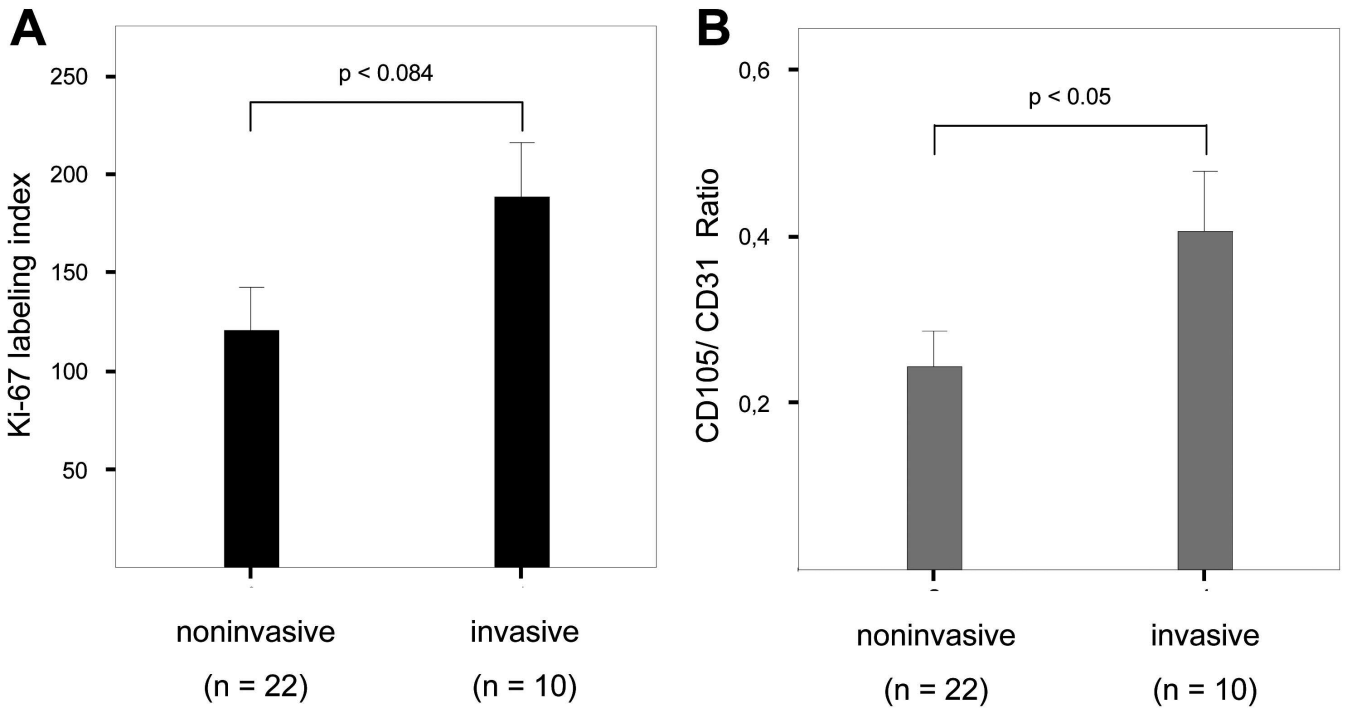

Fig. 2. Quantification of proliferation by the Ki-67 labeling index **(A)** and CD105/CD31 ratio **(B)** in the non-invasive versus invasive group (p<0.084 and p<0.05). Quantification of proliferation by the Ki-67 labeling index **(A)** in the two groups of tumor size, smaller and larger than two centimeters (p<0.05 and p<0.004).

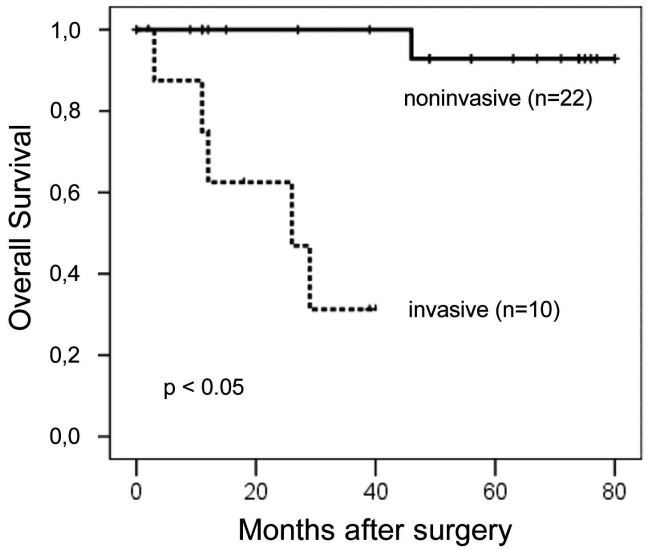

Fig. 3. Kaplan-Meier Analysis of non-invasive versus invasive IPMN of the pancreas ($p<0.05$).

Discussion

The therapeutic strategies for IPMN of the pancreas are still controversial and subject to debate. This is mainly because of the unpredictable behavior of the disease, particularly regarding relapse and transformation into cancer. For this reason we investigated the angiogenesis-rate (AR) in IPMN of the pancreas to characterize the role of neovascularisation in tumorigenesis of this entity.

The pathologic findings and clinical aspects of the 32 patients were in line with patient characteristics of further published articles. Our study confirmed that invasion is a main prognostic marker for a poor outcome (Tanaka, 2004; Gourgiotis et al., 2007).

To investigate the overall proliferative activity of the IPMN's, the Ki-67 labelling index was determined. Based on the postulated adenoma-carcinoma-sequence model in IMPN (Andrejevic-Blant et al., 2007) and its typical growth behavior, overall cell-proliferation is significantly higher in invasive and larger tumors (>2cm).

It is well known that as a consequence of the tumor enlarging new vessels invade and proliferate. In our study, the larger IPMN had a relatively lower CD31 MVD than smaller tumors, suggesting that tumor-growth possibly exceeds neovascularisation and the tumor tissue might be less oxygenized and under-nourished compared to the surrounding healthy tissue.

CD105 and CD31 are both markers for endothelium, but CD105 has a higher specifity for small, developing vessels, i.e. for angiogenesis (Dallas et al., 2008). The impact of CD105 quantification and its potential role in angiogenesis has recently been shown for pancreatic cancer, amongst others (Yoshitomi et al., 2008).

The results of our study show a ubiquitous expression of CD31 in endothelial cells, regardless of the vessel's size or type (arterial, venous or lymphatic). In contrast to this, CD105 was detected only in endothelial cells of small, capillary-like vessels inside and in the surrounding tissue of the tumor. In our series, only few intra- and peritumoral lymphatic vessels were positive for Endoglin-staining. No larger vessels with a three-layered wall are positive for CD105 in our study. These findings confirm an important role of CD105 in early stages of vessel development in the tumor, which has been shown for embryologic angiogenesis (Li et al., 1999). Neovascularisation has an elementary function in the development of growing, malignant tissues (Bergers and Benjamin, 2003).

To determine angiogenesis activity inside a tumor, it is important to know how many vessels preexist and how many are proliferating. Therefore we measured the MVD of all small vessels (CD31) and the MVD of the newly synthesized endothelial cells (CD105) inside the tumor. To determine the growing fraction or neovascularisation activity in IPMN of the pancreas, we calculated a CD105/CD31- ratio.

Because of the different expression pattern of the two molecules, only small sized vessels, which possibly could be stained by anti-CD105 immunohistochemistry, were included in the CD31 microvessel count. Since the goal of the study was to determine the relative amount of growing vessels, the kind of vessels should be similar in both groups. Therefore larger CD31 positive arteries were not taken into consideration. It is under debate whether CD105 expression is immunohistochemical detectable in newly formed lymphatic vessels (Minhajat et al., 2006; Yoshitomi et al., 2008). In our series, all morphologic identifiable small capillary like vessels, which includes the intra- and peritumoral lymphatic vessels, were positive for CD105 staining. Those lymphatic and capillary endothelial cells are hard to differentiate by morphologic criteria only. Because of this, we included the small lymphatic vessels in the count. The rationale behind this decision was that lymphangiogenesis is a well accepted and important component in tumor progression, and of course an integral part of angiogenesis (Alitalo et al., 2005; Choi et al., 2005; Achen and Stacker, 2006; Saad et al., 2006; Schneider et al., 2006).

The results of the AR calculation show a significant association with the Ki-67 labeling index, supporting the principle of synergy between tumor-growth in general and neovascularisation.

Furthermore, the invasive IPMN are not only significantly larger, but have a significantly higher AR compared to noninvasive IPMN. The tumor endothelium of the invading IPMN of the pancreas has a higher rate of proliferation compared to the non invasive IPMN. This illustrates the important role of neovascularisation in the growing, invasive IPMN of the pancreas.

One drawback of this study is the relatively small

number of patients. Further studies are required to confirm the findings and to investigate the underlying molecular mechanisms of tumor progression and neovascularisation and also the role of CD105 and CD31 in IPMN of the pancreas.

In conclusion the results of the study indicate that neovascularisation plays an important role in the tumorigenesis of invasive IPMN of the pancreas. Furthermore, neovascularisation-associated molecules such as CD105 and CD31 might be useful prognostic markers for the disease, and this may have a direct impact on therapy.

Finally, the findings indicate that there may be a potential role for anti-angiogenic therapies as adjuvant treatment in selected patients, particularly for those with invasive IPMN of the pancreas (e.g. irresectable or metastasized IPMN).

Acknowledgements. We thank the patients who willingly and generously provided data and samples for research purposes.

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Accepted April 5, 2010