J Gastroenterol (2013) 48:101–108
DOI 10.1007/s00535-012-0614-z

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Expression of gremlin 1 correlates with increased angiogenesis and progression-free survival in patients with pancreatic neuroendocrine tumors

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Received: 19 October 2011 / Accepted: 9 May 2012 / Published online: 16 June 2012

Abstract
Background Gremlin 1 (GREM1) is a bone morphogenetic protein antagonist and a novel proangiogenic factor. Our aim was to evaluate the prognostic value of GREM1 expression and GREM1-related factors in tumor-associated angiogenesis in pancreatic neuroendocrine tumors (NETs).
Methods The immunohistochemical expression of GREM1 and microvessel density (MVD) were examined in 35 patients with pancreatic NETs and then compared with other clinicopathologic characteristics, including the World Health Organization classification.
Results The presence of expression of GREM1 ($p = 0.016$) and high MVD ($p = 0.020$) were significant and favorable prognostic factors. Moreover, GREM1 expression was significantly associated with high MVD ($p = 0.011$). MVD was significantly higher in well-differentiated NETs than in well-differentiated or poorly differentiated neuroendocrine carcinomas ($p < 0.001$).
Conclusions GREM1 expression was correlated with tumor-associated angiogenesis and was found to be a novel prognostic marker in pancreatic NETS. Our data support a tumor suppressor role of GREM1 in pancreatic NETs.

M.-H. Chen and Y.-C. Yeh contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00535-012-0614-z) contains supplementary material, which is available to authorized users.

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Keywords GREM1 · Pancreatic neuroendocrine tumor · Angiogenesis

Introduction

Pancreatic neuroendocrine tumors (NETs) are rare neoplasms that are characterized by indolent behavior and which have the ability to secrete a variety of peptides and neuroamines, resulting in variable clinical syndromes [1]. The incidence and prevalence of pancreatic NETs have shown a steady increase during the past 30 years [2].

Pancreatic NETs are usually characterized by a high vascular density [3]. However, unlike other solid cancers, high microvessel density (MVD) is a favorable prognostic factor and correlates with well-differentiated pancreatic NETs [4–6]. These observations indicate that angiogenesis increases in low-grade pancreatic NETs, but decreases in high-grade tumors. The mechanism that controls angiogenesis during the malignant transformation of pancreatic NETs is still unclear.

Vascular endothelial growth factor A (VEGF-A) is the most common and potent proangiogenic factor in several cancers. However, the correlation between VEGF-A and pancreatic NETs is controversial, and there is no evidence that it contributes to tumor angiogenesis in pancreatic NETs or that it influences patient survival [4–6]. This absence of evidence has led to the hypothesis that tumor angiogenesis in pancreatic NETs may rely on unidentified angiogenic factor(s). Gremlin 1 (GREM1), a secreted glycoprotein, antagonizes bone morphogenetic proteins (BMPs) 2, 4, and 7, thereby preventing these ligands from interacting with their receptors and resulting in inhibition of transforming growth factor-β signaling [7, 8]. GREM1 is also a novel proangiogenic factor that induces angiogenesis in a BMP-independent manner by activating vascular endothelial growth factors receptor-2 (VEGFR2)-dependent angiogenic responses [9, 10]. The specific biologic role of GREM1, especially angiogenesis, in pancreatic NETs has not yet been explored.

The aim of this study was to examine the expression pattern of GREM1 in pancreatic NETs and to determine its associations with clinicopathologic characteristics, angiogenesis and progression-free survival.

Materials and methods

Patient clinicopathologic data

This study has been approved by the Ethics Committee of Taipei Veterans General Hospital (201009025IC). Clinical data, pathologic data and tissue specimens were obtained through a detailed retrospective review of the medical records of 35 patients with pancreatic NETs who had undergone initial surgical resection between 1985 and 2010 at Taipei Veterans General Hospital [11]. The median age of the patients was 55 years (range 19–76, mean 52.1 years). Surgery consisted of pancreatoduodenectomy in 12 cases, segmental pancreatectomy in one case, distal pancreatectomy in 16 cases and tumor enucleation in six cases. NETs can be classified according to the World Health Organization (WHO) 2000 classification [12] into the following groups: well-differentiated endocrine tumors (WDET), well-differentiated endocrine carcinoma (WDEC), and poorly differentiated endocrine carcinoma (PDEC). For additional analysis, tumors were also classified according to the WHO 2010 classification system [13] into the following groups: neuroendocrine tumor Grade 1 (NET G1), NET G2 and neuroendocrine carcinoma (NEC G3). Follow-up data were available in all cases, and the length of the follow-up ranged from 0.7 to 276.1 months (median 41.1, mean 61.6 months). During the follow-up period, four patients presented with evidence of disease progression and five patients died. However, only one patient's cause of death was tumor-related. The latest survival data were collected on December 31, 2010. The overall survival rate was 78.3 % at 5 years of follow-up and 68.5 % at 10 years of follow-up. The clinicopathologic features of the 35 cases are summarized in Table 1. Several variables, such as presence of lymph node metastases or liver metastases, high WHO 2000 or 2010 grades, high European Neuroendocrine Tumor Society (ENETS) stage, and high Union for International Cancer Control (UICC) stage were closely correlated with shorter survival (Table 1).

Tissue microarray construction and immunohistochemical staining

Hematoxylin and eosin-stained specimens of all 35 samples were reviewed by the pathologist, and the pancreatic tissue microarrays (TMAs) of the NETs were constructed by obtaining three 1-mm-diameter cores from each tumor and the paired adjacent normal pancreatic islets of Langerhans. The hematoxylin and eosin-stained specimens of the constructed TMAs were reviewed and confirmed again by the pathologists (Michael Hsiao and Yi-Chen Yeh). Immunohistochemical staining for GREM1 and microvessel density (MVD) was performed on the TMA slide. The specimens had been fixed in formalin and embedded in paraffin before they were archived. We used the archived specimens for immunohistochemical staining with Bond-Max autostainer (Leica Microsystems, Wetzlar, Germany). Slides were stained with polyclonal anti-gremlin antibody (PAB14845, dilution 1:100; ABNOVA, Taipei. Taiwan) and monoclonal anti-CD34 antibody (clone QBEnd-10,

Table 1 Patients' demographic characteristics and clinicopathologic variables

Characteristics	No. of patients ($n = 35$)	p value for disease-free survival
Age (years)		NS
≥40	26 (74.3 %)	
<40	9 (25.7 %)	
Sex		NS
Male	17 (48.6 %)	
Female	18 (51.4 %)	
VHL disease		NS
VHL+	3 (8.6 %)	
VHL−	32 (91.4 %)	
Functional syndrome		NS
Present	18 (51.4 %)	
Absent	17 (48.6 %)	
Classification according to WHO 2000		<0.001
Well-differentiated tumor	24 (68.6 %)	
Well-differentiated carcinoma	9 (25.7 %)	
Poorly differentiated carcinoma	2 (5.7 %)	
Classification according to WHO 2010		0.001
Neuroendocrine tumor Grade 1	17 (48.6 %)	
Neuroendocrine tumor Grade 2	12 (34.3 %)	
Neuroendocrine cancer Grade 3	2 (5.7 %)	
Missing	4 (11.4 %)	
Size (cm)		NS
<2	12 (34.2 %)	
>2	23 (65.7 %)	
Lymph node status		<0.001
N0	29 (82.9 %)	
N+	6 (17.1 %)	
Liver metastasis		0.035
M0	31 (88.6 %)	
M1	4 (11.4 %)	
ENETS staging		<0.001
I + II	27 (77.1 %)	
III + IV	8 (22.9 %)	
UICC staging		0.035
I + II	31 (88.6 %)	
III + IV	4 (11.4 %)	

VHL von Hippel–Lindau disease, *WHO* World Health Organization, *ENETS* European Neuroendocrine Tumor Society Staging, *NS* not significant, *UICC* Union for International Cancer Control

Data are presented as the number (n) of patients, with the percentage in parenthesis

dilution 1:75; Dako, Glostrup, Denmark). Briefly, specimens from the paraffin-embedded blocks were cut into 5-μm sections. Sections were dewaxed in a 60 °C oven and then deparaffinized in xylene, rehydrated through serial dilutions of alcohol and washed in phosphate-buffered saline (pH 7.2). Immunohistochemical staining was performed on the fully automated Bond-Max autostainer using onboard, heat-induced antigen retrieval in citrate buffer with the ER1 protocol for 20 min and a VBS Refine polymer detection system (Leica Microsystems). Diaminobenzidine was used as the chromogen (Leica Microsystems). The sections were then counterstained with hematoxylin.

Scoring of GREM1 expression

A four-point staining intensity scoring system was employed, as described previously [14], for determining the relative expression of GREM1 in cancer specimens. The staining intensity score ranged from 0 (no expression) to 3 (maximal expression). The results were classified into two groups according to the intensity and extent of staining: (1) GREM1 (−) group, in which no staining was present (staining intensity score = 0); (2) GREM1 (+) group, in which positive staining was detected in <10 % of the cells (staining intensity score = 1), positive immunostaining was present in 10–30 % of cells (staining intensity score = 2) or positive staining was present in >30 % of the cells (staining intensity score = 3). All of the immunohistochemical staining results were reviewed and scored independently by two pathologists (Michael Hsiao and Yi-Chen Yeh). With discrepant results (score 0 and 1) of the same slide, final agreement was obtained after discussion between these two pathologists (consensus). In order to further validate our data, we used an automated image analysis system (Aperio Technology, Vista, CA), which uses a color deconvolution algorithm to visualize GREM1 protein expression in pancreatic NETs [15]. Quantification of immunohistochemical staining was performed with color translation and an automated thresholding algorithm from Aperio Technology.

Evaluation of intratumoral microvessel density

All independent CD34-positive vascular structures were taken into account, irrespective of the presence of an identifiable lumen. The number of CD34-positive structures was counted in five consecutive high-power fields at a magnification of 400× (0.238 mm^2 per field). The MVD for each tumor was calculated as the number of CD34-positive vascular structures per square millimeter. All of the pancreatic NETs were divided into two groups based on the median MVD: (1) low MVD group, in which the MVD was lower than the median MVD; (2) high MVD group, in which the MVD was equal to or higher than the median MVD [6, 16].

Statistical analysis

The correlations between the expression of GREM1, MVD and clinicopathologic variables were analyzed for statistical significance using the chi-square test and Fisher's exact test. The correlation between GREM1 and MVD was also analyzed by the Spearman's non-parametric correlation test. Survival data were analyzed according to the Kaplan–Meier method. The log-rank test was used to compare survival data between groups. A p value of <0.05 was considered to be significant.

Results

The scoring results of GREM1 expression by pathologists was strongly correlated with values obtained using the automated image analysis system

Figure 1a (top) shows representative immunohistochemical staining examples with different GREM1 scores in pancreatic NETs. We also used an automated image analysis system to visualize GREM1 protein expression (Fig. 1a, bottom) in pancreatic NETs [15]. Quantification of both types of immunohistochemical stained specimens is described in the Methods section, and the results showed a strong correlation between manual and automated scoring (Spearman's $\rho = 0.874$, $p < 0.001$).

GREM1 expression in pancreatic neuroendocrine tumors is associated with good prognosis

The prognostic significance of GREM1 expression was determined by assessing its cytoplasmic staining using 33 human pancreatic NET specimens for which there were known clinical follow-up records. All normal islets of Langerhans in non-cancerous tissues demonstrated negative or weak expression of GREM1 [Table 2; Electronic Supplementary Material (ESM) Fig. 1]. The relationships between the levels of GREM1 expression and the clinicopathologic characteristics of pancreatic NETs are summarized in ESM Table S1. Among these specimens, the GREM1 (−) group correlated strongly with reduced progression-free survival relative to the GREM1 (+) group, as shown in Fig. 1b ($p = 0.016$). Our data indicate that loss of GREM1 expression predicted a poor prognosis in terms of pancreatic NETs.

High microvessel density expression in pancreatic neuroendocrine tumors is associated with good prognosis

The prognostic significance of MVD was determined using 31 human pancreatic NET specimens with known clinical follow-up records. Figure 2a shows representative examples of these specimens with different MVDs. All of the pancreatic NETs were divided into two groups based on the median MVD [6]. The high MVD group showed longer progression-free survival compared to that of the low MVD group (Fig. 2b, $p = 0.02$).

GREM1 expression was correlated with high MVD

We next examined the potential associations between GREM1 expression and MVD level. A total of 73.3 % of samples positive for GREM1 expression exhibited a high MVD, whereas 26.7 % of samples negative for GREM1 expression had a high MVD (Fig. 3; $p = 0.011$). GREM1 expression and MVD also showed a positive correlation with a Spearman's $\rho = 0.467$ ($p = 0.009$), as analyzed by the Spearman's non-parametric correlation test. Our data showed that GREM1 expression was significantly correlated with high MVD.

High MVD correlated with well-differentiated pancreatic NETs according to the WHO 2000 classification

Microvessel density was significantly higher in well-differentiated NETs than in well-differentiated or poorly differentiated neuroendocrine carcinomas (Fig. 4; $p < 0.001$). However, no correlation between MVD and pancreatic NETs stratified according to the WHO 2010 classification was observed ($p = 0.116$; data not shown). We also found that GREM1 did not correlate with pancreatic NETs stratified according to the WHO 2000 or WHO 2010 classification ($p = 0.134$, $p = 0.252$; ESM Table S1).

Discussion

In this study, we demonstrated that GREM1 may have potential as a new prognostic marker for pancreatic NET. We found a close correlation between GREM1 expression and favorable prognosis in pancreatic NETs. Because GREM1 is a novel proangiogenic factor [9, 10], we subsequently measured MVD in pancreatic NETs and found that high MVD was a good prognostic factor that correlated with the expression of GREM1 and well-differentiated NETs.

The manual interpretation of immunohistochemical staining remains a subjective process, which may lead to limited statistical confidence due to inter-observer or intra-observer variability. This study used automated quantitative algorithms to analyze GREM1 immunohistochemical data and found a strong correlation between manual and automated scoring (Spearman's $\rho = 0.874$, $p < 0.0001$),

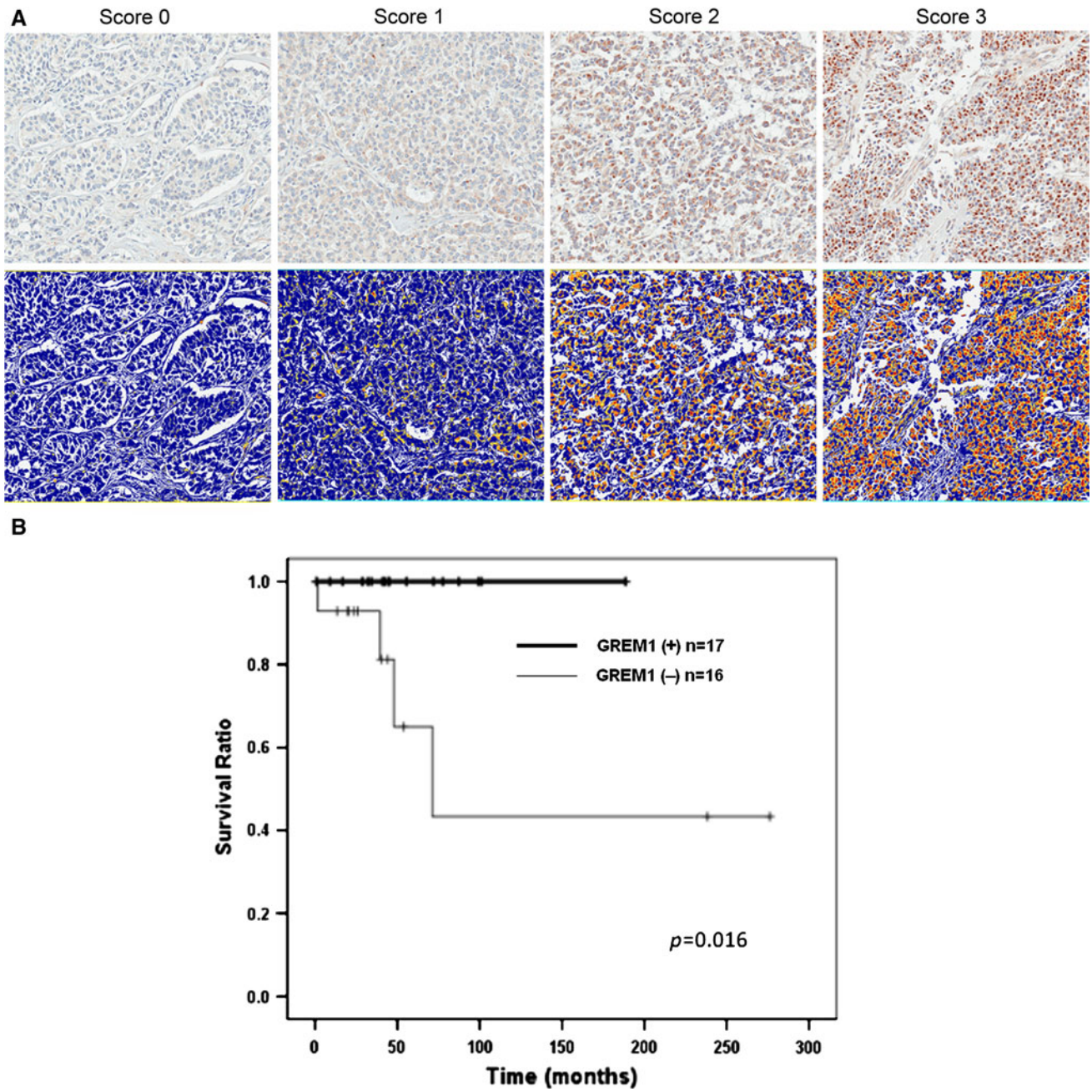


Fig. 1 Gremlin 1 (*GREM1*) is expressed in tumors and correlates with a good prognosis. **a** GREM1 levels in representative pancreatic neuroendocrine tumor (NET) tissues by immunohistochemical staining (*top*) and the color deconvolution algorithm (Aperio Technology; *bottom*). **b** Kaplan–Meier plot of the progression-free survival of 33 patients with pancreatic NETs, stratified by GREM1 expression. Refer to section Scoring of GREM1 expression for a detailed explanation of the GREM1 (−)/GREM1 (+) groups

suggesting that our scoring results were reliable and reproducible.

In order to understand the role of GREM1 in cell proliferation, Curran et al. [17] generated mouse embryonic fibroblasts lacking GREM1. Deletion of GREM1 increased cell proliferation and migration. Similarly, over-expression of GREM1 in an osteoblastic tumor cell line reduced proliferation through transcriptional increases in p21Cip1 in a pathway independent from ERK [18]. Van Vlodrop et al. also reported that GREM1 silencing by region III promoter methylation was significantly associated with higher tumor grade, tumor stage, lower MVD and shorter survival in patients with clear cell renal cell carcinoma [7]. GREM1 has also been found to be silenced by promoter

Table 2 Immunohistochemical analysis of Gremlin 1 expression in islets of Langerhans and pancreatic neuroendocrine tumors

	No. of tissue samples with the indicated GREM1 immunostaining intensity			
	Score 0	Score 1	Score 2	Score 3
Islets of Langerhans	10	8	0	0
Pancreatic NETs	16	10	5	2

NETs Neuroendocrine tumors, *GREM1* Gremlin 1

hypermethylation in several human malignancies [19]. These observations suggest a tumor suppressor role for GREM1.

Angiogenic factors in many types of tumors are related to tumor metastasis, tumor aggressiveness and decreased survival [20, 21]. However, several papers have reported that high MVD is a good prognostic factor in pancreatic NETs and is associated with well-differentiated pancreatic NETs [4, 6]. In addition, Stabile et al. further identified GREM1 as a novel proangiogenic factor [9, 10]. These researchers delivered GREM1 to chicken embryo

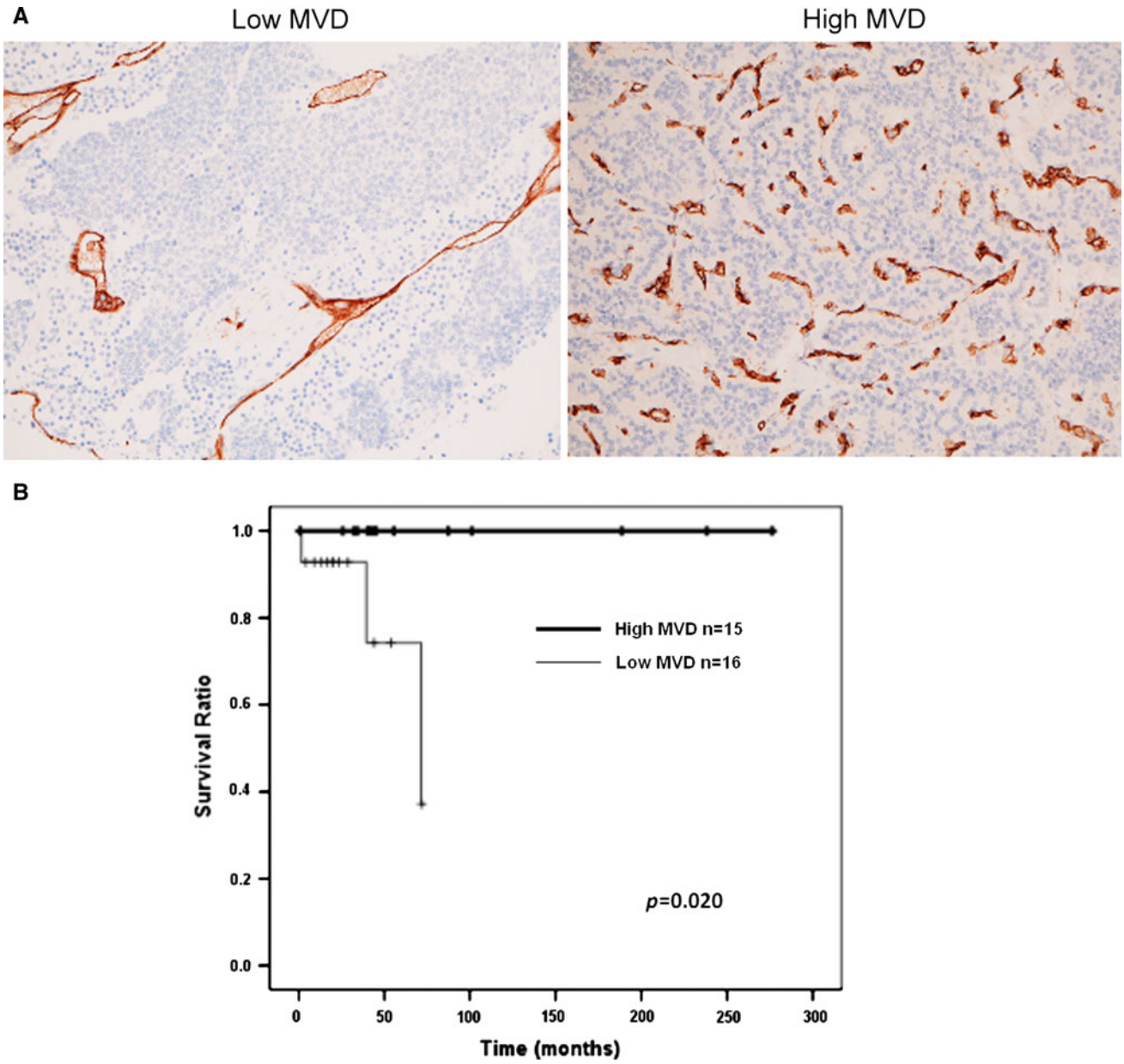


Fig. 2 High microvessel density (*MVD*) correlates with good prognosis. **a** MVD levels in representative pancreatic NET tissues. **b** Kaplan–Meier plot of the progression-free survival of 31 patients with pancreatic NETs, stratified by MVD level

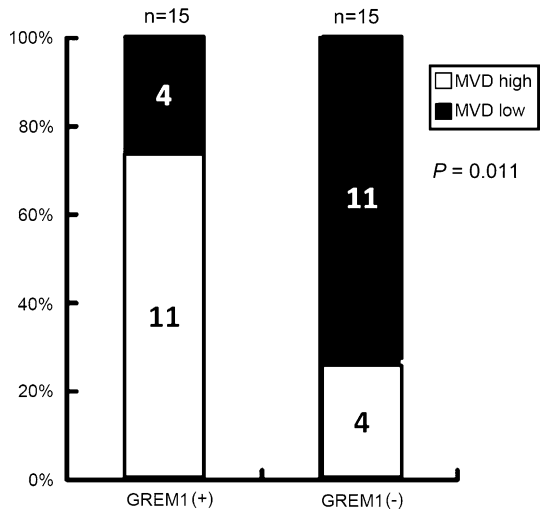
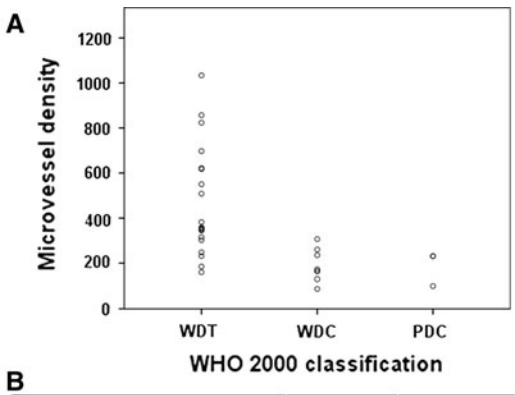


Fig. 3 Relationship between MVD and GREM1 expression. Patients were divided into two groups based on median MVD. The GREM1 (+) group had a higher MVD than the GREM1 (−) group ($p = 0.011$)



B

WHO classification \ MVD	High	Low	*P*-value
Well differential tumor (WDT)	15 (75%)	5 (25%)	<0.001
Well differential carcinoma (WDC)	0	8 (100%)	
Poorly differential carcinoma (PDC)	0	3 (100%)	

Fig. 4 Relationship MVD and the World Health Organization (*WHO*) 2000 classification. **a** MVD increased according to the progression of pancreatic NETs in terms of the WHO 2000 classification. **b** Patients were divided into two groups by median MVD. The high MVD group correlated more strongly with well-differentiated NETs than with well-differentiated or poorly differentiated neuroendocrine carcinoma ($p < 0.001$)

chorioallantoic membrane (CAM), and a potent angiogenic response was observed in the GREM1 implants when compared with that of vehicle-treated embryos. The number of macroscopic blood vessels was also significantly higher in GREM1 implants. In our study, both GREM1 expression and high MVD were good prognostic factors in pancreatic NETs, and there was a significant correlation between these two factors (Figs. 2b, 3). Taken together, our data indicate that GREM1 expression plays a tumor suppressor role and may participate in tumor-associated angiogenesis in pancreatic NETs.

Normal endocrine tissues, such as pancreatic islets of Langerhans, are characterized by high vascular density. However, all normal islets of Langerhans in our study demonstrated negative or very weak expression of GREM1, suggesting that GREM1 is only involved in tumor-associated angiogenesis in pancreatic NETs (Table 2; ESM Fig. 1). A similar phenomenon was also reported by Stabile et al. [10], who observed strong GREM1 immunoreactivity in the endothelial cells of lung tumor samples, but not in non-neoplastic lung tissue [10].

Other angiogenic factors have been studied in pancreatic NETs. The role of VEGF-A in pancreatic NETs is controversial, and there is no evidence that it contributes to increased patient survival [4–6]. CXCL-12, which is a known CXC chemokine, is also a candidate molecule associated with angiogenesis in pancreatic NETs [6]. Another potential factor is angiopoietin-2 (Ang-2), which is a ligand of the endothelial tyrosine kinase, Tie2. Ang-2 overexpression stimulates neoangiogenesis in orthotopic pancreatic NET xenografts, promotes disease progression of NETs and is regarded as an adverse prognostic marker [22].

In the WHO 2000 classification [12], NETs were categorized into WDET, WDEC, and PDEC based on tumor size, local invasion of adjacent organs, angioinvasion, perineural invasion, Ki-67 proliferation index and the presence of metastases. However, in the WHO 2010 classification [13], gastroenteropancreatic NETs were categorized into NET G1, NET G2 and NEC G3 based on the Ki-67 proliferation index. In our study, we observed that high MVD was correlated with pancreatic NETs stratified according to the WHO 2000 classification, but not the WHO 2010 classification. This finding suggests that MVD was not correlated with the tumor proliferation index, but was influenced by other factors, such as tumor invasion or metastasis.

The main limitation in this study was the small sample size ($n = 35$) and low event rate in the progression-free-survival (PFS) analysis. Pancreatic NETs are rare malignancies, and most patients are diagnosed with distant metastasis. It was thus difficult to collect samples. All cases in this study were resected pancreatic NETs with good prognosis, which accounts for the low event rate of PFS and overall survival. In fact, only one patient died of this tumor, and so it was not possible to perform a meaningful evaluation of the significance of GREM1 in the OS analysis. The role of GREM1 in pancreatic NETs requires further validation in future studies.

In conclusion, our results indicate that GREM1 may have potential as a novel prognostic biomarker and as a candidate tumor suppressor gene in pancreatic NETs. GREM1 is a secreted glycoprotein and could therefore be

tested in serum samples as a non-invasive biomarker of pancreatic NETs. GREM1 is also shown to be a proangiogenic factor that is possibly involved in tumor-associated angiogenesis.

Acknowledgments This research was supported by Grants from Taipei Veterans General Hospital (101DHA0100657, 100DHA0100480, 100DHA0100489 and 101DHA0100653), Center of Excellence for Cancer Research at Taipei Veterans General Hospital (DOH100-TD-C-111-007), Taiwan Clinical Oncology Research Foundation, the National Science Council (NSC100-2627-B-010-005), and the Ministry of Education, Aim for the Top University Plan (National Yang-Ming University).

Conflict of interest None.

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