Endoplasmic Reticulum Stress in Pancreatic Neuroendocrine Tumors is Linked to Clinicopathological Parameters and Possible Epigenetic Regulations

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Abstract. *Background: Endoplasmic reticulum (ER) stress is a highly-conserved cellular defense mechanism in response to perturbations of ER function. The role of ER stress in pancreatic neuroendocrine tumors (pNET) still remains unclear. Materials and Methods: We analyzed the protein expression pattern of the four key players of ER stress, (chaperone binding imunoglobluin protein (BiP), C/EBP homologous protein (CHOP), activating transcription factor 4 (ATF4) and caspase 4) as well as histone deacetylases (HDACs) by a tissue microarray (TMA) of 49 human pNET resected between 1997 and 2013 following, extensive clinicopathological characterization. Results: Immunohisto-chemical profiling revealed a significant up-regulation of BiP, ATF4, CHOP and caspase 4 in pNET cases compared to normal controls. Correlated to clinicopathological parameters especially BiP expression could be linked to higher grading and proliferation as well as to lower survival probability. Finally, expression of ER stress markers correlated with HDAC expression in situ and pharmalogical inhibition by panobinostat significantly reduced cell viability in vitro. Conclusion: Up-regulation of ER stress in pNET indicates the presence and engagement of ER stress signaling in this tumor entity demonstrating another possible anticancer therapy option in pNET.*

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Pancreatic neuroendocrine tumors (pNET) are a very rare and heterogeneous group of malignant disorders encompassing 1-2% of all pancreatic malignancies with an incidence of <1.0 /100,000 cases per year (1). Non-functional pNET remain asymptomatic for a long time while functional pNET (2) cause symptoms by hypersecretion of hormones or bioamines, hence termed insulinoma (45%), gastrinoma (20%), glucagonoma (13%), VIP (vasoactive intestinal peptide)oma (10%), and somatostatinoma (5%) (3). ER stress is a cellular response to perturbations of ER function (4): ER homeostasis can be altered by intensive protein synthesis triggering a stuck of protein maturation processes, decreased function of ER chaperones and alterations of ER calcium stores. All these circumstances lead to ER stress, ultimately resulting in accumulation of unfolded or misfolded proteins within the ER (5). Several cellular mechanisms contribute to reduction of ER stress: initiated by the ER-transmembrane transducers inositol-requiring 1α (IRE1α), PKR-like ER kinase (PERK) and activating transcription factor 6α (ATF6α) (5, 6), the unfolded protein response (UPR) increases protein folding capacity, rapid turnover of misfolded proteins and a slowdown of *de novo* protein synthesis (5, 6). Under normal conditions, IRE1α, PERK and ATF6α are kept inactive by the ER chaperone binding immunoglobulin protein (BiP). During accumulation of unfolded proteins, BiP dissociates from the UPR transducers (7). As a result, activated PERK phosphorylates a subunit of the eukaryotic translation initiation factor 2α (eIF2α), thus slowing-down global mRNA translation and reducing the amount of unfolded proteins. Additionally, this mechanism enhances the expression of ATF4 (5, 8) which in turn induces the expression of C/EBP homologous protein [CHOP, also known as growth arrest-and DNA damage-inducible gene 153 (*GADD153*)] (9). If cells are not able to overcome ER stress,

Table I. *Characteristics of patients with pancreatic neuroendocrine tumors (pNET).*

n (%)	49 (100.0)
Female	30 (61.2)
Male	19 (38.8)
Age (mean±SD [min-max])	60.0±14.5 [14.2-82.7]
Female	57.6±16.2
Male	63.8±10.7
Size (cm, mean±SD [min-max])	3.0±2.6 [0.5-12.0]
Localization n (%)	
Unknown	3 (6.1)
Head	19 (38.8)
Corpus	9 (18.4)
Cauda	18 (36.7)
TNM[a]	
T1	19 (38.8)
T2	11 (22.4)
T3	16 (32.7)
T4	3 (6.1)
N0	33 (67.3)
N1	16 (32.7)
M0	39 (79.6)
M1	10 (20.4)
Grading[b] [Mitotic[c]/proliferation[d] rate (mean±SD)]	
G1	28 (57.1) [1.5±1.3/1.3±0.6]
G2	14 (28.6) [7.3±7.7/5.2±2.1]
G3	7 (14.3) [76.7±33.0/63.2±14.6]
Hormone activity[e]: no/yes	28 (57.1)/20 (40.9)

[a] According to current TNM (7th edition, 2010) (27), [b] according to the guidelines of the current TNM based on mitotic or proliferation activity, [c] H&E associated mitotic activity per 10 high-power fields, [d] Ki-67 associated proliferation index calculated as percentage per 2,000 tumor cells, [e] Hormone activity is measured by immunohistochemistry (see details in material and methods; One PNET case could not be characterized due to the fact that the tumor content within the paraffin black was exhausted). T, Tumor (extent of the tumor); N, node (absence/presence and extent of regional lymph node metastasis); M, metastasis (absence/presence of distant metastasis); G, grading; SD, standard deviation.

a cell demise program is initiated leading to cell death (7), mediated, in part, by CHOP (6, 8) that represses the expression of the anti-apoptotic B-cell lymphoma 2 gene (*Bcl-2*) (10). In addition, IRE1α activates tumor necrosis factor receptor-associated factor 2 (TRAF2), that causes *via* c-Jun N-terminal kinases/stress-activated protein kinase (JNK/SAPK) activation of ER-specific caspases (11). Human caspase 4 is a member of the caspase 1 sub-family and was found to be activated specifically by ER stress inducers (12). Once caspase 4 is activated, all cellular events will act to promote cell death.

Our previous study showed that ER stress can be induced by panobinostat, a pan-(H)DAC inhibitor (HDACI), leading to cell death pathway in hepatocellular carcinoma (HCC) cells (13). HDACs are a group of epigenetic enzymes that usually cause chromatin compaction and epigenetic gene silencing (14) and comprise of four sub-classes (I, IIA/B, III, IV) (15).

As ER stress-related pathways might represent promising therapeutic anticancer targets, the aim of the present study was to analyze pNET tumor samples for signs and extent of ER stress and related apoptotic cell death. Therefore, we investigated the protein expression of the ER stress-related factors BiP, ATF4, CHOP and caspase 4 as well as the expression of the HDAC proteins in 4 pNET tissue microarrays. Moreover, we investigated the cytotoxic effects of panobinostat on the pNET cell line BON1.

Material and Methods

Ethics statement. The present study was conducted according to National and Institutional guidelines of the Paracelsus Medical University Salzburg/Salzburg County Hospital as well as in accordance with the declaration of Helsinki (1964); the anonymized samples are exclusively available for research purposes in retrospective studies. All analyses on human pNET samples were approved by the local ethics committee (415-EP/73/408-2014).

Clinical and pathological characterization of pNET cases. We analyzed 49 pNET cases surgically resected between 1997 and 2013 and archived at the Institute of Pathology, Paracelsus Medical University, Salzburg, Austria. All cases were extensively characterized for clinicopathological parameters including sex, age, size, localization, type of surgery, tumor grade and stage. Neuroendocrine differentiation was investigated by immunohistochemical staining for synaptophysin (mouse monoclonal, ready-to-use, Dako, Vienna, Austria) and Chromogranin A (mouse monoclonal, dilution 1:400; Dako) on 5-μm-thick formalin-fixed, paraffin embedded (FFPE) sections.

Grading of pNET cases. Based on conventional 5-μm hematoxylin-eosin-stained FFPE sections, the mitotic activity of the pNET cases were assessed by counting mitoses in 10 consecutive high-power fields (HPF), according to published guidelines (16) on a Leica DM 2000 microscope (Leica Mikrosysteme, Vienna, Austria) by two independent investigators (KE, DN). Additionally, the mitotic and proliferative activity was investigated by Ki-67 and phosphohistone-H3 (PHH3) immunohistochemistry, as described in detail previously (17).

Assessment of pNET hormone activity. All specimens were routinely analyzed for immunohistochemical expression of calcitonin, gastrin, glucagon, insulin, serotonin, somatostatin and VIP on whole-tumor slides. Finally, the expression of somatostatin receptor 2 (SSTR-2) was investigated by immunohistochemistry (IHC) according to the scoring system proposed by Volante *et al.* (18).

Immunohistochemistry and processing for markers of ER stress (BiP, ATF4, CHOP and caspase 4) and HDACs (1-6, 8-11 and Sirt1). In order to simplify the investigations and ensure comparability of the

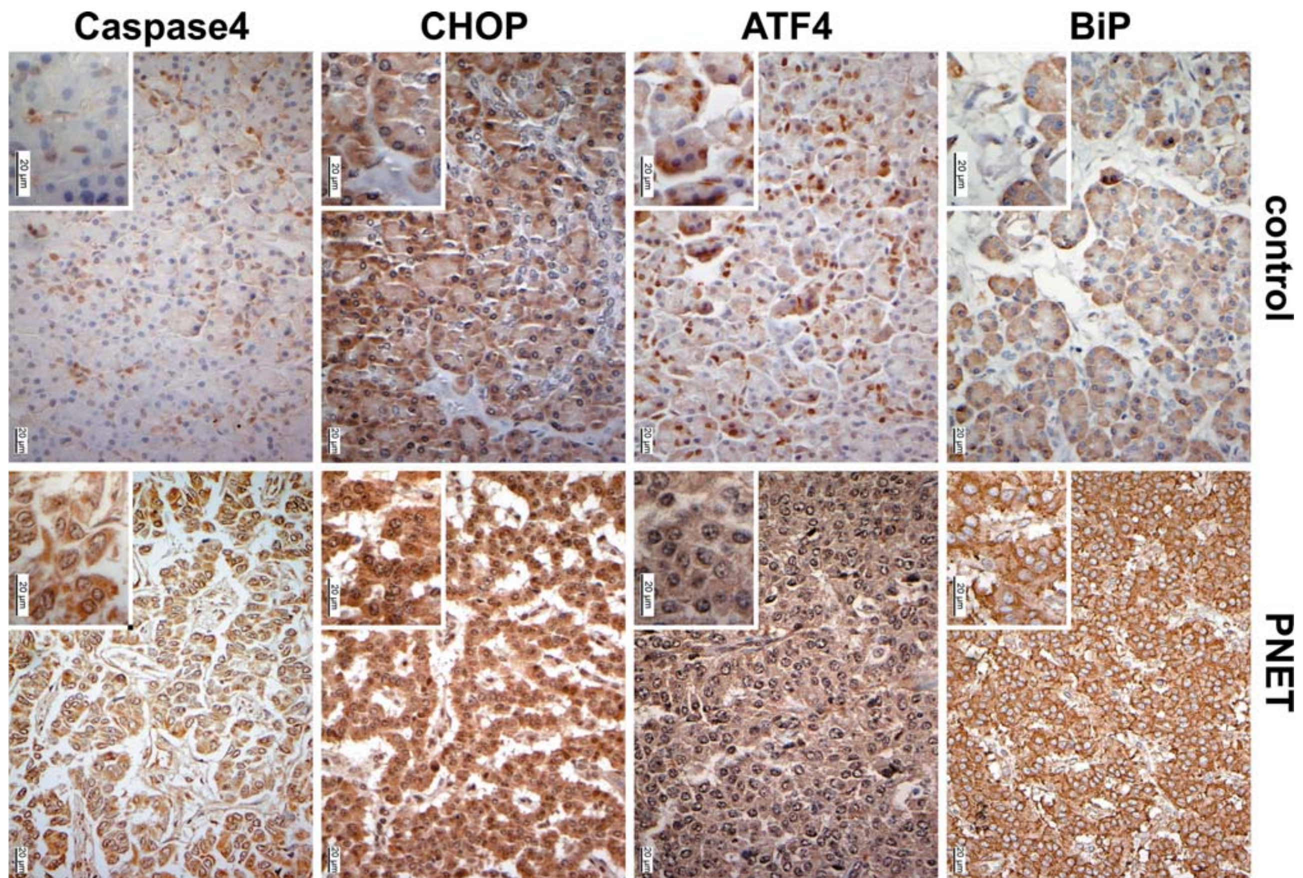

Figure 1. *Representative immunohistochemical expression pattern of ER stress components. BiP, ATF4 and CHOP as well as caspase 4 in control tissue (upper) compared with pNET (lower) showing increased expression levels of BiP, ATF4 and CHOP as well as caspase 4 in tumor compared to normal control tissue. Additionally, a shift from cytoplasma to nuclear was seen for ATF4 (insert) and CHOP (insert) (original magnification: 400x, inserts 630x).*

immunohistochemical signals, four tissue microarrays (TMA) were performed. As far as possible, each pNET case was represented on a TMA with tumor tissue as well as adjacent normal pancreas tissue (19). Each TMA was cut into 5-µm sections, raised on adhesive glass slides and dried at 60˚C for 1 h. De-paraffination, antigen retrieval, immunostaining, counter staining, dehydration and cover slip application as well as pre-treatment were performed using standardized routine IHC protocols. Immunohistochemical staining was performed on a Dako Autostainer Plus combined with the EnVision Plus System using primary mouse or rabbit antibodies. In case of a primary goat antibody, a secondary enzyme conjugated antibody was used. Sources of all applied primary antibodies (including catalog-number, clone species, dilution, incubation and pre-treatment) are available on request.

Interpretation and scoring of the IHC. Examination of each pNET case within the TMAs was carried out semi-quantitatively by counting the number of stained cells and assessing the intensity of the staining. Afterwards an immunoreactivity score (IRS; 0-300) was calculated by multiplying the scores for intensity (0-3) and the stained cells (0-100%) as previously published (20).

Cell culture and reagents. BON1 cell-line, representing the gold-standard *in vitro* model for the study of pNET, was kindly provided by Dr. Thaddeus Till Wissniowsky from the Division of Gastroenterology, University Hospital Marburg (Marburg, Germany). BON1 cells were cultured in DMEM (Dulbecco's modified Eagle's medium, Sigma-Aldrich, Munich, Germany) supplemented with 10% fetal bovine serum, penicillin (107 U/l) and streptomycin (10 mg/l; Biochrom, Berlin Germany) at 37˚C in a humidified atmosphere containing 5% CO_2. Panobinostat was provided by Novartis Pharma AG (Basel, Switzerland) and prepared as previously described (13).

Real-time cell viability analysis. The xCELLigence RTCA SP system (Roche Applied Science, Mannheim, Germany) was used as previously described (21-23) for real-time analysis of the cellular response of BON1 cells following incubation with 1 nM up to 100 µM panobinostat (each triplicates). Fourty-eight hours later the cells have been seeded in a gold 96-well microplate (E-Plate 96. ACEA Biosciences Inc, San Diego, USA). The cell index indicating attachment and adherence of cells to the microplate's electrode was measured continuously each 15 min for 80 h. Data analysis was performed using the RTCA Software v1.2.1. (Roche Applied Science).

Statistical analysis. Statistical analysis was performed with SPSS 20.0 (IBM Corporation, New York, USA). The Wilcoxon´s signed-rank test/Student's *t*-test and univariate ANOVA (analysis of variance) were

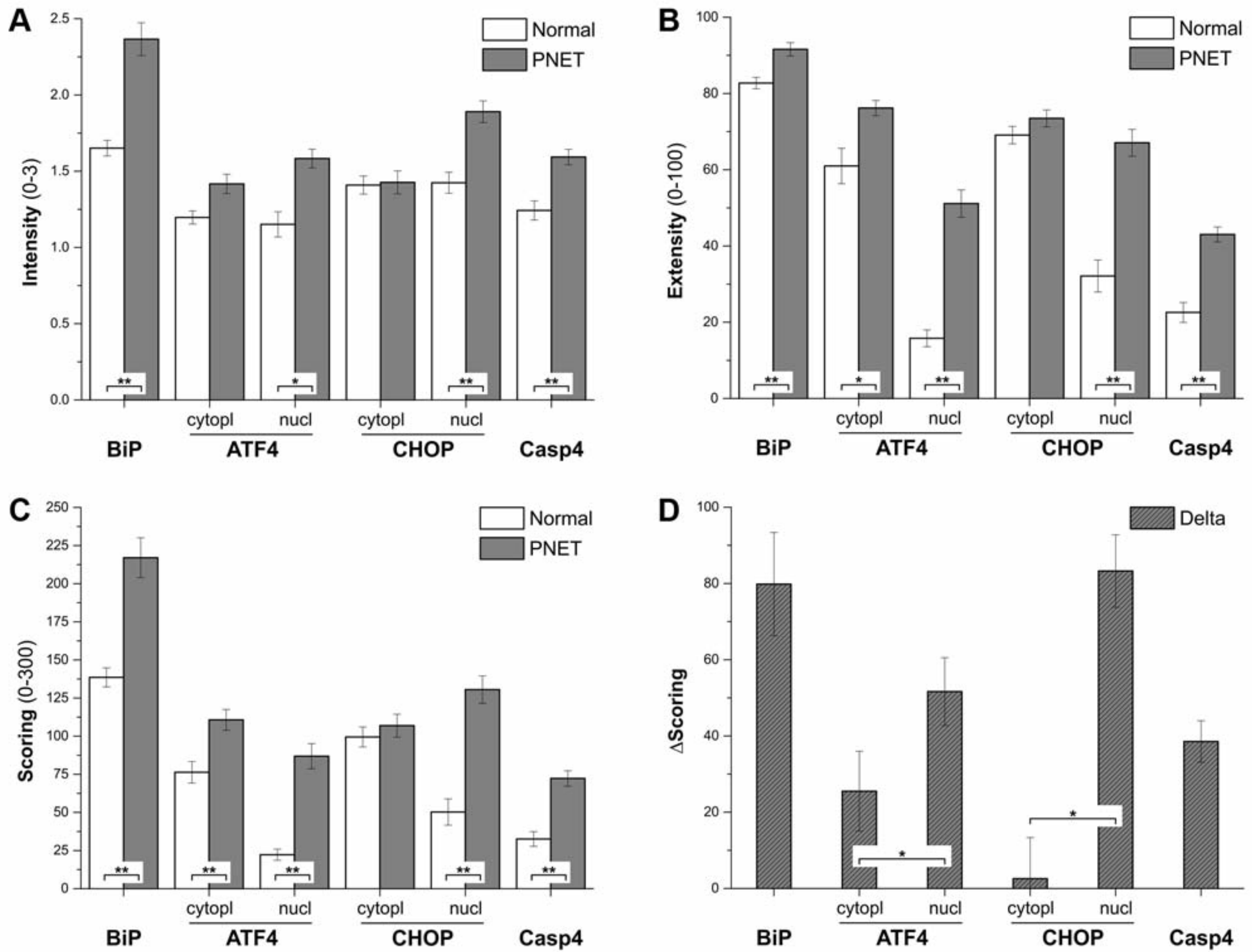

Figure 2. *Quantitative analysis of BiP, ATF4 and CHOP as well as caspase 4 in control tissue compared with pNET. The levels of staining intensity (A), extensity (B) as well as the immunohistochemical scoring system (C) (see Materials & Methods) are given as mean±SEM. Cytopl, cytoplasmatic; nucl, nuclear. *p<0.05, **p<0.01 (t-Test).*

used to test for differences between two and more groups of tissue samples, respectively. Kendall's rank two-tailed, Spearman rank correlation test and a linear regression analysis were used for correlation analysis. For survival analysis, cases with missing date of death were excluded. Univariate survival analysis was performed using the Kaplan-Meier method comparing the survival curves with the log-rank test. For multivariate survival analysis, the Cox proportional hazards model was used. For all calculations, $p<0.05$ and $p<0.01$ was considered as significant or highly significant, respectively.

Results

Clinical characteristics of patients with pNET. As listed in Table I, 49 cases (n=29 (59.2%) females *versus* n=20 (40.8%) males with a mean age of 60.0±14.5 years) with pNET (40.8% immunohistochemically endocrine-positive) were included in the study. The pNET showed a mean size of 3.0±2.6 cm and were mainly located in the head (n=19 (38.8%)) and cauda (n=18 (36.7%)) of the pancreas. The majority of the pNET cases were of T1 (n=19 (38.8%)) and T3-status (n=16 (32.7%)) showing locoregional lymph-node metastasis in 16 cases (32.7%) and distant metastasis in 10 cases (20.4%). Finally, the number of pNET cases decreased with grading: G1 with 28 (57.1%), G2 with 14 (28.6%) to G3 with 7 pNET cases (14.3%).

Immunohistochemical expression of ER stress-associated proteins. As shown in Figures 1 and 2, immunohistochemical scoring revealed a significant up-regulation of BiP, ATF4 (predominantly nuclear) and CHOP (predominantly nuclear) as well as caspase 4 in all pNET cases compared to normal pancreatic control tissues ($p<0.05$, Student's *t*-Test). Interestingly, a "basal" expression of the mentioned markers was also observed in normal control tissue of the pancreas. In detail, the highest intensity levels of ER stress-related proteins were found for BiP and CHOP followed by caspase 4 and

Table II. *Expression score levels of BiP, ATF4, CHOP, and caspase 4 in pNET.*

	BiP	ATF4 (Mean±SEM)		CHOP (Mean±SEM)		Caspase 4
	(Mean±SEM)	cytoplasma	nucleus	cytoplasma	nucleus	(Mean±SEM)
T1	215.7±18.7	118.1±12.2	**105.8±15.2[b]**	102.8±11.6	**146.8±9.3[c]**	76.6±8.0
T2	**167.7±30.2[a]**	115.2±13.1	77.6±13.6	102.5±17.0	123.2±19.2	63.5±10.8
T-Low	197.3±16.6	117.1±8.9	95.7±11.0	102.7±9.5	137.4±9.5	71.5±6.4
T3	**259.8±19.2[a]**	106.2±11.9	**69.3±13.3[b]**	116.5±13.8	130.3±19.7	73.7±8.8
T4	202.5±60.2	78.3±17.4	95.0±27.5	100.0±30.1	**74.1±32.2[c]**	71.6±26.7
T-High	249.0±19.1	101.3±10.4	73.8±11.9	113.4±12.2	119.8±17.6	73.3±8.2
N0	215.1±15.6	108.9±8.3	83.6±9.1	106.0±8.6	129.4±10.3	72.8±6.4
N1	221.6±24.9	114.2±12.4	93.3±17.8	108.9±15.3	133.1±18.7	71.1±8.0
M0	208.4±15.1	111.8±7.9	86.6±9.8	108.5±8.9	132.3±9.9	70.9±5.4
M1	248.3±24.2	107.0±14.0	87.5±14.9	100.8±13.0	124.1±21.9	77.5±13.2
G1	**192.6±20.6[d]**	116.6±9.3	**91.8±8.8[e]**	102.6±8.3	130.5±11.8	68.9±7.2
G2	227.2±18.5	116.5±12.3	80.5±15.1	99.1±14.6	135.4±16.0	78.6±8.3
G-Low	204.8±14.9	116.6±7.3	88.1±7.6	101.3±7.4	132.3±9.4	72.5±5.4
G3	**268.7±17.3[d]**	85.6±15.2	**81.2±30.2[e]**	129.6±22.7	123.1±26.3	71.0±13.2
HA-No	235.7±15.4	107.9±8.8	78.6±12.3	108.6±11.1	130.4±12.6	70.6±7.0
HA-Yes	200.7±21.0	113.3±11.4	96.3±10.9	104.5±9.7	130.6±12.9	75.2±7.5

Values are given by mean±SEM depending on TNM, grading and hormone activity. Bold fields highlights the significant differences (p<0.05) within the same column indicated by "a" to "e" using ANOVA. HA, Hormone activity is measured by immunohistochemistry; T, tumor (extent of the tumor); N, node (absence/presence and extent of regional lymph node metastasis); M, metastasis (absence/presence of distant metastasis); G, grading.

ATF4 while highest staining extensity was found for BiP followed by ATF, CHOP and caspase 4. Finally, the highest difference between normal and tumor tissue was detected for CHOP and BiP. Comparing the cytoplasmatic and nuclear differences for ATF4 and CHOP, higher nuclear differences were observed for CHOP, whereas higher cytoplasmatic differences were found for ATF4 (p<0.05, *t*-Test).

Associations of ER stress factors and clinicopathological findings. Table II shows the expression levels of BiP, ATF4 and CHOP and caspase 4 in relation to TNM, grading and pNET-associated hormone activity. A heterogeneous expression of BiP was observed for different T-stages, whereby low T-stages (T1 and T2) showed lower expression of BiP compared to higher T-stages (T3 and T4) reaching significant level for T2 *versus* T3 (p=0.01, ANOVA). BiP levels were higher in cases with positive lymph nodes, distant metastasis and hormone inactivity as well as increased significantly with the tumor grade (G1 *versus* G3, p=0.029, ANOVA).

The cytoplasmatic and nuclear expression of ATF4 decreased with increasing T-stages (nuclear ATF4 between T1 and T3: p=0.05, ANOVA). Higher levels of ATF4 were found in cases with lymph node metastasis and hormone activity, whereby lower levels were seen in cases with distant metastasis and increased grading with a significant difference between pNET cases with grading G1 and G3 (p=0.05, ANOVA). Analysis of CHOP revealed a heterogeneous expression for T-stages (with significant difference between T1 and T4 stage, p=0.05, ANOVA) and grading as well as similar expression levels for N-stages and status of hormone activity. Finally, the expression of caspase 4 was not significantly associated with TNM stages or with grading. As only grading was directly associated with BiP expression, we correlated BiP with mitotic and proliferation activity, measured by conventional HE staining, as well as PHH3 and Ki-67 immunohistochemistry. As shown in Figure 3, the intensity levels, extension levels, as well as the calculated IRS of BiP correlated significantly with mitotic count based on HE-staining and immunohistochemistry for PHH3 as well as with the conducted overall HE- or PHH3 linked grading (r≥0.255, p≤0.05; Kendall's rank two-tailed and Spearman rank correlation test). Interestingly, the correlation analysis revealed only a significant association between expression levels of BiP in normal bordered pancreatic tissue to the Ki-67 associated proliferation activity (r=0,251, p=0.05, Kendall's rank two-tailed and Spearman rank correlation test) in contrast to the expression levels of BIP in pNET. Therefore, a linear regression analysis was carried out (as shown in Figure 3) demonstrating increasing levels of BiP with enhanced morphologically and immunohistochemically detected mitosis in pNET cases, whereby the linear regression model reached

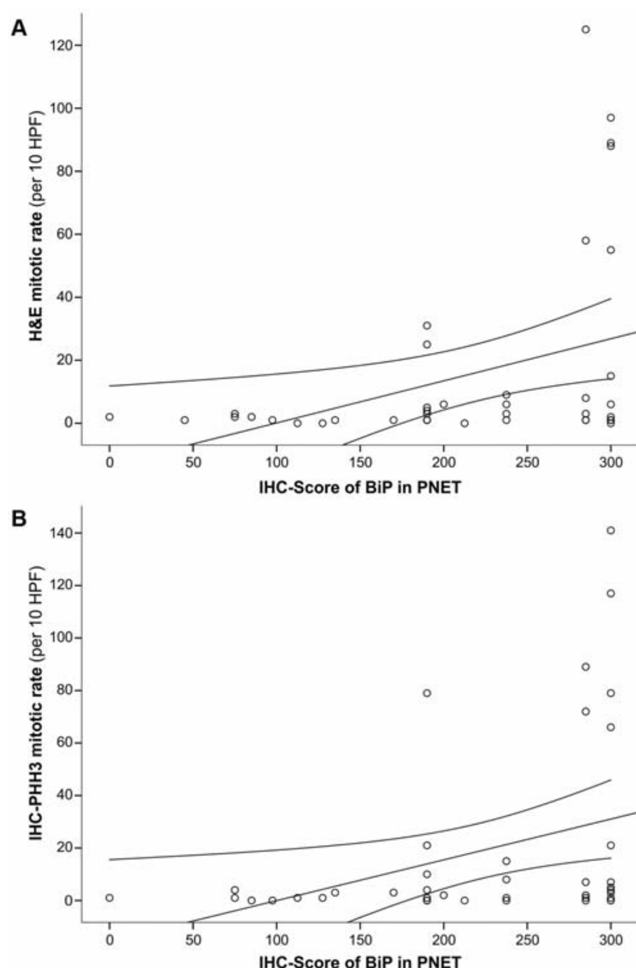

Figure 3. *Linear regression analysis of BiP with H&E-associated and PHH3-associated mitosis rate. The mitotic rates based on HE staining (A) and immunohistochemistry for PHH3 (B) is plotted against BiP IHC scores. The confidence interval of the linear regression line indicates the increasing variation of BiP score level especially in cases with a mitotic index smaller than 20 per 10 HPF referring to NET grade G1 and grade G2 cases.*

significance levels for both applied detection methods (R_{HE}=0.368 (ANOVA p=0.016), R_{PHH3}=0.355 (ANOVA p=0.023)). Confidence intervals of the linear regression line indicate the highest variation of BiP score levels in cases with a mitotic index smaller than 20 per 10 HPF referring to NET grade G1 and grade G2 cases.

Impact of ER stress-related proteins on overall survival. Overall survival analysis revealed a mean survival time of 122.6 months (CI=90.6-154.5 months) using the Kaplan-Meier method. Known prognostically-relevant clinicopathological parameters significantly impacted on overall survival (log-rank test): grading (p<0.001, Figure 4A), pN status (p<0.001, Figure 4B) and pM status (p=0.021, Figure 4C). The Cox proportional hazards model for all clinicopathological parameters, as well as ER stress factors indicated pN and pM stages as independent significant prognostic markers (Cox regression model backward Wald method, Omnibus Tests of Model Coefficients, χ^2=38.4, p<0.001). As the Cox-regression analysis indicated no significant results for ER stress factors, the expression of BiP, ATF4, CHOP and caspase 4 was categorized into low- and high-expressing cases according to mean expression levels. With this stratification, the univariate Kaplan-Meier survival analysis indicated a tendency for BiP (p=0.222, log-rank test), ATF4 (p=0.344, log-rank test) and CHOP (p=0.191, log-rank test), whereby higher expression of BiP is linked to a worse outcome. In contrast to that, higher expression of ATF4 and CHOP is associated with better outcome (Figure 4C, D and E). For caspase 4 (p=0.857, data not shown) no reasonable tendency could be found.

Effect of pan-HDACI panobinostat on BON1 cell viability. Figure 5 shows that panobinostat caused a block of cell viability already at 1 nM. 10 nM panobinostat was determined as half-maximal inhibitory concentration (IC_{50}) and it was able to drastically reduce the cell viability, showing a 10-times higher efficacy than previously shown (13, 21).

The consecutive correlation analysis of members of ER Stress and HDACs revealed a significant positive association between BiP and HDAC3, HDAC6 and Sirt1 (r>0.31, p<0.05) and between caspase 4 and HDAC5 (r=0.328, p=0.042) as well as a negative linkage between CHOP and HDAC5, HDAC10 and Sirt1 (r<-0.373, p<0.05; Kendall's rank two-tailed and Spearman rank correlation test).

Discussion

In the current study, we analyzed the protein expression pattern of ER stress-associated factors BiP, ATF4, CHOP and caspase 4 in pNET tumors using semi-quantitative immunohistochemical scoring. pNET are a rare tumor entity with aggressive and fatal clinical course, in some cases. Due to sparse insight into pathogenetic pathways and limited therapeutic options, the rationale of the study was to investigate the ER stress pathway in pNET.

Solid tumors are often embedded in a very unsuspectable microenvironment. Due to high proliferation rate of these tumors, the microenvironment rapidly reaches low pH-values, low oxygen tension, and low nutrient supply. All these factors stroke the cellular microenvironment causing accumulation of misfolded proteins that lead to ER stress. Several previous studies investigated an association between ER stress components and the development of different types of solid cancers such as breast, brain, lung, liver, colon, prostate and skin cancer as well as hematological malignancies (22).

Our current work revealed, for the first time, that all investigated ER stress components could be detected in normal pancreatic tissue and they showed a significant

increase of expression in pNET ($p<0.05$). In particular, the highest scores were observed for BiP followed by CHOP, ATF4 and finally caspase 4 - thus indicating the central role of BiP in ER stress and tumorigenesis. Continually, BiP, also known as 78 kDa glucose-regulated protein (GRP78), is shown to be over-expressed in a variety of different cancer entities (23-25). BiP sustains tumor cell survival by suppressing the expression of CHOP and therefore preventing ER stress-mediated apoptosis (26). Our analysis revealed a significant association between BiP expression and tumor stage as well as tumor grading according to the current TNM staging system (16, 27). Furthermore, linear association between mitotic activity and BiP expression suggests a direct correlation of ER stress and tumor cell turnover, as recently shown in breast cancer and malignant gliomas (28, 29) –suggesting that BiP expression could function as a biomarker of cancer progression (30). ATF4 and CHOP are significantly linked to tumor stage only, whereby the expression levels were decreasing with higher T-stages. ATF4, downstream target of the PERK cascade related to the UPR, functions, amongst others, as an activating transcription factor for pro-survival genes and high ATF4 expression levels can be found in different solid tumors, especially in response to hypoxic stress (31, 32). CHOP is the key regulator of ER stress-induced apoptosis but it is also capable of regulation of genes promoting cell survival and proliferation (33). Interestingly, we were able to confirm that higher expression of ATF4 and CHOP is tendentially linked to better outcome, whereby higher expression of BiP is associated with worse outcome. These findings support the known paradox that ER stress, depending on the circumstances, both promotes and inhibits carcinogenesis (34-36). Detailed analysis of the non-canonical ER stress pathway as well as the connection between ER stress and autophagy could bring more insight in these biological processes and clarify, in the future, the main role exerted by ER stress in carcinogenesis mechanisms.

To date, everolimus and sunitinib are the only worldwide approved and registered drugs for the treatment of pNET. Sunitinib is an inhibitor of vascular endothelial growth factor (VEGF) and platelet derived growth factor-inhibitor (PDGF) –two factors known for tumor-promoting properties in pNET (37). Everolimus recently showed significantly prolonged progression-free survival in patients with advanced pNET (38). Everolimus, by inhibiting mechanistic Target of Rapamycin (mTOR), a serine-theronine kinase, could impede the insulin-like growth factor-1 (IGF-1) cascade leading to block cell growth, proliferation and angiogenesis in pNET (39, 40). However, mTOR does also exert a role during ER stress: Ye *et al*. demonstrated the existence of a crosstalk between mTOR and eIF2αS51P (41). More recently, Rajesh *et al*. showed that either genetic or pharmacological inhibition of mTOR complex 2 (mTORC2) inactivates Akt leading to activation of the PERK-eIF2αS51P arm (42). Therefore, mTOR inhibition should be further investigated during ER stress and in relation to pharmacological effects as well as to specific clinical end-points such as recurrence, metastasis and survival.

Another possible therapeutic approach to influence ER stress is based on epigenetic modifications induced by HDAC inhibitor (HDACI) treatment. Beside HDACI's effects on cell death, cell-cycle arrest and gain-of-function of tumor suppressor genes (43), they do also interact with non-histone proteins leading to an accumulation of misfolded proteins and ER stress-related cell death (44).

Our intensive immunhistochemical investigations of HDACs and ER stress on the consecutive TMA slides revealed an interesting significant positive association between HDAC3, HDAC6 and Sirt1 with BiP possibly linking epigenetic and ER stress. Several previous studies could prove a connection between HDACs and ER stress members, including the BiP-CHOP-ATF4 axis (45).

BiP is a major anti-apoptotic factor and a representative of the pro-survival arm of the UPR. BiP is able to bind and keep inactivate pro-apoptotic factors like caspase 7 and supress CHOP expression, thus maintaining ER integrity and homeostasis through autophagy regulation (46). Furthermore, BiP is a major effector of drug resistance in cancer (30). In 2009, Baumeister *et al*. showed, in cell cultures and xenografts, that HDAC1 is a repressor of BiP promotor activity. Further on, this study indicated that lowering the BiP levels significantly increases the apoptotic effects of HDACI (47).

It was shown that inhibition of HDAC6, member of HDAC class IIb, induces cell death mediated by etoposide, a topoisomerase II inhibitor, or doxorubicin or SAHA, a pan-HDACI. Interestingly, HDAC6 is able to de-acetylate non-histone proteins like tubulin (48). Namdar *et al*. showed that a combination of tubacin and SAHA inhibited the catalytic effect of HDAC6 and, at the same time, caused a strong increase of the expression fold of CHOP (49). Recently, Kikuchi *et al*. showed that inhibition of HDAC4 by TMP269, a selective class IIa HDACI, and induction of ER stress by carfilzomib led to significant upregulation of ATF4 and CHOP in multiple myeloma cells (45).

Our group has already shown that panobinostat, a pan-DAC inhibitor, is able to trigger ER stress-related cell death in HCC cell lines (13). In the current study, we showed that panobinostat is able to block and reduce cell viability in BON1 cells, an *in vitro* model of pancreatic neuroendocrine tumors. Together with the above-mentioned findings, these data suggest alternative therapeutic approaches for pNET, based on the observed association of ER stress-related factors, HDAC expression and relevant clinicopathological parameter such as proliferation status, grading, staging and survival. This supports the idea that the combination of HDACI and ER stress-triggering agents could presents a new therapeutic option for the treatment of pNET.

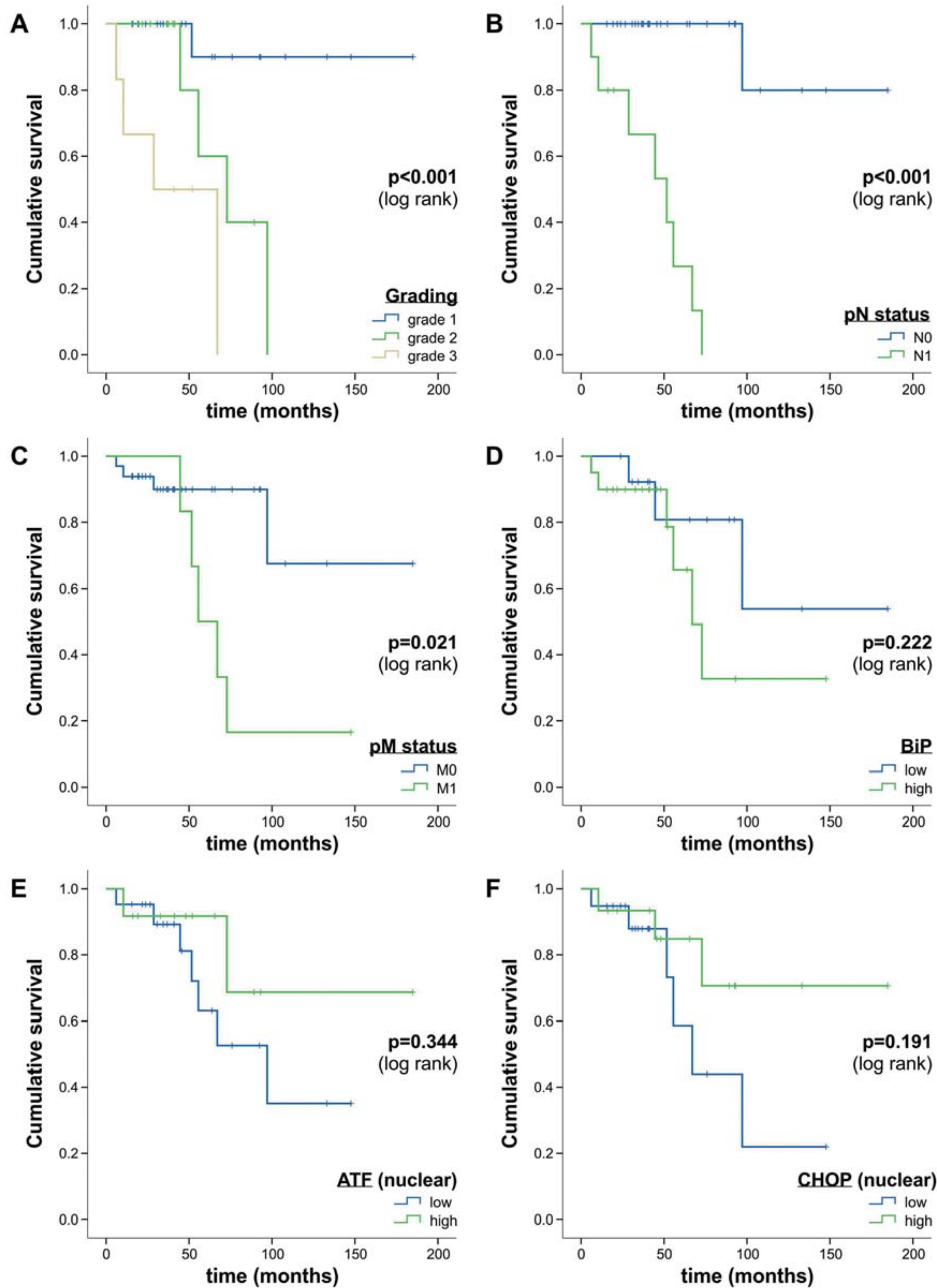

Figure 4. *Survival analysis of patients with pNET. The Kaplan-Meier survival analysis (using the log-rank test) was performed for grading (A, p<0.001), pN (B, p<0.001), pM-stage (C, p=0.021) indicating significant differences for these groups of patients. When expression of BiP (D), ATF4 (E) and CHOP (F) was categorized into low and high levels, univariate survival analysis indicated a tendency for BiP (p=0.222), ATF4 (p=0.344) and CHOP (p=0.191), whereby higher expression of BiP is associated with worse outcome-in contrast to ATF4 and CHOP where higher expression is linked to better outcome.*

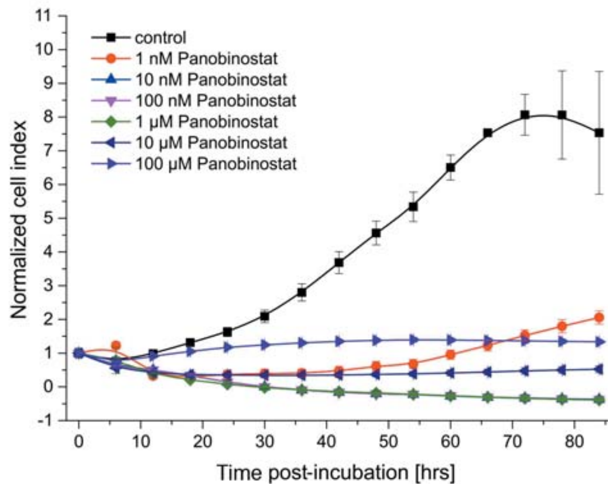

Figure 5. *Cytotoxic effects of panobinostat on BON1 pNET cell line. The cell viability was continuously measured using the xCELLigence system (Roche) for 80 h post-incubation with different concentrations of panobinostat. The cell index stands for overall viability based on the extent of cell attachment.*

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