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ORIGINAL ARTICLE

Expression of $p27^{Kip1}$ and $p18^{Ink4c}$ in human multiple endocrine neoplasia type 1-related pancreatic neuroendocrine tumors

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Abstract

Purpose Pancreatic neuroendocrine tumors are a major manifestation of multiple endocrine neoplasia type 1 (MEN1). This tumor syndrome is caused by germline mutations in *MEN1*, encoding menin. Insight into pathogenesis of these tumors might lead to new biomarkers and therapeutic targets for these patients. Several lines of evidence point towards a role for $p27^{Kip1}$ and $p18^{Ink4c}$ in MEN1-related tumor development in animal models for MEN1, but their contribution to human MEN1-related pancreatic neuroendocrine tumor development is not known.

Methods In this study, we characterized protein expression of $p27^{Kip1}$ and $p18^{Ink4c}$ in human MEN1-related PanNETs by immunohistochemistry. From the nationwide DutchMEN1 Study Group database including > 90% of the Dutch MEN1 population, MEN1-patients, who underwent pancreatic surgery, were selected. A tissue micro-array was constructed with available paraffin tissue blocks, and PanNETs from 61 MEN1 patients were eligible for analysis.

Results Expression of $p27^{Kip1}$ was high in 57 (93%) PanNETs and 67% of the tumors showed low expression of $p18^{Ink4c}$ (67.3%). No association was found between expression of either $p27^{Kip1}$ or $p18^{Ink4c}$ and clinic-pathological characteristics.

Conclusions These findings indicate that loss of $p18^{Ink4c}$, but not $p27^{Kip1}$, is a common event in the development of MEN1-related PanNETs. Restoration of $p18^{Ink4c}$ function through CDK4/6 inhibitors could be a therapeutic option for MEN1-related PanNETs.

Keywords Multiple endocrine neoplasia type 1 · Pancreatic neuroendocrine tumors · Menin · $p27^{Kip1}$ · $p18^{Ink4c}$

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Introduction

Pancreatic neuroendocrine tumors (PanNETs) are a major manifestation of multiple endocrine neoplasia type 1 (MEN1). MEN1 is an inherited tumor syndrome, also characterized by parathyroid and pituitary adenomas and other neuroendocrine tumor types [1]. Pancreatic NETs are an important cause of morbidity and mortality in MEN1 patients [2]. Follow-up and treatment of MEN1 patients with PanNETs is complicated. The only potentially curative therapy for MEN1-related PanNETs is surgery, which often leads to short- and long-term complications. Timing and extent of surgery is clinically challenging, due to tumor multifocality, young age at diagnosis and MEN1-related co-morbidity. Furthermore, little is known about the natural course of these mostly slowly growing PanNETs and prognostic factors are lacking [2]. Current guidelines recommend considering surgery for hormonally active (functioning) PanNETs. Furthermore, surgery is recommended for tumors ≥ 1 cm and rapidly growing tumors, but evidence supporting this recommendation is limited [1]. Insight into pathogenesis of MEN1-related PanNETs might lead to novel biomarkers and therapeutic targets for these patients.

MEN1 is caused by germline mutations in the tumor suppressor *MEN1*, encoding the protein menin. In accordance with Knudson's second hit hypothesis, loss of heterozygosity (LOH) of *MEN1* is an early event in MEN1-related PanNET development [2, 3]. Aggressive disease in patients with *MEN1* mutations leading to truncated menin protein has been reported [4–8]. However, evidence for a genotype–phenotype correlation is limited and it is likely that additional events are involved in development of the aggressive phenotype. Recently, it was shown that *MEN1* mutations also occur in 44% of sporadic PanNETs [9]. One of the best-characterized functions of menin is its role in the activation of gene transcription through chromatin modification [10]. It has been shown in different (non-) endocrine cell systems, that menin is a stable subunit of the histone methyltransferase MLL1 and MLL2 complexes [11–13]. These large protein assemblies activate transcription of their target genes via trimethylation of lysine 4 of histone H3 (H3K4me3) [14]. Known targets of the menin-MLL1/2 complexes in a mouse model system include *CDKN1B* and *CDKN2C* encoding the cyclin-dependent kinase inhibitors (CDKIs) $p27^{Kip1}$ and $p18^{Ink4c}$, respectively [15]. Malfunction of $p27^{Kip1}$ and $p18^{Ink4c}$ contributes to uncontrolled growth and cancer progression in several epithelial human cancers [16].

Evidence for a role of $p27^{Kip1}$ and $p18^{Ink4c}$ in both non-MEN1-related and MEN1-related PanNET development initially came from mouse models [17–19]. Evidence for the involvement of $p27^{Kip1}$ in human PanNET development comes from studies focusing on sporadic PanNETs. Germline mutations in *CDKN1B* were found in patients with a MEN1-like syndrome, which has been named MEN4 [20, 21]. Interestingly, an aggressive course of disease was seen more frequently in MEN1 patients with a specific *CDKN1B* polymorphism compared to patients with the wild-type variant of this gene [22]. It can be hypothesized that the side by side existence of MEN1 mutations and this *CDKN1B* polymorphism negatively influences the function of $p27^{Kip1}$ by further reducing its expression. Less evidence is available for $p18^{Ink4c}$ in human endocrine tumor development and, to the best of our knowledge no mutations in *CDKN2C* in either hereditary or sporadic PanNETs have been described.

In summary, reports from animal models and recent genetic findings are supportive for a role of $p27^{Kip1}$ and $p18^{Ink4c}$ in MEN1-related PanNET development, but it is not known whether alterations of these factors actually contribute to PanNET tumorigenesis in MEN1 patients. We investigated whether low expression of both $p27^{Kip1}$ and $p18^{Ink4c}$ are common hallmarks of MEN1-related PanNETs.

Materials and methods

Case selection

MEN1 patients, who underwent pancreatic surgery for PanNETs between 1985 and February 2013, were selected using the longitudinal DutchMEN1 Study Group (DMSG) database covering > 90% of the Dutch MEN1 population. All MEN1 patients included in this database were diagnosed according to clinical practice guidelines [1], were aged 16 years and older, and were under treatment in one of the university medical centers (UMCs) in The Netherlands. The Medical Ethical Committees of all UMCs in the Netherlands approved the study protocol for data collection. This database has been described in detail elsewhere [23]. Clinical data used in this study are extracted from the DMSG database.

From the selected cases, formalin-fixed paraffin-embedded (FFPE) tissue blocks containing the largest PanNET per patient (according to pathology reports) were collected from the archives of Pathology departments throughout the Netherlands in collaboration with "the nationwide network and registry of histo- and cytopathology in the Netherlands" (PALGA) [24].

This study was performed according to national guidelines with respect to the use of 'excess tissue' and ethical approval for this study was obtained from the scientific committee of PALGA and of the Pathology department of the UMC Utrecht.

Clinical definitions

Both insulinomas and non-functioning PanNETs were included. Definition of an insulinoma was a positive 72-h prolonged supervised fast prior to resection of the PanNET. Further subdivision into type of functioning tumors was not made.

Patients were considered to have liver metastases when (1) this diagnosis was confirmed by pathological examination or (2) radiological examination was positive for liver metastases. Radiology was positive when reports of CT- and/or MRI scans lesions suspicious for liver metastases were described on consecutive radiological exams. Each case with liver metastases was evaluated in detail by an expert panel (WdH, GV and MV), which decided per case whether liver metastases were PanNET-related or not PanNET-related. End of follow-up was defined as either the time of diagnosis of PanNET-related liver metastases, non-PanNET-related liver metastases, death or end of data collection.

Construction of tissue microarray

Formalin-fixed paraffin-embedded (FFPE) tissue blocks were used for the construction of a tissue microarray (TMA). Representative tumor regions were marked on hematoxylin & eosin (H&E)-stained slides by a pathologist. From these regions, three tissue cores with a diameter of 0.6 mm were punched out of the corresponding paraffin block and transferred into a TMA paraffin block using a TMA machine (TMA grand master, 3D Histec, Budapest, Hungary). Normal pancreatic tissue was included from every patient when possible. The layout of the TMA was determined in advance with the TMA software (3D Histec, Budapest, Hungary).

Immunohistochemistry

TMA slides (thickness 4 μm) were stained for all markers by immunohistochemistry (IHC) as detailed below. Supplementary Table 1 shows the details of the experimental staining methods regarding antibodies used, antigen-retrieval method, antibody dilutions and incubation time. Antibodies used in this study have been used for immunohistochemistry previously [25–27]. For all stainings, sections were deparaffinized in xylene for 10 min followed by dehydration through graded alcohols. Endogenous peroxidase activity was blocked for 15 min in a buffer solution of pH 5.8 (containing 8.32 g citric acid, 12.52 g disodium hydrogen phosphate dihydrate, 2 g sodium azide in 1 l of water) with hydrogen peroxide (0.3%). After antigen retrieval for 20 min, a cooling-down period of 20 min was followed by incubation with protein block (Protein Block Serum Free, Dakocytomation) and/or the primary antibody. Slides were incubated with HRP-containing secondary antibodies and peroxidase reactivity was developed by 3,3′-diaminobenzidine. Slides were counterstained with Mayer's hematoxylin, rehydrated in graded alcohols and cleared in xylene. In between steps, slides were washed with phosphate-buffered saline. Stainings for CgA, synaptophysin and MIB-1 (Ki-67) were performed according to standardized protocols, using the automated IHC staining system Ventana Bench Mark ultra. Pancreatic NETs were included in our analysis upon positive staining for neuroendocrine markers. Cases with negative staining for these markers were excluded from the analysis ($n = 3$).

Immunohistochemical scoring

The TMAs were co-jointly scored by two observers and a third observer was consulted in case of uncertainty. The observers were blinded to clinicopathological features. All PanNETs were graded according to the WHO classification system, based on both the Ki-67 labeling index (Ki-67 LI) and mitotic count of whole tissue slides [28]. Classification is as follows: WHO G1 tumors: Ki67 LI ≤ 2 and mitosis $< 2/10$ high power fields (HPF); WHO G2 tumors: Ki67 LI 3–20 and/or mitoses 2–20/10 HPF; WHO G3 tumors Ki67 LI > 20 and/or mitosis $> 20/10$ HPF [29].

Each PanNET core was labeled as either positive (when $> 30\%$ positive nuclei) or negative ($\leq 30\%$ positive nuclei) for menin staining (Fig. 1a–c). Expression of $p27^{Kip1}$ was regarded as low or high, with $\leq 50\%$ or $> 50\%$ of the nuclei being positive, respectively, as described [16, 30] (Fig. 1d–e). Expression of $p18^{Ink4c}$ was scored as low ($\leq 2\%$ positive nuclei), intermediate (3–20% positive nuclei) or high ($\geq 20\%$ positive nuclei) as previously described [31] (Fig. 1f–h). The average of the three cores per PanNET was calculated whenever possible and used for analysis.

Statistical analysis

Statistical analyses were performed using the IBM SPSS Statistics for Windows (Version 22, Armonk, NY: IBM Corp). The Fisher's exact test, the Mann–Whitney *U* test and the Kruskal–Wallis one-way analysis of variance by rank test were used when appropriate. Results were considered to be statistically significant if $p < 0.05$.

Results

Clinical characteristics

Seventy patients were selected from the DMSG database. FFPE blocks obtaining the largest PanNET were available from 61 patients (87%) and these were included in this study. Patient characteristics are summarized in Table 1.

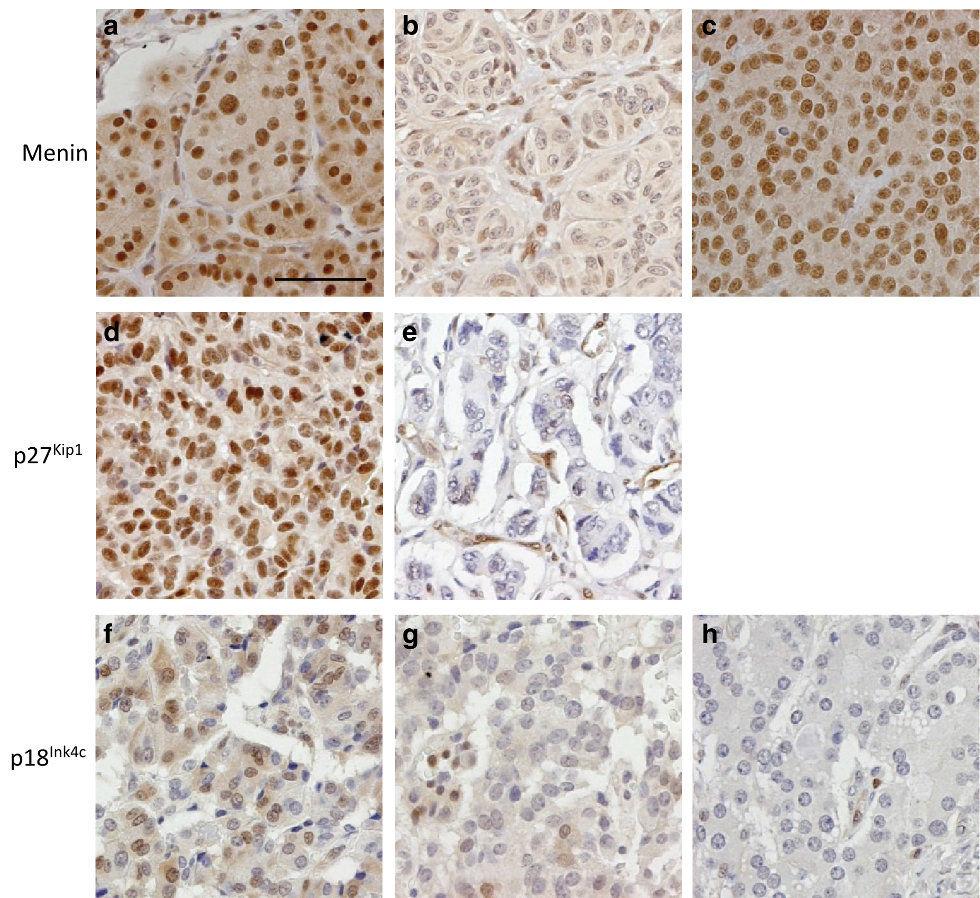

Fig. 1 Representative examples of immunohistochemical stainings for menin, $p27^{Kip1}$ and $p18^{Ink4c}$ performed on pancreatic neuroendocrine tumors (PanNETs). Menin staining positive in a pancreatic islet in a MEN1 patient (**a**) and negative in a PanNET (size 3.2 cm) of the same patient (**b**). Sporadic PanNET showing positive menin staining (**c**). High p27Kip1 expression (**d**) and low p27Kip1 expression (**e**) in MEN1-related PanNETs (size 2.2 and 4.5 cm respectively). High (**f**), intermediate (**g**), low (**h**) $p18^{Ink4c}$ expression in MEN1-related PanNETs (size 3, 1.5 and 3, respectively). Scale bar representing 50 µm

The median age of the patient group is 41 years (20–81). Insulinomas are seen in 23% of the patients ($n = 14$) and non-functioning PanNETs in 75% ($n = 46$). Median PanNET size of the insulinomas was 2.1 cm (0.7–3.5) and 2.9 (0.3–20) in non-functioning PanNETs. Most PanNETs were graded as WHO G1 tumors (85%). In total, nine patients (15%) developed liver metastases after a median follow-up time of 5.8 years (0–28.5); one patient with an insulinoma developed liver metastases and eight patients with non-functioning PanNETs.

Immunohistochemical analysis of menin, $p27^{Kip1}$ and $p18^{Ink4c}$

Results are summarized in Table 2. Menin staining was negative in 52 MEN1-related PanNETs (85%) and eight PanNETs were menin positive (13%). In five of these patients (63%), there was only a clinical basis for MEN1 diagnosis (two or more MEN1 manifestations) [1]. In these five patients, no *MEN1* mutation could be found and no first degree relative had the MEN1 syndrome.

Only 7.1% ($n = 4$) of the PanNETs displayed low $p27^{Kip1}$ expression; all these PanNETs were non-functioning PanNETs. Within the non-functioning PanNETs, no significant associations were seen between $p27^{Kip1}$ expression and clinical and pathological characteristics. However, 22% of the WHO G2 PanNETs had low expression (2/9) compared to 4% of the WHO G1 PanNETs (2/52) ($p = 0.072$).

Most PanNETs showed low $p18^{Ink4c}$ expression ($n = 41$; 67%), intermediate expression was seen in 7 PanNETs (12%) and high expression was seen in 12 PanNETs (20%). No significant associations with clinical and pathological characteristics were found. We also determined the percentage of $p18^{Ink4c}$ positive cells in normal-appearing pancreatic islets in TMA cores from MEN1 patients, available in 26 cases. Eight cases (30%) showed no $p18^{Ink4c}$ expression in the islets. Seven of these cases also lacked $p18^{Ink4c}$ expression in the PanNET and one case displayed high $p18^{Ink4c}$ expression. In these 26 cases, the average of $p18^{Ink4c}$-positive islet cells in normal tissue was 27.6% and in PanNETs 10.8% (reduction by 16.8%).

Discussion

In this study, we analyzed the expression of the cell cycle regulators $p27^{Kip1}$ and $p18^{Ink4c}$ in MEN1-related PanNETs in an unselected cohort of MEN1 patients from the

Table 1 Clinical characteristics of MEN1 patients and characteristics of pancreatic neuroendocrine tumors included in this study

	Total ($n = 61^a$)	Insulinoma ($n = 14$)	Non-functioning PanNET ($n = 46$)
Gender, *n* (%)			
Male	30 (49)	3 (21)	26 (57)
Female	31 (51)	11 (79)	20 (43)
Age at surgery PanNET			
Years, median (range)	41 (20–81)	33 (20–81)	41.5 (20–60)
Tumor size, cm			
Median (range)	2.5 (0.3–20)	2.1 (0.7–3.5)	2.9 (0.3–20)
Unknown	5	0	5
Tumor size, *n* (%)			
≤ 2 cm	26 (43)	7 (50)	18 (39)
> 2 cm	32 (52)	7 (50)	25 (54)
Unknown	3 (5)	0	3 (7)
WHO grade PanNET, *n* (%)			
G1	52 (85)	13 (93)	38 (83)
G2	9 (15)	1 (7)	8 (17)
Liver metastases			
Synchronous	2	0	2
Metachronous	7	1	6
None	52	13	38
Follow-up, years			
Median (range)	5.8 (0–28.5)	5.8 (0.3–17.8)	5.8 (0–28.5)

[a] In one case it was not known whether the PanNET was an insulinoma or non-functioning PanNET

Table 2 Results of immunohistochemical stainings for menin, $p27^{Kip1}$ and $p18^{Ink4c}$ in pancreatic neuroendocrine tumors in MEN1

	Total ($n = 61^a$)	Insulinoma ($n = 14$)	Non-functioning PanNET ($n = 46$)
Menin expression, *n* (%)			
Positive	8 (13)	4 (29)	4 (9)
Negative	52 (85)	10 (71)	41 (89)
Unknown	1	0	1 (2)
$p27^{Kip1}$ expression, *n* (%)			
Low	4 (7)	0 (0)	4 (9)
High	57 (93)	14 (100)	42 (91)
$p18^{Ink4c}$ expression, *n* (%)			
Low	41 (67)	6 (43)	34 (74)
Intermediate	7 (12)	3 (21)	4 (9)
High	12 (20)	5 (36)	7 (15)
Unknown	1	0	1

[a] In one case is was not known whether the PanNET was an insulinoma or non-functioning PanNET

Netherlands. The genes encoding these regulators have been described as target genes for menin-MLL1/2 histone methyltransferase complexes. Our results demonstrate that low expression of p27Kip1 is not a hallmark of MEN1-related PanNETs in humans and suggests a role for $p27^{Kip1}$ in preventing tumor progression in MEN1 patients.

The high expression of $p27^{Kip1}$ in 93% of the PanNETs in our MEN1 cohort was not expected. Most published data from cell systems and mouse models focusing on *CDKN1B* as a target gene for regulation by the menin-MLL1/2 complexes point towards a loss of $p27^{Kip1}$ as an important and initial event in PanNET development [15, 19]. Interestingly, Bai et al. failed to find a genetic interaction between *Men1* and $p27^{Kip1}$ in mice, but a clear explanation for this was not offered [32]. Previously, $p27^{Kip1}$ expression in human MEN1-related PanNETs has been studied in a small number of PanNETs. An increase in the number of $p27^{Kip1}$ negative cells in PanNETs compared to normal islets was seen (20.0 versus 5.4%) in a small subset of MEN1-related tumors ($n = 7$) [15]. Another study did not observe a loss of $p27^{Kip1}$ expression at mRNA level in a small number of PanNETs with mutations in *MEN1*, including four MEN1-related PanNETs [33]. Our data show high $p27^{Kip1}$ expression in most of the MEN1-related PanNETs, which casts doubts on the direct activation role of menin in $p27^{Kip1}$ mRNA expression. We propose that this may be related to differences in *CDKN1B* ($p27^{Kip1}$) gene regulation between mouse and human pancreatic cells. As for the tumor suppressor role of $p27^{Kip1}$ in human PanNETs, we cannot exclude the possibility of a partial loss (not detectable by IHC) of $p27^{Kip1}$ or loss of function of $p27^{Kip1}$ by other mechanisms. Interestingly, low expression of $p27^{Kip1}$ was more frequent in WHO G2

PanNETs. The Ki-67 labeling index in most MEN1-related PanNETs from our cohort was low. Ki-67 is a marker for proliferation and its low labeling index is consistent with the high expression of the negative regulator of cell cycle progression $p27^{Kip1}$.

Low expression of $p18^{Ink4c}$ was seen in the majority of MEN1-related PanNETs (67.3%). This suggests that the loss of $p18^{Ink4c}$ is a common and possibly early event in MEN1-related PanNET development, which is in line with data obtained in cell lines and mouse studies. Additionally, a decrease in the percentage of $p18^{Ink4c}$-positive cells between tumor cells and normal pancreatic islet cells was seen. Some normal-appearing pancreatic islets were negative for $p18^{Ink4c}$. An explanation might be the fact that tumor formation was already initiated in these islets as it is known, that it can be difficult to distinguish normal islets from microadenomas in pancreatic tissue from MEN1 patients. In the light of the development of CDK-inhibiting compounds [34], our finding that $p18^{Ink4c}$ expression is low in the majority of MEN1-related PanNETs, is of particular interest. Especially, CDK4/6 inhibitors might be considered as a therapeutic option in MEN1 patients and further research on this is needed.

More than 1300 (mostly inactivating) germline mutations spread throughout the coding region of the *MEN1* gene have been described [35]. LOH of the tumor suppressor *MEN1* is a common and early event in MEN1-related PanNET development, which is also supported by our menin expression data. In only 8 (13%) MEN1-related PanNETs, menin expression was retained. In five of these patients, *MEN1* mutation analysis was negative (no *MEN1* mutation found ($n = 4$), unclassified variant ($n = 1$)). This is consistent with positive menin staining and suggests that other gene mutations are involved in the MEN1-like syndrome in these patients. The antibody used in our study recognizes the epitope mapping to a region between residue 575 and the C-terminus of menin. The germline mutations of the remaining three menin positive MEN1-related tumors were missense mutations (c.116T > G(p.Leu39Trp) and c.550G > C(p.Glu184Gln)) and an in-frame deletion (c.358_360del(p.Lys120del)), which may explain positive menin staining in these cases.

In conclusion, normal *MEN1* expression is not a prerequisite for expression of the human *CDKN1B* ($p27^{Kip1}$) gene in human pancreatic tissues. Loss of *CDKN1B* ($p27^{Kip1}$) expression does not appear to be an initiating event for PanNET formation in MEN1 patients. In contrast, our results support a model in which the *CDKN2C* ($p18^{Ink4c}$) gene is regulated by menin in PanNETs from MEN1 patients. Our study shows that cell cycle regulation is a potential therapeutic target in MEN1-related PanNETs and further research is needed to evaluate CDK-inhibiting drugs as a therapeutic option in these patients.

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Complicance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent For this type of study formal consent is not required.

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