DNA methylation profiling in MEN1-related pancreatic neuroendocrine tumors reveals a potential epigenetic target for treatment

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Abstract

Objective: Epigenetic changes contribute to pancreatic neuroendocrine tumor (PanNET) development. Hypermethylation of promoter DNA as a cause of tumor suppressor gene silencing is a well-established oncogenic mechanism that is potentially reversible and therefore an interesting therapeutic target. Multiple endocrine neoplasia type 1 (MEN1) is the most frequent cause of inherited PanNETs. The aim of this study was to determine promoter methylation profiles in MEN1-related PanNETs.

Design and methods: Methylation-specific multiplex ligation-dependent probe amplification was used to assess promoter methylation of 56 tumor suppressor genes in MEN1-related ($n=61$) and sporadic ($n=34$) PanNETs. Differences in cumulative methylation index (CMI), individual methylation percentages and frequency of promoter hypermethylation between subgroups were analyzed.

Results: We found promoter methylation of a large number of potential tumor suppressor genes. CMI (median CMI: 912 vs 876, $P=0.207$) was the same in MEN1-related and sporadic PanNETs. We found higher methylation percentages of *CASP8* in MEN1-related PanNETs (median: 59% vs 16.5%, $P=0.002$). In MEN1-related non-functioning PanNETs, the CMI was higher in larger PanNETs (>2 cm) (median: 969.5 vs 838.5; $P=0.021$) and in PanNETs with liver metastases (median: 1036 vs 869; $P=0.013$). Hypermethylation of *MGMT2* was more frequent in non-functioning PanNETs compared to insulinomas (median: 44.7% vs 8.3%; $P=0.022$). Hypermethylation of the Von Hippel–Lindau gene promoter was observed in one MEN1-related PanNET and was associated with loss of protein expression.

Conclusion: Promoter hypermethylation is a frequent event in MEN1-related and sporadic PanNETs. Targeting DNA methylation could be of therapeutic value in MEN1 patients with advanced PanNETs.

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Introduction

Hypermethylation of CpG sites in promoter regions of tumor suppressor genes, leading to gene silencing, is a well-characterized epigenetic process in the development of a variety of cancers (1).

Because DNA methylation is potentially reversible, it may serve as an interesting therapeutic target (2). In fact, the DNA methyl transferase inhibitors decitabine and azacitidine are approved to treat hematological cancers (3). Promoter hypermethylation of selected genes has been reported in sporadic pancreatic neuroendocrine tumors (PanNETs) (4). Although the estimated incidence of PanNETs in several population-based studies is <1% (5), the incidence has increased considerably over the last decades (6). The usual tumor suppressor and oncogenes involved in development of other solid tumors are infrequently mutated or deleted in PanNETs (7). In contrast, whole exome sequencing has revealed that genes encoding proteins involved in chromatin remodeling and modification are commonly mutated in sporadic PanNETs (8). Somatic inactivating mutations of the *MEN1* gene, encoding the tumor suppressor menin, were seen in 37–44% of sporadic PanNETs (8, 9). One of the best-characterized functions of menin is to activate gene transcription through chromatin modification. In addition to genetic changes caused by mutations of the *MEN1* gene, other epigenetic mechanisms such as DNA methylation may contribute to PanNET tumorigenesis in MEN1 (4). MEN1 is the most frequent cause of inherited PanNETs and is caused by germ-line mutations in *MEN1* (10). In accordance with Knudson's second hit hypothesis, loss of heterozygosity (LOH) of the *MEN1* gene is an early event in PanNET development in MEN1 patients (11, 12, 13). PanNETs are an important cause of death in MEN1, but little is known about prognostic factors and the best therapeutic strategies (14). Unraveling the biological processes and oncogenic pathways involved in tumorigenesis in MEN1 patients is an important step toward the development of prognostic markers as well as therapeutic targets. Therefore, the aim of this study was to determine promoter methylation profiles in MEN1-related PanNETs.

Subjects and methods

Patients and clinical definitions

Both MEN1-related and sporadic well-differentiated PanNETs were included in this study. Selection of

PanNET tissue from MEN1 patients has been described extensively before (15). In summary, the longitudinal DutchMEN1 Study Group (DMSG) database, which covers >90% of the Dutch MEN1 population aged 16 years and older, was used. Patients, who had undergone surgery for PanNETs between 1985 and the end of 2013, were included. Formalin-fixed paraffin-embedded tissue blocks from the largest PanNET per patient were collected in collaboration with 'the nationwide network and registry of histo- and cytopathology in the Netherlands' (PALGA) (16). Clinical data were collected until December 2013.

Sporadic PanNETs were selected from the database of the UMC Utrecht tissue bank. Patients with a recorded history suggestive of a hereditary endocrine tumor syndrome were excluded. The Medical Ethics Committee of the UMC Utrecht concluded that the Medical Research Involving Human Subjects Act (WMO) was not applicable to this retrospective study. The study was performed according to national guidelines with respect to the use of excess tissue for scientific research. Patients with objections against this use were not included. Ethical approval for the research protocol was obtained from the Medical Ethics Committee of the UMC Utrecht.

WHO grade was based on both the Ki67 labeling index (LI) and the mitotic count (17, 18). Tumor size as mentioned in the pathology reports was used and dichotomized at 2 cm. Definition of insulinoma and PanNET-related liver metastases in MEN1 patients has been described extensively before (14). Data on the liver metastases were not available for sporadic cases. We included 61 MEN1 PanNETs and 34 sporadic PanNETs. Baseline characteristics are shown in Tables 1 and 2. Median age of MEN1 patients was lower compared to sporadic patients (41 vs 57.5 years, $P \leq 0.001$) and PanNETs from sporadic patients were more frequently WHO G2 tumors (35.3% vs 16.4%, $P = 0.045$).

DNA extraction

Tumor areas with at least 30% tumor cells were identified on H&E slides. Corresponding areas were micro-dissected from deparaffinized 5 μ m slides and suspended in direct lysis buffer (10 mM Tris-HCl pH 8.0/0.1 mM EDTA). After overnight incubation with proteinase K (10 mg/mL; Sigma Aldrich) at 56°C, followed by boiling for 10 min, samples were centrifuged and DNA was extracted. DNA concentrations were measured with a spectrophotometer (NanoDrop ND-1000, Thermo Scientific).

Table 1 Clinical and pathological characteristics: patients with MEN1-related or sporadic PanNETs.

	MEN1 (n=61)	Sporadic (n=28)	P value
Gender, n (%)			
Male	29 (47.5)	18 (52.9)	0.672
Female	32 (52.5)	16 (47.1)	
Age at PanNET resection in years*	41 (20–60)	57.5 (27–81)	0.000
PanNET size, n (%)			
≤2 cm	25 (41)	21 (61.8)	0.130
>2 cm	33 (54.1)	13 (38.2)	
Unknown	3 (4.9)	0	
WHO grade, n (%)			
G1	51 (83.6)	22 (64.7)	0.045
G2	10 (16.4)	12 (35.3)	
Type, n (%)			
Insulinoma	13 (21.3)	11 (32.4)	0.326
Other	47 (77)	23 (67.6)	
Unknown	1 (1.6)	0	

NA, not applicable; PanNET, pancreatic neuroendocrine tumor.

*Values presented as median (range)

Methylation-specific multiplex ligation-dependent probe amplification

The principle of methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) has been described before (19). SALSA MS-MLPA tumor suppressor probe mixes ME001-C2, ME002-C1 and ME004-A1 were used in this study, and MS-MLPA was performed according to the manufacturer's protocol (MRC Holland). The three tumor suppressor gene probe mixes contained 27, 26 and 31 HhaI-sensitive probes respectively and 14, 15 and 9 reference probes (without the HhaI restriction site). In this study, 56 different tumor suppressor genes were included. [Supplementary Table 1](#) (see section on [supplementary data](#) given at the end of this article) shows an overview of essential information regarding the genes included in the three probe mixes ([Supplementary Table 1](#)).

For each MS-MLPA reaction, at least 50 ng DNA was used. All runs were performed in a Veriti 96 Well Thermo Cycler (Applied Biosystems). A water sample, a positive control (A549 cells or MCF-7 cells treated with M.SssI DNA methyltransferase) and negative controls (derived from human blood) were included in every MLPA run. PCR fragments were separated by capillary electrophoresis (ABI 3730 capillary sequencer, Applied Biosystems). Genemapper software version 4.1 (Applied Biosystems) and Coffalyser.NET analysis software (MRC Holland) were used to calculate methylation status.

Table 2 Clinical and pathological characteristics: patients with MEN1-related NF-PanNET or insulinoma.

	NF-PanNET (n=47)	Insulinoma (n=13)	P value
Gender, n (%)			
Male	26 (55.3)	2 (15.4)	0.013
Female	21 (44.7)	11 (84.6)	
Age at PanNET resection in years*	42 (20–60)	31 (20–46)	0.002
PanNET size, n (%)			
≤2 cm	18 (38.3)	6 (46.2)	0.759
>2 cm	26 (55.3)	7 (53.8)	
Unknown	3 (6.4)	0	
WHO grade, n (%)			
G1	39 (83)	11 (84.6)	1
G2	8 (17)	2 (15.4)	
Liver metastases, n (%)			
Yes	8 (17)	1 (7.7)	0.668
No	38 (80.9)	12 (92.3)	
Unknown	1 (2.1)	0	
Follow up in years*	5.75 (0–28.5)	5.75 (1.25–17.75)	0.475

NA, not applicable; NF-PanNET, non-functioning PanNET; PanNET, pancreatic neuroendocrine tumor.

*Values are presented as median (range)

Immunohistochemistry

Immunohistochemistry for menin in sporadic PanNETs was performed on tissue micro-array slides including a subset of the sporadic cohort using a polyclonal antibody for menin (Bethyl Laboratories, A300-105A, 1:1600, ARS pH 5.8 pre-treatment and incubation at room temperature for 1 h). Menin staining was considered either positive (>30% positive nuclei) or negative (≤30% positive nuclei) (15).

For detection of pVHL, a monoclonal antibody was used (BD, clone Ig32, Cat.no. 556347, 1:200, ARS pH6 pre-treatment and overnight incubation at 4°C).

Briefly, 4 µm sections were deparaffinized and blocked for endogenous peroxidase activity by immersion in 0.3% H₂O₂ in methanol. Antigen retrieval (indicated as ARS) was performed in sodium citrate buffer (0.01 M/pH 6.0) for 20 min at 100°C. Nonspecific binding sites were blocked using Protein Block Serum free (DAKO), followed by primary antibody incubation. Antibody binding was visualized using the BrightVision+poly-HRP detection system (ImmunoLogic, Diemen, The Netherlands), with 3,3-diamino-benzidine as chromogen (DAB, Sigma). Sections were counterstained with hematoxylin, dehydrated and coverslipped using Pertex.

Statistics

The cumulative methylation index (CMI) was calculated as the sum of the methylation percentage of all genes as described before (20). The Mann–Whitney *U* test was performed to compare CMI and methylation of individual genes between MEN1-related and sporadic PanNETs. Furthermore, sporadic PanNETs were subdivided into menin staining-positive and menin staining-negative tumors. The Kruskal–Wallis test was used to compare MEN1-related PanNETs with menin-positive and -negative sporadic PanNETs. Within the MEN1-related PanNETs, subgroups analysis for differences in CMI and methylation of individual genes was performed. Spearman correlation coefficient was calculated for the association between methylation data and age when appropriate. In a different (dichotomous) approach, 15% methylation was defined as the cut-off value for promoter hypermethylation. This value was previously determined based on titration experiments in cell lines and has been used before in similar studies (21, 22, 23). The Fisher exact test was applied to compare frequency of hypermethylation for individual genes of one subgroup with another. Two-sided *P* values <0.05 were considered to be statistically significant. All statistical analyses were performed using IBM SPSS Statistics 23.0.

Results

The CMI was calculated and differences in methylation percentages patterns for individual genes between subgroups were analyzed (continuous data analysis). This showed no significant differences in the CMI of MEN1-related and sporadic PanNETs (median CMI: 912 vs 876; *P*=0.207). In MEN1 subgroup analysis of non-functioning PanNETs, CMI was higher in larger PanNETs (>2cm) (median: 969.5 vs 838.5; *P*=0.021) and higher in PanNETs with liver metastases (median 1036 vs 869; *P*=0.013).

When comparing methylation percentages at individual gene loci, higher percentages of *CASP8* methylation were found in MEN1-related tumors compared to sporadic PanNETs (median: 59% vs 16.5%; *P*=0.002). The methylation percentage of *CASP8* in menin-negative sporadic PanNETs was comparable to MEN1-related PanNETs, but lower in menin-positive sporadic PanNETs (respectively median 59% vs 44% vs 19%; *P*=0.046) (Fig. 1). MEN1-related insulinomas showed a higher methylation percentage than non-functioning PanNETs for *RASSF1_1* (median: 70% vs 47%; *P*=0.001). There was no correlation between *RASSF1_1*

Figure 1

Promoter methylation of *CASP8*. (A) In MEN1-related (*n*=61) and sporadic PanNETs (*n*=34, *P*=0.002). (B) In MEN1-related (*n*=61) and sporadic PanNETs after subdividing sporadic PanNETs into menin-negative (*n*=10) and menin-positive tumors (*n*=13), (*P*=0.046). Dotted line represents cut-off value for hypermethylation (15%).

methylation percentage and age (Spearman correlation coefficient -0.097 ; *P*=0.348). Other relevant differences in quantitative methylation of individual genes between MEN1 subgroups were not observed. In the dichotomous analysis, we found that both in MEN1-related and sporadic PanNETs, a large number of potential tumor suppressor showed promoter hypermethylation (Fig. 2). In general, the frequency of hypermethylation of individual genes was comparable between these two groups, except for *CASP8* (77% vs 50%; *P*=0.011), *RASSF1_1* (98.4% vs 79.4%,

Figure 2

Promoter hypermethylation of genes in both MEN1-related and sporadic PanNETs. Cut-off value for promoter hypermethylation was 15%. Only gene loci with hypermethylation in >10% of the MEN1-related tumors are shown.

$P=0.003$) and *RASSF1_2* (98.4% vs 82.4%; $P=0.008$). In these cases, hypermethylation was more frequent in MEN1-related PanNETs. Promoter hypermethylation of *MEN1* (2 different probes included) was not seen in MEN1-related or in sporadic PanNETs. Analysis of MEN1 subgroups revealed that hypermethylation of *MGMT_2* is more frequently seen in non-functioning PanNETs compared to insulinomas (44.7% vs 8.3%; $P=0.022$) (Fig. 3). There was no correlation between *MGMT_2* methylation percentage and age (Spearman correlation coefficient 0.187; $P=0.071$).

Interestingly, in one grade 2 MEN1-related PanNET, the methylation percentage of *VHL* was remarkably high (*VHL_2* 73% and *VHL_1* 36%). Hypermethylation of *VHL* was not seen in any other MEN1 PanNET. Immunohistochemistry showed loss of VHL expression in this specific tumor, whereas expression was normal in another PanNET of the same patient that showed no *VHL* promoter hypermethylation (Fig. 4).

Discussion

We present the first promoter hypermethylation analysis of tumor suppressor genes in MEN1-related PanNETs. In this study, we included a comprehensive series of

Figure 3

Promoter methylation of *MGMT* in MEN1-related insulinomas and non-functioning PanNETs. Dotted line represents the 15% cut-off value for hypermethylation ($P=0.022$).

Figure 4

Promoter hypermethylation and protein expression of VHL differs between different PanNETs of the same MEN1 patient. (A) VHL protein expression is lost in the PanNET with promoter hypermethylation of *VHL*. (B) In another PanNET of the same patient without promoter hypermethylation of VHL, protein expression is retained. Magnification 200 $\times$.

well-characterized MEN1-related PanNETs and studied DNA methylation of a large number of tumor suppressor genes. We show that hypermethylation of CpG sites in promoter regions of tumor suppressor genes is a common event in both sporadic and MEN1-related PanNETs. This suggests that similar DNA methylation mechanisms play a role in both MEN1-dependent and -independent tumors (8). We found differences in frequency and in levels of *CASP8* methylation between MEN1-related and sporadic tumors. In this light, the finding that methylation percentages of *CASP8* are higher in MEN1-related PanNETs and in sporadic PanNETs negative for menin staining compared to sporadic PanNETs with retained menin expression, is of specific interest. *CASP8* encodes the caspase-8 protein with a central role in activation of apoptosis (24). Inactivation of *CASP8* by DNA promoter methylation is a well-described event in development and progression of various tumor types such as neuroblastoma, glioblastoma multiforme and pheochromocytoma (25, 26, 27). Recently, Stefanoli and coworkers observed a high frequency of *CASP8* DNA promoter hypermethylation in sporadic PanNETs, which is in line with our results (31). It has been shown before in murine embryonic embryoblasts that menin suppresses tumor formation by inducing apoptosis via caspase 8 expression (28). Menin might prevent the *CASP8* promoter from being methylated, possibly by inducing histone H3, lysine 4 (H3K4) methylation, which is known to counteract DNA methyltransferase activity (29). The high frequency of DNA promoter hypermethylation of the *CASP8* promoter in MEN1-related PanNETs supports this hypothesis.

In MEN1 patients, cumulative DNA promoter methylation was found to be higher in large PanNETs and in PanNETs concurrent with liver metastases. These results

imply that there is a role of DNA promoter methylation in tumor progression. DNA promoter methylation could, therefore, also be a therapeutic target in MEN1 patients with advanced PanNETs.

The tumor suppressor gene *RASSF1* is considered to be the most frequently altered gene by DNA promoter methylation in sporadic PanNETs to date (4). This gene is involved in induction of cell cycle arrest and is inactivated by methylation in various cancers (30). DNA promoter hypermethylation of this gene has been reported before in 63–83% of sporadic (predominantly non-functioning) PanNETs (31, 32, 33, 34), which is comparable to the frequency of hypermethylation in the sporadic PanNETs included in our study. Interestingly, in MEN1, nearly all PanNETs are hypermethylated at the *RASSF1* promoter (99% and 97% for *RASSF1_2* and *RASSF1_1* respectively) indicating that this is a frequent molecular event in MEN1-associated PanNET development. *RASSF1* median methylation percentages were higher in insulinomas compared to non-functioning PanNETs in MEN1. Indeed, it has been shown before that loss of menin promotes β -cell proliferation by influencing the RAS signaling pathway (including *RASSF1*), but the exact mechanism remains unclear (35).

As in sporadic PanNETs, DNA promoter hypermethylation of *MGMT* was frequent in MEN1 tumors, especially in non-functioning PanNETs. Our results are in line with a previously reported hypermethylation frequency of *MGMT* in sporadic non-functioning PanNETs of approximately 40% (32). The *MGMT* gene product O⁶-methylguanine DNA-methyltransferase provides resistance to treatment with alkylating agents such as temozolomide (36). *MGMT* promoter methylation could increase sensitivity to temozolomide and serve as a predictive biomarker of response to chemotherapy in MEN1 patients with advanced disease (37).

In one MEN1-related PanNET (WHO grade 2), a high methylation percentage of the *VHL* promoter was found together with loss of VHL protein expression. It has been reported that somatic *VHL* mutations and LOH of chromosomal locus 3p (with the site of *VHL*) may be involved in PanNET development and/or progression in MEN1 (38, 39). DNA promoter hypermethylation of *VHL* has previously been described in 6% of sporadic PanNETs (40). To our knowledge, this is the first report of *VHL* promoter hypermethylation in a MEN1 PanNET.

The importance of epigenetics in progression of PanNETs was demonstrated before. Mutations in the chromatin remodelers *DAXX* (death domain-associated protein) and *ATRX* (alpha thalassemia/mental retardation

syndrome X-linked) occur in 43% of the sporadic cases (8). Mutations in these genes correlate well with loss of nuclear expression by IHC and this predicts metastatic disease and poor survival in patients with PanNETs (41). Also in MEN1 PanNETs, loss of nuclear expression of *DAXX* or *ATRX* is a late event (42). Analysis of our data according to *DAXX*/*ATRX* expression status in MEN1-related PanNETs did not reveal effects on the cumulative methylation nor on the methylation status of the *CASP8*, *MGMT* and *VHL* genes (data not shown). In summary, DNA promoter hypermethylation of tumor suppressor genes is a common event in MEN1-related and sporadic PanNETs. Promoter methylation may contribute to PanNET development and progression in MEN1. Targeting DNA methylation could be of therapeutic value in MEN1 patients with PanNETs.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/EJE-18-0195>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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