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DNA METHYLATION PROFILING IDENTIFIES DIFFERENT PROGNOSTIC CLUSTERS OF PANCREATIC NEUROENDOCRINE TUMORS

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INTRODUCTION

Pancreatic neuroendocrine tumors: classification and TNM staging

Pancreatic neuroendocrine tumors (PanNETs) are a heterogeneous group of relatively rare neoplasms whose yearly incidence is 1 case/100,000 people and represent approximately 1-2% of all pancreatic tumors [1]. Although uncommon, they represent a disease with growing incidence and high prevalence [2]. This phenomenon probably reflects either a higher knowledge of this tumor type by the pathology or the wider availability of suitable and efficient radiological and nuclear medicine diagnostic tools [3]. The incidence in relatively old autopsy studies has been reported as high as 1.5%, even if in these reported series the tumors were clinically unrecognized, often asymptomatic and usually small (<1 cm in size) lesions.

PanNETs show no significant gender predilection and occur at all ages, with a peak incidence between 30 and 60 years [4-6]. Although most PanNETs occur sporadically, approximately 5-10% of these neoplasms have a hereditary background and they may be a part of two main hereditary syndromes: MEN-1 syndrome (Multiple Endocrine Neoplasia, Type 1) and VHL syndrome (Von Hippel Lindau).

Since the beginning of the last century, when the first report of a tumor believed to originate from the endocrine pancreas was published [7], several investigators have tried to elucidate the clinico-pathological and molecular characteristics of these neoplasms. Because different methodological approaches have been used to classify these tumors, a variety of nomenclatures have been proposed and they often created some misunderstanding among pathologists and clinicians [8].

Clinically, the most used classification of PanNETs is based on the presence/absence of abnormal hormone production and this approach identifies two broad categories of functioning or nonfunctioning tumors. Nearly 30-40% of PanNETs are not hormone-producing and are classified as “nonfunctioning ” and are typically diagnosed when their sizes cause compression or invasion of adjacent organs or when they give rise to liver or lymph node metastases. Conversely, the remaining 60-70% of PanNETs are classified as “functioning” and include preferentially insulinomas, glucagonomas, somatostatinomas, gastrinomas and VIP-omas (Table 1).

Table 1. Functional Classification of PanNETs [1]

%	Type	Behavior
5-10	Nonfunctioning, clinically silent	Mostly benign
50	Insulinomas	90% benign
20	Other functioning tumors: gastrinoma, glucagonoma, somatostatinoma, carcinoid, Cushing's tumors, etc	50-90% malignant low grade
20	Nonfunctioning, locally symptomatic tumors	70-80% malignant low grade
1-5	Small cell carcinoma with poor endocrine differentiation	All malignant, high grade

In 1995, a group of endocrine pathologists [9] proposed a revised classification of neuroendocrine tumors of the lung, gut and pancreas. The purpose of this classification was to identify clinical and morphological features that were helpful in delineating categories of tumors with different prognoses. The criteria proposed by the international group of pathologists in 1995 together with various more reliable prognostic parameters identified in the last years [10-16], represented the basis for the 2010 WHO classification of PanNETs [17].

Currently, the general histological classification of PanNETs comprises the two major categories of well/moderately differentiated and poorly differentiated PanNETs. Although several clinico-pathological features (presence and type of the endocrine syndrome, tumor size, invasion of nearby tissue, pattern of growth with prevalence of broad solid areas, presence of necrosis, cellular atypia, high proliferative index, vascular invasion, and presence of bands forming fibrosis) have been demonstrated to correlate with tumor aggressiveness and patients' prognosis, in the more recent WHO classification of PanNETs they are divided into three main categories on the basis of the mitotic count and Ki67 proliferative index [18]. PanNET G1 are characterized by low mitotic and proliferative status (< 2 mitoses X 10 HPF, and < 2% Ki67 index), PanNET G2 by mitotic count between 2 and 20 mitoses X 10 HPF and Ki67 index between 3% and 20%. Poorly differentiated neuroendocrine carcinomas, now simply defined as neuroendocrine carcinomas (NEC, G3), show a high proliferation including > 20 mitoses X 10 HPF and > 20% Ki67. From a morphological point of view, PanNET G1 and G2 are characterized by tumor cell monomorphism, absent or mild nuclear atypia, together with the frequently observed trabecular structure. NECs show a mostly solid

structure either organized in large, poorly defined aggregates often with central necrosis, or diffuse sheets of cells with multiple minute foci of necrosis. They are highly invasive, invariably presenting with metastases to lymph nodes, liver and distant organs (Figure 1).

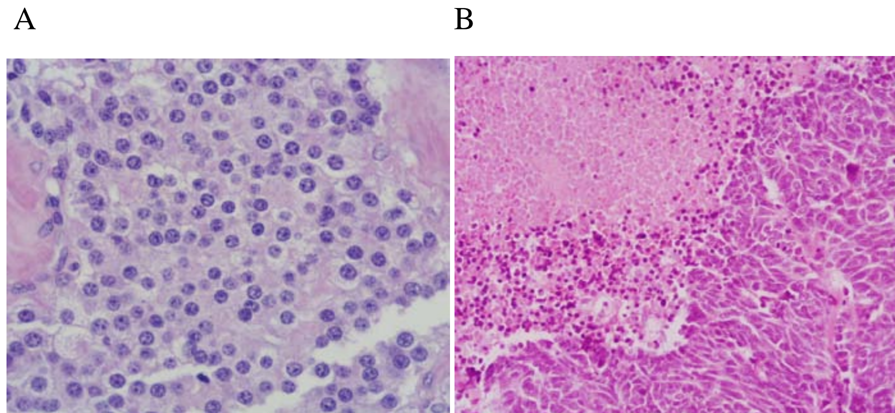


Figure 1: A) Glucagonoma of the pancreas: tumor cells are well differentiated and form trabecular structures. B) Poorly differentiated endocrine carcinoma of the pancreas composed of cells with severe nuclear atypia forming solid sheets. On the left top an area of necrosis is well evident.

In addition to the WHO classifications, a TNM staging must be included in routine sign out of PanNETs (Table 2). A TNM staging system for neuroendocrine neoplasms of the pancreas was proposed for the first time in 2006 by the European Neuroendocrine Tumor Society (ENETS) [19]. More recently, the International Union for Cancer Control developed a TNM staging system, which is now endorsed by both the American Joint Cancer Committee and the World Health Organization (UICC/AJCC/WHO 2010 TNM) [17, 20, 21]. Table 2 summaries T and stage definitions according to ENETS and to UICC/AJCC/WHO. The presence of two different TNM systems raised concerns of potential confusion in patient management [22, 23]. Recently, Rindi *et al.* suggested the ENETS TNM staging is superior to the UICC/AJCC/ TNM staging system and support its use in the clinical practice [24].

Table 2. T and stage definitions in the European Neuroendocrine Tumor Society (ENETS) and the International Union for Cancer Control/American Joint Cancer Committee/World Health Organization (UICC/AJCC/WHO) 2010 TNM staging system [24]

Definitions	**ENETS TNM**	**UICC/AJCC/WHO 2010 TNM**
T definition		
T1	Limited to the pancreas, < 2 cm	Limited to the pamcreas, <=2 cm in greatest dimension
T2	Limited to the pancreas, 2-4 cm	Limited to the pancreas, >2 cm in greatest dimension
T3	Limited to the pancreas, >4 cm or invading duodenum or bile duct	Beyond the pancreas but without involvement of the superior mesenteric artery
T4	Tumor invading adjacent organs (stomach, spleen, colon, adrenal gland) or the wall of large vessels (celiac axis or the superior mesenteric artery)	Involvement of the celiac axis or the superior mesenteric artery (unresectable tumor)
Stage definition		NA
Stage I	T1, N0, M0	NA
Stage IIa	T2, N0, M0	NA
Stage IIb	T3, N0, M0	NA
Stage IIIa	T4, N0, M0	NA
Stage IIIb	Any T, Any N1, M0	NA
Stage IV	Any T, Any N, M1	NA
Stage IA	NA	T1, N0, M0
Stage IB	NA	T2, N0, M0
Stage IIA	NA	T3, N0, M0
Stage IIB	NA	T1-T3, N1, M0
Stage III	NA	T4, Any N, M0
Stage IV	NA	Any T, Any N, M1

Legend: NA= not applicable

Molecular genetics of sporadic PanNETs

Genetic abnormalities have been investigated to elucidate the pathogenesis of PanNETs as well as to identify new biological markers with diagnostic and clinical value.
For hereditary PanNETs, alterations of *MEN1, VHL, TSC2* and *NF1* are the main factors that drive tumorigenesis. The genetic defect causing each of these inherited PanNET syndromes has been elucidated and considerable advances have been made in understanding the cellular roles of the various altered proteins [25].

In contrast, the genetic alterations involved in the onset and progression of sporadic PanNETS are poorly understood, although in recent years several studies have been carried out to gain insights into the genetic basis of these tumors. To date, two main approaches have been used to study sporadic PanNETs: 1) mutation analysis of the susceptibility genes associated with hereditary PanNETs or screening of the most commonly mutated genes in pancreatic ductal adenocarcinomas (PDACs); 2) study of chromosomal gains and losses to identify candidate loci involved in the development of these tumors. In Table 3 we summarize a comprehensive review of the papers so far published on this subject.

Several studies demonstrated that approximately 20-40% of sporadic PanNETs had somatic inactivating mutations in *MEN1,* which encodes menin, a component of histone methyltransferase complex [26-33]. *MEN1* mutation was frequently associated with gastrinoma and glucagonoma, but was rare in insulinoma and nonfunctioning PanNETs. Recently, whole-exome sequencing of PanNETs confirmed that *MEN1* is the most frequently mutated gene in these tumors [34]. In addition this study demonstrated that PanNETs exhibited frequent mutations in other genes involved in chromatin remodeling complex such as *DAXX* (death-domain-associated protein) and *ATRX* (α−thalassemia/mental retardation syndrome X-linked). Overall, somatic mutations in PanNETs were identified in *MEN1* (44%), *DAXX* (25%), *ATRX* (18%), *PTEN* (7.3%), *TSC2* (8.8%), *PIK3CA* (1.4%). Other oncosuppressor genes such as *VHL, RB1, BRCA2* [35-41] and common oncogenes including *myc, fos, c-erbB-2* and *sis* [42-45] have been examined in PanNETs but no genetic alterations were observed.

Exome sequencing of PanNETs demonstrated that there were important differences between the genetic landscapes of PanNETs and those of pancreatic ductal adenocarcinomas (PDACs). First, there were 60% fewer genes mutated per tumor in

PanNETs than in PDACs, with a mean of 16 mutations per tumor among PanNETs. Second, the genes most commonly affected by mutations in PDACs (*KRAS*, *TGF-β* pathway, *CDKN2A* and *TP53*) were rarely altered in PanNETs and *vice versa*. Third, the spectrum of mutations in PDAC and in PanNET was different, with C-to-T transitions more common in PDACs than in PanNETs and C-to-G tranversions more common in PanNETs than in PDACs. This suggests that mutations in PanNETs and PDACs arise through different mechanisms, perhaps because of exposure to different environmental carcinogens or through the action of different DNA-repair pathways.

Table 3. Summary of gene mutations and losses of putative Tumor Suppressor Genes in PanNETs.

MUTATIONAL ANALYSES			
GENE	**Mutation**	**Range %**	**References**
MEN-1 gene (11q13) mutation	78/285 (27%)	0-50%	[26-34]
DAXX gene (6p21.3) mutation	37%		[34]
ATRX gene (Xq21.1) mutation	18%		[34]
PTEN gene (10q23) mutation	9/101 (9%)	7-13%	[34, 46]
TSC2 gene (16p13.3) mutation	9%		[34]
PIK3CA gene (3q26.3) mutation	1.4%		[34]
VHL gene (3p25.3) mutation	0/39 (0%)		[34, 35, 38, 39]
RB1 gene (13q14.2) mutation	0/39 (0%)		[37]
BRCA2 gene (13q12-q13) mutation	0/5 (0%)		[40]
TP53 gene (17p13.1) mutation	2/39 (4%)	0-5%	[41, 47-51]
KRAS (12p12.1) gene mutation	0/80 (0%)		[34, 47, 50-52]
CDKN2A (9p21) p16 gene mutation	0/14 (0%)		[51, 53-55]
DPC4/SMAD4 gene (18q21.1) mutation	0/45 (0%)		[51, 56-58]
TIMP-3 gene (22q12.3) mutation	2/31 (5%)	0-5%	[34, 59]
HDAC II gene (6q21) mutation	0/10 (0%)		[60]
CDKN2B p15 gene mutation	0/18 (0%)		[61]
Other common oncogenes *myc, fos, c-erbB-2*	0%		[50, 51, 62]
CHROMOSOMAL ABNORMALITIES			
CHROMOSOMAL POSITION	**Loss**	**Range %**	**References**
1p	55/351 (16 %)	4-44%	[63-71]
1q	31/221 (15%)	4-44%	[63, 67, 68, 71-73]
2p-2q	61/372 (17%)	4-44%	[63, 65, 67, 68, 70-72]
3p	215/620 (35%)	5-100%	[35-37, 39, 41, 58, 63, 65-68, 70, 72-78]
3q	24/191 (13%)	4-40%	[36, 63, 72, 73, 75, 76]
4p-4q	10/212 (5%)	0-5%	[63, 66, 68, 75]
5q	2/44 (5%)	0-14%	[66, 68, 75, 79]
6p	16/184 (9%)	5-20%	[63, 68, 70, 72, 73, 76]
6q	110/331 (33%)	6-66%	[36, 63, 65, 67-69, 71, 72, 76, 80]
7q	3/77 (4%)	0-11%	[41, 63, 68, 73]
8p-8q	23/289 (8%)	4-40%	[63, 65, 66, 68, 70, 72, 76]
9p-9q	26/234 (11%)	0-57%	[53-55, 61, 63, 67, 68, 70, 73, 75, 81]
10p-10q	45/278 (16%)	6-44%	[46, 63, 66-68, 70, 72, 76]
11p	54/294 (18%)	4-54%	[63, 65, 67-69, 71, 75, 76]
11q	178/596 (30%)	4-73%	[26, 30, 36, 41, 58, 59, 63, 65-67, 69-73, 75, 76, 82, 83]
12p-12q	10/126 (8%)	0-6%	[63, 68, 71, 75]
13p-13q	8/187 (4%)	0-15%	[35, 37, 41, 63, 65, 68, 72, 75]
15q	22/148 (15%)	0-27%	[36, 63, 70-72]
16p-16q	52/290 (18%)	4-40%	[63, 65, 66, 70-72, 75, 84]
17p-17q	15/85 (18%)	6-35%	[41, 63, 66, 67]
18p-18q	47/395 (12%)	0-27%	[41, 56-58, 63, 65-68, 70, 73, 75, 84, 85]
19p-19q	1/40 (3%)	0-3%	[66, 75]
20q	8/41 (20%)	20-50%	[65, 67]
21p-21q	26/80 (32%)	12-47%	[63, 67, 70, 72]
22p-22q	93/320 (29%)	0-96%	[36, 63, 66, 68-73, 75, 86, 87]
Xp	39/231 (17%)	4-32%	[63, 65, 71, 76, 88]
Xq	35/220 (16%)	4-27%	[63, 69, 71, 76]
Y LOH	22/84 (26%)	7-45%	[63, 69, 76]

In recent years, comprehensive genome-wide approaches such as Comparative Genomic Hybridization (CGH), high resolution allelotyping and gene expression analyses have revealed candidate loci for genes involved in the development of PanNETs but these have not been substantiated through genetic or functional analyses.

Most of the genome-wide studies demonstrated that genetic alterations seem to accumulate during tumor progression: the total number of genomic changes per tumor appears to be associated with both the tumor size and the stage of the disease [69]. These results point toward a tumor suppressor pathway impairment and chromosomal instability as important mechanisms associated with malignancy in PanNETs. The alterations described are not randomly distributed on chromosomes but are particularly common in distinct chromosomal regions. Gains are common on 4pq (17% of the tumors) 5q (25%), 7pq (41%), 9q (28%), 12q (23%), 14q (32%), 17pq (31%) and 20q (27%), whereas genomic losses frequently occur on 1p (16%), 3p (35%), 6q (33%), 10pq (16%), 11q (30%), Y (26%) and X (17%).

Loss of heterozygosity studies on PanNETs indicated different chromosomal regions such as 1p, 3p, Xp and 6q, whose deletion is associated with more aggressive behavior [35, 47, 63, 68, 76]. Allelic deletion on 11q is frequently found in sporadic PanNETs [28, 31, 67] and combining data from all studies referred, it appears that the LOH rate is usually 2-3 times higher than the frequency of mutations of the *MEN1* gene.

Some differences have been reported among PanNETs considering both type and number of DNA alterations. Insulinomas exhibit a lower number of genomic alterations than other PanNETs and they frequently show gain of 9q32 and loss of 22q13.1, which appear to be early genetic events in these tumors [71]. By contrast, they rarely show 3p and 6q losses associated with malignancy [77, 80]. Malignant insulinomas, in contrast, harbor a large number of chromosomal alterations similar to that seen in other types of malignant PanNETs [49]. In pancreatic gastrinomas, only limited chromosomal imbalances are encountered. Losses at 3p and 18q21 occur in approximately 33% and 22% of cases, respectively [65, 69].

Nonfunctioning PanNETs (NF-PanNETs) in general harbor higher numbers of chromosomal gains and losses than functioning tumors. These genetic aberrations occur in chromosomal loci frequently involved in malignant tumors [73]. In a recent high

resolution allelotyping analysis of only NF-PanNETs, Rigaud *et al.*. [67] underlined the existence of two different allelotypes among NF-PanNETs: one aneuploid or multiploid with a high degree of large chromosomal allelic deletions and the second, diploid, showing a small number of scattered losses with no apparent specific localization. In this study, survival analysis showed that no specific chromosomal alteration was associated with outcome, whereas ploidy status is an independent factor adding prognostic information to that given by the proliferative index evaluated with Ki67 immunohistochemistry.

DNA methylation changes in PanNETs

Malignant cells show major disruptions in DNA methylation patterns when compared with normal cells [89]. The pattern of DNA methylation changes substantially when cells became malignant, as a result of two major phenomena. Firstly, global genomic hypomethylation has been linked to the induction of chromosomal instability and hypothesized to contribute to oncogenesis by activation of oncogenes (such as *c-MYC* and *H-RAS*), and of latent retrotrasposons [90, 91]. Secondly, hypermethylation of tumor-suppressor genes contributes to a selective advantage of the cancer cell and therefore may represent one of the steps in the sequence of events leading to malignancy. DNA methylation patterns are becoming increasingly attractive tools in the management of cancer patients. DNA methylation is being examined as a powerful markers for early diagnosis of cancer, and for disease monitoring during and after treatment [92-98]. DNA methylation patterns change during the process of tumorigenesis and these changes appear to be events contributing directly to the transformed phenotype [99]. Promoter hypermethylation has been shown to be associated with adverse prognosis in a variety of malignancies, including prostate, colon, breast, non-small cell lung cancer, and non-Hodgkin lymphoma [100-103].

To date very few studies have been published about DNA methylation changes in PanNETs and there is no information about the role of gene promoter-specific hypermethylation as well as global hypomethylation in the development and progression of these tumors.

Much of what is known today about this subject was gained through studies focused on aberrant hypermethylation of single or few candidate genes. Several specific CpG-island-associated genes are frequently methylated in PanNETs, namely *RASSF1A*, *CDKN2A, HIC1, RARβ, APC, MGMT, ER* [78, 79, 104-107]. The cumulative methylation frequency ranges from only a few percent to more than 70% for some of these genes. These methylation rates often differ substantially depending on the study population, tumor histology, and/or methodology used to assess CpG island methylation. In table 4 we provide a brief overview of the current literature summarizing the panel of genes investigated, the methylation rates and the methods used.

RASSF1A gene silencing by DNA methylation has been reported as a major event in PanNETs with methylation rates ranging from 63% to 100% [78, 79, 104-107]. This gene works as tumor suppressor in the RAS pathway, regulating proliferation, inducing apoptosis and stabilizing microtubules [108-110]. Ras-association domain family 1 gene has seven isoforms produced by alternative splicing and expressed from two different promoters. Recently, Malpeli *et al.*. demonstrated that the isoform *RASSF1A* is down regulated in PanNETs compared to normal pancreas and that the overall extent of *RASSF1A* methylation in PanNETs correlated inversely with its expression [104]. Moreover, some authors reported the common occurrence of RASSF1A methylation in normal pancreas adjacent to PanNETs suggesting that epigenetic change might arise early in the onset of PanNETs [111-113].

Another gene frequently investigate in PanNETs for methylation status is *CDKN2A* [53, 54, 61, 79, 105, 106, 114, 115], a cyclin dependent kinase 4 inhibitor that causes retinoblastoma protein to stay in its active form and arrest the cell cycle at G1-S transition. Muscarella *et al.*. [53] reported the presence of *CDKN2A/p16* promoter hypermethylation or homozygous deletion in a limited number of pancreatic gastrinomas and in nonfunctioning PanNETs (a total of 14 cases investigated). These data were confirmed by Lubomierski *et al.*. [81] who reported loss of expression of at least one of the tumor suppressor genes *CDKN2A/p16, CDKN2B/p15 and CDKN2D/p14* localized as a gene cluster at 9p21. mRNA transcripts of these genes were lost most frequently in nonfunctioning PanNETs (57%) and less commonly in insulinomas (30%) and gastrinomas (22%).

Arnold *et al.*. [79, 107] reported high frequency of epigenetic inactivation of *HIC*-1 (Hypermethylated In Cancer 1) ranging from 83% to 93%. HIC-1 encodes a zinc-finger transcriptional factor [116] that regulates p53-dependent apoptotic DNA-damage response [117]. The role of HIC-1 as a TSG is not completely understood and further studies are needed to elucidate its function in the pathogenesis of PanNETs.

Although most of the analyses of DNA methylation patterns in PanNETs were single-gene studies, three studies analyzed the methylation status of many genes in order to define a methylation profile of these tumors [105, 107, 114]. In the first study, Chan *et al.* [114] analysed the methylation status of 14 genes in 11 PanNETs and in 16 gastrointestinal neuroendocrine tumors demonstrating different methylation profiles in

the two subsets of tumors. House *et al.* [105] investigated aberrant methylation of 11 candidate tumor suppressor genes in forty-eight PanNETs and found hypermethylation of at least one gene in 87% of PanNETs. In decreasing order of frequency, the five most commonly methylated genes were: *RASSF1A* (75%), *CDKN2A* (40%), *MGMT* (40%), *RAR-β* (25%), and *MLH1* (23%). Moreover, the authors demonstrated that aberrant methylation of 3 or more genes predicted both decreased patient survival at 5 years and tumor recurrence within 24 months from the time of surgery. In agreement with these results, Arnold *et al.*. [107] analysed the methylation status of 11 genes in 46 PanNETs confirming that hypermethylation of tumor suppressor genes was more common in PanNETs with high proliferation index (Ki67>10%) and that a CpG island methylator phenotype (CIMP phenotype) [118] showed a trend towards worse survival.

Besides gene-specific methylation, global hypomethylation in PanNETs and gastrointestinal neuroendocrine tumors has been recently investigated [119, 120] using LINE-1 methylation status as a surrogate marker for genome-wide methylation. These studies showed that LINE-1 hypomethylation is common in neuroendocrine tumors compared with normal tissue and is associated with malignant behavior.

Table 4 Review of gene promoter-specific hypermethylation in PanNETs.

Gene	Frequency of gene methylation (%)	Range	Methods	References
RASSF1A	125/161 (77.6%)	63% - 100%	M/P	[78, 79, 104-107]
CDKN2A(p16)	32/135 (23.7%)	0% - 75%	M	[53, 54, 79, 105, 106, 114, 115]
HIC1	48/52 (92%)	83% - 93%	M	[79, 107]
APC	39/104 (37.5%)	21% - 70%	M	[79, 105, 107]
MGMT	33/131 (25.2%)	0% - 40%	M/C	[79, 105-107, 114]
MLH1	11/58 (19%)	0% - 23%	M	[79, 89]
TIMP-3	8/112 (7.1%)	0% - 44%	M	[59, 105, 107]
MEN1	10/55 (18.2%)	11% - 19%	M	[79, 107]
CDKN2A(p14)	8/75 (10.7%)	0% - 44%	M	[105, 106, 114]
RARb	12/59 (20.3%)	0% - 25%	M/C	[105, 114]
CDH1	12/92 (13%)	2% - 23%	M	[105, 107]
ER	7/11 (64%)	64%	M/C	[114]
P73	8/48 (17%)	17%	M	[105]
CDKN2B(p15)	2/18 (11%)	11%	P	[61]
COX2	1/11 (9%)	9%	M/C	[114]
THBS1	1/11 (9%)	9%	M/C	[114]
RUNX	3/46 (7%)	7%	M	[107]
VHL	2/35 (6%)	6%	M	[38]
GSTp	0/48 (0%)	0%	M	[105]
PTEN	0/48 (0%)	0%	M	[107]

Legend: M: Methylation Specific PCR (MSP); C: COBRA; P: Pyrosequencing

AIM

The aims of our work were to investigate the occurrence and relevance of gene promoter-specific hypermethylation and global hypomethylation in 58 PanNETs in order to correlate the methylation profiles with DNA copy number alterations (CNAs) and with the clinico-pathologic features of the tumors.

In particular our purposes were:

1. To study the methylation status of 33 tumor suppressor genes and copy number alterations (CNA) of 53 genes in an archival series of formalin fixed and paraffin embedded (FFPE) tumor tissues by MS-MLPA (Methylation-specific multiplex ligation-dependent probe amplification).
2. to validate MS-MLPA results for the methylation status of *MLH1, MGMT, THBS1, WT-1, CASP-8*, and *CDKN2A (p16)* genes using additional independent techniques (methylation specific PCR (MSP), bisulfite pyrosequencing and bisulfite cycle sequencing) to test the robustness of MS-MLPA assay in gene hypermethylation analyses.
3. To optimize and apply a quantitative bisulfite pyrosequencing assay to measure CpG methylation of Long Interspersed Nuclear Elements -1 (LINE-1) in order to evaluate the occurrence of global DNA hypomethylation in PanNETs compared with normal pancreas.
4. To evaluate whether a Two dimensional unsupervised hierarchical clustering can help to define gene promoter specific hypermethylation profiles of PanNETs. Moreover we verified if a small subset of hypermethylated markers led to identify specific methylation profiles of PanNETs by using Receiver Operator Characteristic (ROC) curves.
5. To correlate DNA hypermethylation clusters with LINE-1 hypomethylation, DNA CNAs and with the clinic-pathologic features of the tumors in order to define clinic-pathologic and molecular subsets of PanNETs.

MATERIALS AND METHODS

Case selection

Surgical specimens of 58 well characterized pancreatic neuroendocrine tumors (PanNETs) encompassing all the main histologic and functional types (Table 5) were selected from our previously-reported series of 155 tumors [13]. Selection depended on the availability of enough material to perform molecular analyses coupled with complete clinicopathologic information including age, endocrine and non endocrine symptoms, tumor size, mitotic and Ki67 proliferative index, vascular and perineural invasion, presence of metastases at the time of diagnosis, evidence of local invasion at surgery, perioperative mortality, and evidence of local recurrence or distant metastases during follow-up. Tumor grade and stage were performed according to the ENETS scheme [18, 19]. This study was performed in agreement with the clinical standards laid down in the 1975 Declaration of Helsinki and its revision in 1983, and according to the rules of the Ethics Committee of the Ospedale di Circolo, Varese, Italy.

Table 5. Main clinicopathologic features of PanNETs

Number of cases	**58**
Gender	
Male	*29*
Female	*29*
Age (years)	
Mean	*49*
Range	*18-79*
Diameter (cm)	
Mean	*4.7*
Range	*0.3-18*
Site°	
Head	*21*
Body	*18*
Tail	*12*
Genetic	
MEN1	*2*
Sporadic	*56*
Syndrome	
Nonfunctioning	*28*
Insulinoma	*23*
Glucagonoma	*2*
Zolliger Ellison	*2*
VIPoma	*2*
Cushing	*1*
Grading	
G1	*28*
G2	*30*
G3	*0*
Angioinvasion	
Absent	*26*
Present	*31*
Neuroinvasion	
Absent	*23*
Present	*15*
Staging*	
Stage I	*7*
Stage II	*20*
Stage III	*12*
Stage IV	*7*
Follow-up^	
AFD	*37*
AWD	*6*
DOD	*12*
DOC	*1*

°: for 8 cases information regarding site was not available; *: for 12 enucleated tumors lymph nodes were not available for histologic evaluation so the pN was not detected; ^ two patients died immediately after surgery. MEN 1= multiple endocrine neoplasia syndrome type 1, AFD: alive freed from disease, AWD= alive without disease, DOD= dead for disease, DOC= dead for other causes

DNA extraction and evaluation of amplifiability

DNA samples were obtained from FFPE tissues using representative 8-µm sections. Three sections of every specimen were treated twice with xylene, and then washed twice with ethanol. DNA was extracted using QIAamp® DNA FFPE Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Neoplastic areas were manually microdissected for DNA extraction and contained at least 70% of tumor cells, to minimize contamination by normal cell. Each DNA sample was evaluated for integrity and amplifiability by BIOMED-2 multiplex PCR in order to correlate DNA fragmentation with MS-MLPA reproducibility. Briefly, as illustrated in Figure 2 five pairs of control gene PCR primers were used to amplify products of exactly 100, 200, 300, 400 and 600 bp from the following four target genes: *TBXAS1* (exon 9), *RAG1* (exon 2), *PLZF* (exon 1), *AF4* (exon 11), *AF4* (exon 3). DNA fragmentation and MS-MLPA reproducibility were correlated (data not shown). Only with at least 200 bp fragmentation showed reproducible results and were used in MS-MLPA analysis.

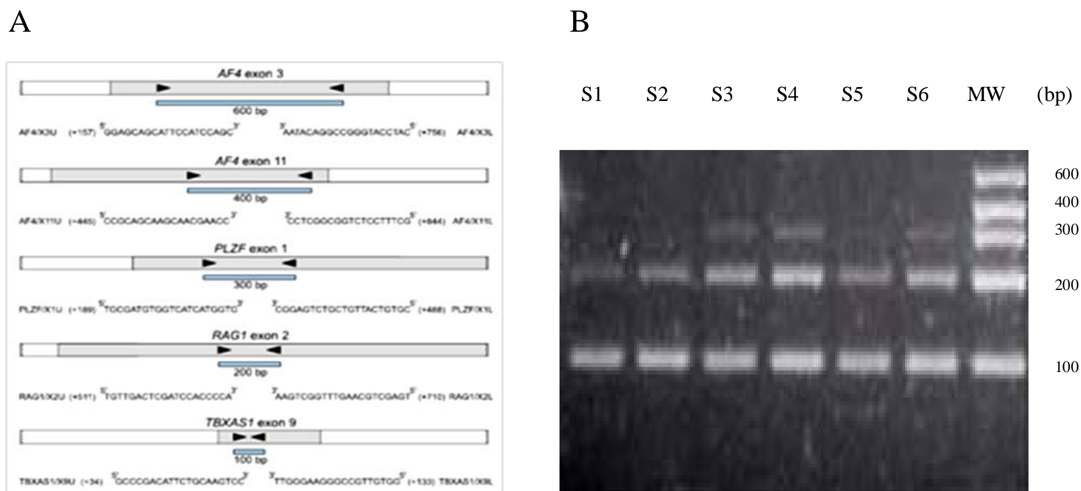


Figure 2: Control multiplex PCR for the assessment of amplifiability and integrity of DNA samples from FFPE tissues. A) Schematic diagram of five control genes and the five primer sets for obtaining PCR products of 600, 400, 300, 200 and 100 bp. B) FFPE DNAs (S1-S6) generated PCR products ranging from 200 to 300 bps. This result illustrates the average level of amplifiability of FFPE DNAs observed in our study.

Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) analysis

MS-MLPA analysis was performed on all the 58 PanNETs reported in Table 1 using the ME001 MS-MLPA Tumor suppressor-1 Kit and the ME002 MS-MLPA Tumor suppressor-2 Kit (MRC-Holland, Amsterdam, The Netherlands). Using these two kits a total of 33 tumor suppressor genes were analysed for aberrant promoter methylation and 53 genes for copy number alterations (CNAs). All these genes are frequently silenced by methylation in tumors of different sites as well as they harbour frequently genetic alterations during tumorigenesis. Table 6 lists all the genes examined for the methylation analysis and for copy number analysis and reports their chromosomal position, sequence accession number and the amplicon size obtained by MS-MLPA analysis.

Table 6. List of the genes examined by MS-MLPA analysis

Number	Gene	Chromosomal position	Reference sequence	Amplicon size
1	***TP73***	1p36.3	NM_005427.2	400*, 238**
2	*MSH6*	2p16	NM_000179	328**
3	***CASP8***	2q33-q34	NM_001080125.1	265*
4	***FHIT***	3p14.2	NM_002012	409*
5	***MLH1***	3p22.3	NM_000249	167*, 463*
6	***RASSF1***	3p21.3	NM_007182	328*, 382*
7	*CTNNB1*	3p21	NM_001904.3	472*
8	***RARB***	3p24	NM_000965	193*, 453**
9	***VHL***	3p25.3	NM_000551	355*, 265**
10	*CASR*	3q21.1	NM_000388.3	483*, 483**
11	*IL-2*	4q26-q27	NM_000586	445**
12	***APC***	5q21-q22	NM_000038	148*, 337**
13	***ESR1***	6q24-q27	NM_000125	373*, 301**
14	*PARK2*	6q25.2-q27	NM_4562.2	154*
15	*CDK6*	7q21-q22	NM_001259.6	310*, 310**
16	*CFTR*	7q31-q32	NM_000492	154**
17	***PAX5***	9p13.2	NM_016734	208**
18	***CDKN2A (p14)***	9p21	NM_058195.2	161*
19	***CDKN2A (p16)***	9p21	NM_058195	427**
20	*CDKN2B*	9p21	NM_004936.3	211*
21	***DAPK1***	9q34.1	NM_004938	346*
22	*PTCH1*	9q22.1-q31	NM_000264	175**
23	*CELF2*	10p13	NM_015413.1	364*
24	*CREM*	10p12.1-p11.1	NM_181571.1	136*, 136**
25	***PTEN***	10q23	NM_000314	292*, 183**, 373**
26	***MGMT***	10q26	NM_002412	191**, 346**
27	***CD44***	11p13	NM_000610	319*, 462**
28	***WT1***	11p13	NM_000378	247**
29	***PAX6***	11p13	NM_001604	409**
30	***GSTP1***	11q13.2	NM_000852	454*, 273**
31	***ATM***	11q22-q23	NM_000051	184*, 160**, 418**
32	***CADM1***	11q23.2	NM_014333	427*, 364**
33	***CDKN1B***	12p13.1-p12	NM_004064	274*
34	*TNFRSF1A*	12p13.2	NM_001065.2	175*, 445*
35	*PAH*	12q22-q24.2	NM_000277	229*, 229**
36	***CHFR***	12q24.33	NM_001161344	238*, 292**
37	***BRCA2***	13q12-q13	NM_000059	301*, 418*, 148**
38	***RB1***	13q14.2	NM_000321	319**, 472**
39	*MLH3*	14q24.3	NM_014381.2	202*, 202**
40	***THBS1***	15q15	NM_003246	355**
41	***PYCARD***	16p11.2	NM_013258	398**
42	*TSC2*	16p13.3	NM_000548	281*, 281**
43	*CDH1*	16q22.1	NM_004360.3	337*
44	***CDH13***	16q23.3	NM_001257	436*, 219**
45	*PMP22*	17p12	NM_000304	256**
46	***TP53***	17p13.1	NM_000546	168**
47	***HIC1***	17p13.3	NM_006497.3	220*
48	***BRCA1***	17q21.31	NM_007294.3	246*, 140**
49	*BCL2*	18q21.3	NM_000633.2	256*
50	***STK11***	19p13.3	NM_000455	382**
51	*KLK3*	19q13.41	NM_145864	390*, 390**
52	***GATA5***	20q13.33	NM_080473	434**
53	***TIMP-3***	22q12.3	NM_000362	142*

Legend: All the 53 genes were analyzed for copy number alterations. Gene names in bold highlight the 33 genes analysed for aberrant methylation. (*): PCR products obtained with ME001- MS-MLPA kit; (**): PCR products obtained with ME002- MS-MLPA kit; Twenty-five genes were examined with at least two MS-MLPA probes, due to a partial overlapping between the probe mixes contained in the two kits. For these genes a mean value of raw MS-MLPA results was calculated.

MS-MLPA technique was described by Nygren *et al.* [121] and the main steps of the protocol are reported in Figure 3.

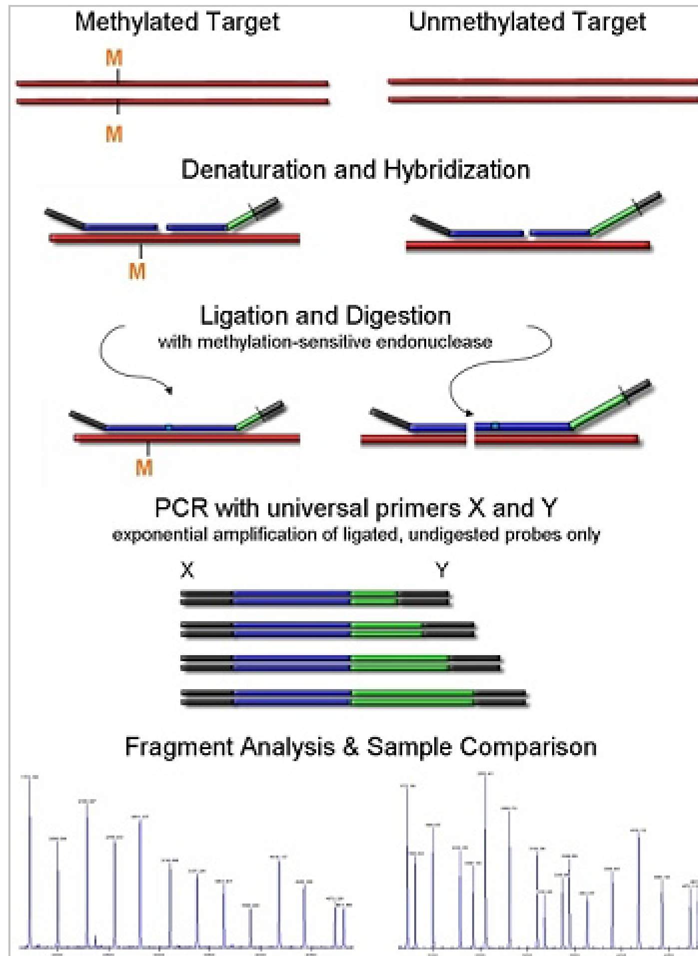


Figure 3 Diagram of the steps of MS-MLPA procedure. The MS-MLPA procedure can be divided in five steps: 1. DNA denaturation and hybridisation of MLPA probes; 2. ligation and digestion; 3. PCR; 4. separation of amplification products by capillary electrophoresis and 5. data analysis.

According to the manufacturer's instructions the probe-mix was added to 50-250 ng of denatured DNA (98°C for 5 minutes) and allowed to hybridize for 16 hours at 60°C. After hybridization of the MLPA probes to the denatured DNA, the reaction was equally divided in two tubes. One tube is designed for the detection of the alterations in the copy number and the other tube for analyses of the aberrant DNA methylation. Ligase mix was added to the first vial, and Ligase-Digestion mix to the second vial. Since the MS-MLPA probes contain a motif for the methylation-sensitive restriction enzyme *HhaI*, the DNA methylation status was determined by restriction digestion by addition of *HhaI* to the second tube.

Samples were incubated for 30 minutes at 48°C, then the HhaI enzyme is inactivated by denaturation at 95°C for 1 minute. Since the unmethylated sequences are cut by the restriction enzyme, this process results in the ligation of the methylated sequences only. Eight microliters of the two aliquots are then amplified in a 25 µl of PCR reaction using a Veriti thermal cycler (Applied Biosystems, Foster City, USA) with this thermal protocol: 35 cycles of denaturation at 95 °C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 1 min with a final extension of 20 min at 72°C. Aliquots of 2.5 µl of the PCR reaction were combined with 0.5 µl TAMRA internal size standard (Applied Biosystems, Foster City, USA) and 13.5 µl of deionized formamide. After denaturation, PCR products were separated by electrophoresis on an ABI 310 capillary sequencer and Genemapper analysis v.4.0 (Applied Biosystems). Values corresponding to peak size (base pairs) and peak height were used for further data processing by Coffalyser V7 software (MRC-Holland). All MS-MLPA reactions were performed at least two times.
Copy number analysis was performed using MS-MLPA results from undigested samples, comparing each tumor sample with three reference DNA samples (normal DNA isolated from leukocytes and included in each analysis). The data generated in the undigested sample were first normalised intra-sample by dividing the signal of each probe by the signal of every reference probe in that sample, thus creating as many ratios per probe as there are reference probes. Relative copy number or Dosage Quotient (DQ) was obtained by comparing this ratio with the same ratio obtained from reference DNA sample (inter-sample normalisation). Since DQ values obtained with normal DNA samples were always between 0.7 and 1.3, loss or gain of a specific region was scored in tumor DNAs when DQ was <0.5 or >1.5, respectively.

The methylation profile of a sample was assessed by calculating a Methylation dosage Ratio (MR) following this calculation: $MR=(Px/Pctrl)_{Dig}/(Px/Pctrl)_{Undig}$ where Px is the peak area of a given target probe, Pctrl is the sum of the peak areas of all control probes, Dig stands for *HhaI* digested sample, and Undig stands for undigested sample. Aberrant methylation was scored as a categorical variable using a specific MR threshold for each gene corresponding to the highest level of accuracy of the test, as previously reported [122].

Validation of MS-MLPA results

Validation of MS-MLPA results was possible for *MLH1, THBS1, MGMT, WT-1* and *CASP-8* genes using three different methods commonly employed for DNA methylation analysis, namely methylation-specific PCR (MSP) [123] , bisulfite pyrosequencing [124] and bisulfite cycle-sequencing [125, 126] on tumor samples from different body sites. Table 7 summarizes the sequence of the primers used for DNA methylation analyses. designed with the PyroMark Software Assay.

MS-PCR was applied to analyse *MLH1* methylation status following the original method developed by Herman *et al.* [123]. Bisulfite modification of genomic DNA (300 ng) was performed with an EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Bisulfite-converted DNA was amplified separately using the primers specific for methylated and unmethylated sequences of *MLH1* (Table 7). A single round of fluorescent PCR was performed using 5 μl of the bisulfite-converted DNA in a 15 μl reaction containing 1.5 μl of 10X buffer (Roche, Mannheim, Germany), 0.3 μM primer pairs, 200 μM dNTPs and 2U DNA polymerase (Roche, Mannheim, Germany). Thermal cycling conditions were: 5 min at 95°C, 10 cycles of 94°C/50 s, specific T annealing/50 s, 72 °C/50 s and 25 cycles of 89°C/30 s, specific T annealing/30 s and 72°C/30 s. The fluorescently labeled PCR products were electrophoresed on an Applied Biosystems 310 automated DNA sequencer (Applied Biosystems, Milan, Italy) and the fluorescent signals from the differently sized alleles were recorded and analyzed using Genescan software (version 2.1) (Applied Biosystems, Milan, Italy). All MSP data have been obtained from at least two independent modifications of DNA and we scored as positive only signals detectable in repeated experiments. Bisulfite pyrosequencing and bisulfite cycle-sequencing were used to confirm methylation patterns of *THBS-1, WT-1, MGMT* and *CASP-8* genes using primers designed by PyroMark Assay Software (Table 7).

PCR products for *THBS1* and *MGMT* genes were analysed by pyrosequencing using PyroGold reagents on a PyroMark Q96 ID system (Qiagen). The PCR product was bound to Sepharose HP (Amersham Biosciences, Uppsala, Sweden) and the Sepharose beads containing the immobilized PCR product were prepared for pyrosequencing according to the manufacturer's instructions. Pyrogram outputs were analyzed by the Pyromark Q24 software using the Allele Quantification software

(Qiagen) to determine the percentage of methylated alleles at each CpG site. Aberrant methylation of *THBS1* and *MGMT* was scored when the mean percentage value of methylated alleles was higher than 10%. This threshold value corresponded to the mean percentage of methylated alleles plus 3 times the standard deviation observed in 10 normal unmethylated DNA samples.

PCR products for *WT-1* and *CASP-8* genes were analysed by cycle-sequencing using Big Dye Terminator mix version 1.1, on ABI 310 (Applied Biosystems, CA, USA). Sequencing electropherograms were analysed by Sequencing Analysis software (version 5.3, Applied Biosystems, CA, USA).

Table 7. Validation of MS-MLPA results using methylation-specific PCR (MSP), bisulfite pyrosequencing, bisulfite cycle-sequencing.

GENE	Method	Primer sequences	Number of samples examined	Overall concordance with MS-MLPA results
MLH1	Methylation-Specific PCR (MSP)	MET-Fw: 5'HEX-ACGTAGACGTTTTATTAGGGTCGC-3' MET-Rv: 5'-CCTCATCGTAACTACCCGCG-3' UNMET-Fw: 5'FAM- TTTTGATGTAGATGTTTTATTAGGGTTGT-3' UNMET-Rv: 5'-ACCACCTCATCATAACTACCCACA-3'	102	95% (97/102)
THBS-1	Pyrosequencing	Fw: 5'-GTTTATTGGTAGGAGGAATTTTTAGGAA-3' Rv: 5' biotin -CCCTAAACTCCCAAACCAACTC-3' Seq: 5'-AGGAATGAGAGAGTTTTTTTAAAAG-3'	30	90% (27/30)
MGMT	Pyrosequencing	Fw: 5'biotin-GGATATGTTGGGATAGTT-3' Rv: 5'-AAACTAAACAACACCTAAA-3' Seq: 5'-CCCAAACACTCACCAAA-3'	96	96% (92/96)
WT-1	Cycle Sequencing	Fw: 5'-GGTTAAGAAGGGGAGGTGG-3' Rv: 5'-AACAACCTCCTCTTCAACC-3'	30	100% (30/30)
CASP-8	Cycle Sequencing	Fw: 5'-TGGAGTTAGTATAAATGTTTTTTAATAAAG-3' Rv: 5'-ACCCAATTTCCAACCATTCA-3'	30	100% (30/30)

LINE-1 PCR and pyrosequencing

The methylation status of LINE-1 was evaluated on the 58 PanNETs and eight samples of normal pancreas by using bisulfite-PCR and pyrosequencing LINE-1 assay was designed toward a consensus LINE-1 sequence (GenBank accession number M80343.1) and allowed to quantify the percentage of 5-methylated cytosines (%5mC) in five consecutive CpG sites (Figure 4). PCR was performed in a 50 µl reaction volume that included: 2 pmol of forward primer, 2 pmol of reverse biotinylated primer, 5 ul of bisulfite-treated genomic DNA, 1.25 Units of GoTaq DNA polymerase, 1X GoTaq Flexi Buffer (Promega, Madison, WI, USA), 200 µM dNTPs. Thermal cycling conditions were: 3 min at 95°C, 45 cycles at 95 °C/25 s, 50°C/25 s, 72 °C/25 s followed by a final extension at 72°C for 5 min.

Fully methylated and unmethylated DNA (Millipore, Billerica MA, USA) were used as positive and negative controls in each experiment. Reproducibility was confirmed by analyzing all the samples in duplicate with a maximum of within-sample coefficients of variation equal to 5% (range 2%-5%).

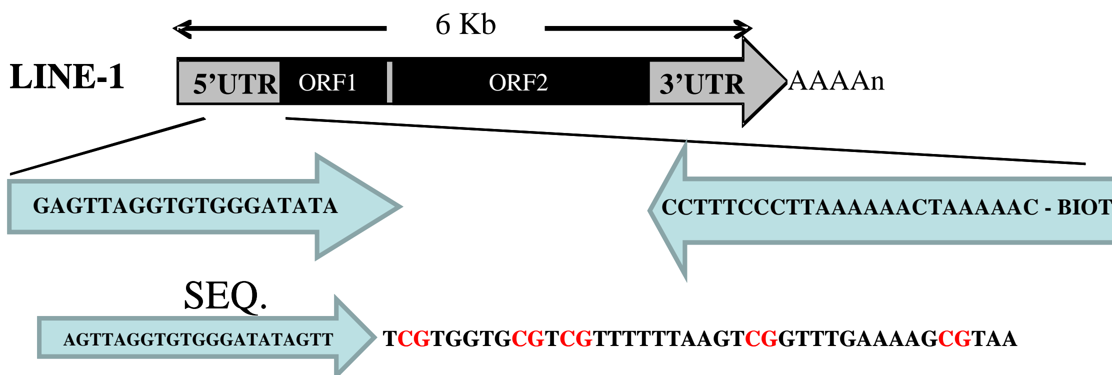


Figure 4: Diagram of the pyrosequencing assay used to measure LINE-1 promoter methylation in PanNETs and samples of normal pancreas. Arrows indicate the sequence and the position of the primers used for the bisulfate PCR (145 bp) and pyrosequencing. The percentage of 5-methylated cytosines was measured in the five consecutive CpG sites highlighted in red.

Statistical analysis

Methylation matrix was generated codifying as 1/0 the presence/absence of methylation of a specific gene in a specific patient. Then, the final matrix has as many rows as the number of patients and as many columns as the number of genes. A two way agglomerative cluster analysis (on genes and patients) has been performed on this matrix using complete linkage and Jaccard index distance metric. Given the dichotomous nature of our data Jaccard index was selected as the best dissimilarity measure. Three different groups of patients were emerged from the clustering. Chi-squared tests were applied to identify possible associations between these groups and all the clinical variables. To investigate if these three groups had different survival rates we performed univariable and multivariate survival analysis. Specifically in the univariable case we used Kaplan-Mayer survival curves estimation followed by the log-rank test, while in the multivariate analysis we used the Cox's proportional hazard model. In both cases we found a statistically significant difference among groups. Anova test was performed to evaluate the difference in the percentage of CNAs and of LINE-1 methylation among the three groups.

RESULTS

Validation of MS-MLPA

Validation of MS-MLPA results was possible for *MLH1, MGMT, THBS1, WT-1, CASP-8*, and *CDKN2A (p16)* genes, using additional independent techniques: methylation-specific PCR (MSP), bisulfite pyrosequencing, bisulfite and cycle sequencing. As is evident from the comparative analyses reported in Table 7, there was a good concordance between MS-MLPA and the other three methods based on bisulfite conversion of DNA. In particular, comparing the MS-MLPA and MSP methods, there was agreement between the two approaches in 95 % of the samples (97 of 102 cases). All five discordant samples showed methylation of *MLH1* using MSP but were negative in the MS-MLPA analysis. Bisulfite cycle sequencing confirmed MS-MLPA results in 100 % of tumor samples analyzed for *WT-1* and for *CASP-8* methylation. Analogously, MS-MLPA and bisulfite pyrosequencing showed good concordance in *MGMT* and *THBS1* methylation analysis, confirming the same results in 96 % and in 90 % of cases, respectively. The only four discordant cases were included among the five discordant samples comparing MS-MLPA and MSP methods: they were MSI tumors, negative for the protein expression, methylated using MSP method but negative in MS-MLPA analysis.

DNA hypermethylation profiling identifies different prognostic clusters of PanNETs

MS-MLPA analysis of the 58 PanNETs reported in Figure 5 demonstrated that a subset of 16 PanNETs (28%) was characterized by a concerted hypermethylation at a large number of genes (more than 25% of the 33 promoters examined) and that this phenotype was positively correlated with poor prognosis (p=0.02). Unsupervised hierarchical clustering provided the best performance in subtype classification of PanNETs distinguishing three specific methylation profiles that were strongly associated with prognosis at univariable analysis (p=0.004; Figure 5). Cluster 3 (20 patients) was positively correlated with poor prognosis compared to cluster 1 and cluster 2 (13 and 25 patients, respectively) and showed significant higher levels of methylation in the following genes: *DAPK1, TIMP3, PAX5, HIC1, CADM1, PYCARD, ESR1, VHL, RARB, WT1* (p<0.001). Samples in cluster 1 displayed a very homogeneous profile being almost completely unmethylated, while PanNETs in cluster 2 showed high frequency of hypermethylation in few genes, such as *CASP8, GSTP1* and *RASSF1*. Of note, these three genes were commonly methylated also in cluster 3.

Receiver operating characteristic (ROC) curves were generated showing the sensitivity versus 1-specificity of all the methylated genes in order to define the genes that most significantly distinguished PanNETs in cluster 3 from PanNETs in clusters 1 and 2. The areas under the ROC curves (AUCs) ranged from 0.96 to 1.00 for the best combinations of genes (Figure 6). In particular, hypermethylation of *DAPK1*, *TIMP-3* and *PAX5* exhibited 100% sensitivity and 100% specificity in identifying PanNETs in cluster 3.

We next analysed the clinico-pathological profiles of the three DNA methylation clusters and Table 3 summarizes the tumor characteristics in each class. There were no statistically significant differences in age, gender, tumor size, proliferation index, degree of differentiation among the three clusters. However, consistent with the higher mortality of cluster 3 patients, there was a significant difference in the proportion of stages IV in cluster 3 compared with cluster 1 and 2 (33% *versus* 4% of patients, respectively; p=0.04) Moreover, cluster 3 included mainly nonfunctioning PanNETs and fewer insulinomas compared with cluster 1 and 2 (p=0.08).

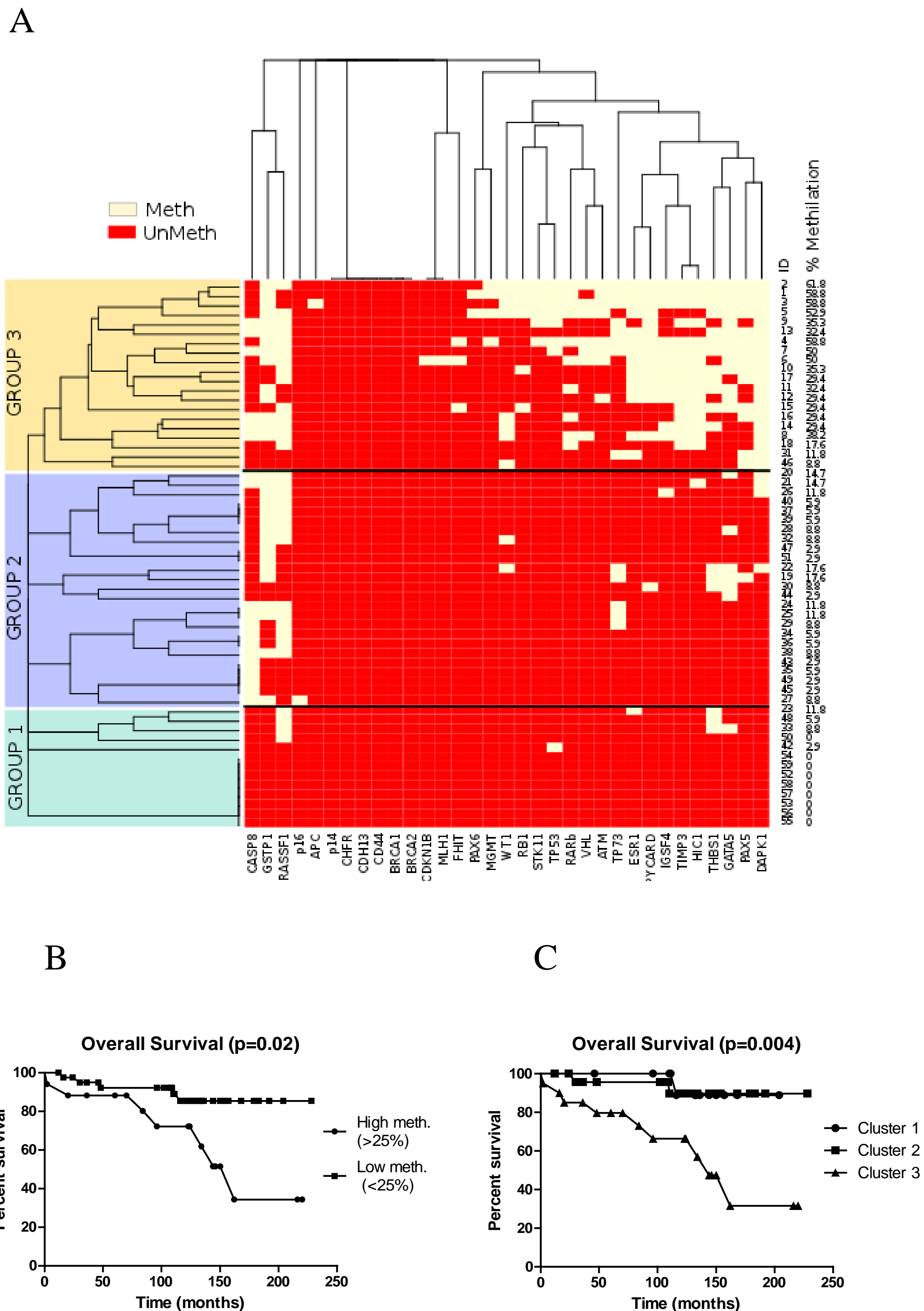


Figure 5: Unsupervised hierarchical clustering of the 58 PanNETs according to their methylation profiles. A) Heatmap with cases orientated along the horizontal axis and genes orientated along the vertical axis. B) Kaplan-Meier curve indicating that PanNETs with high levels of gene methylation (more than 25% of the 33 promoters examined) show worse prognosis than tumors with low levels of methylation (P=0.02). C) Kaplan-Meier curve indicating that Cluster 3 was positively correlated with poor prognosis compared to cluster 1 and cluster 2

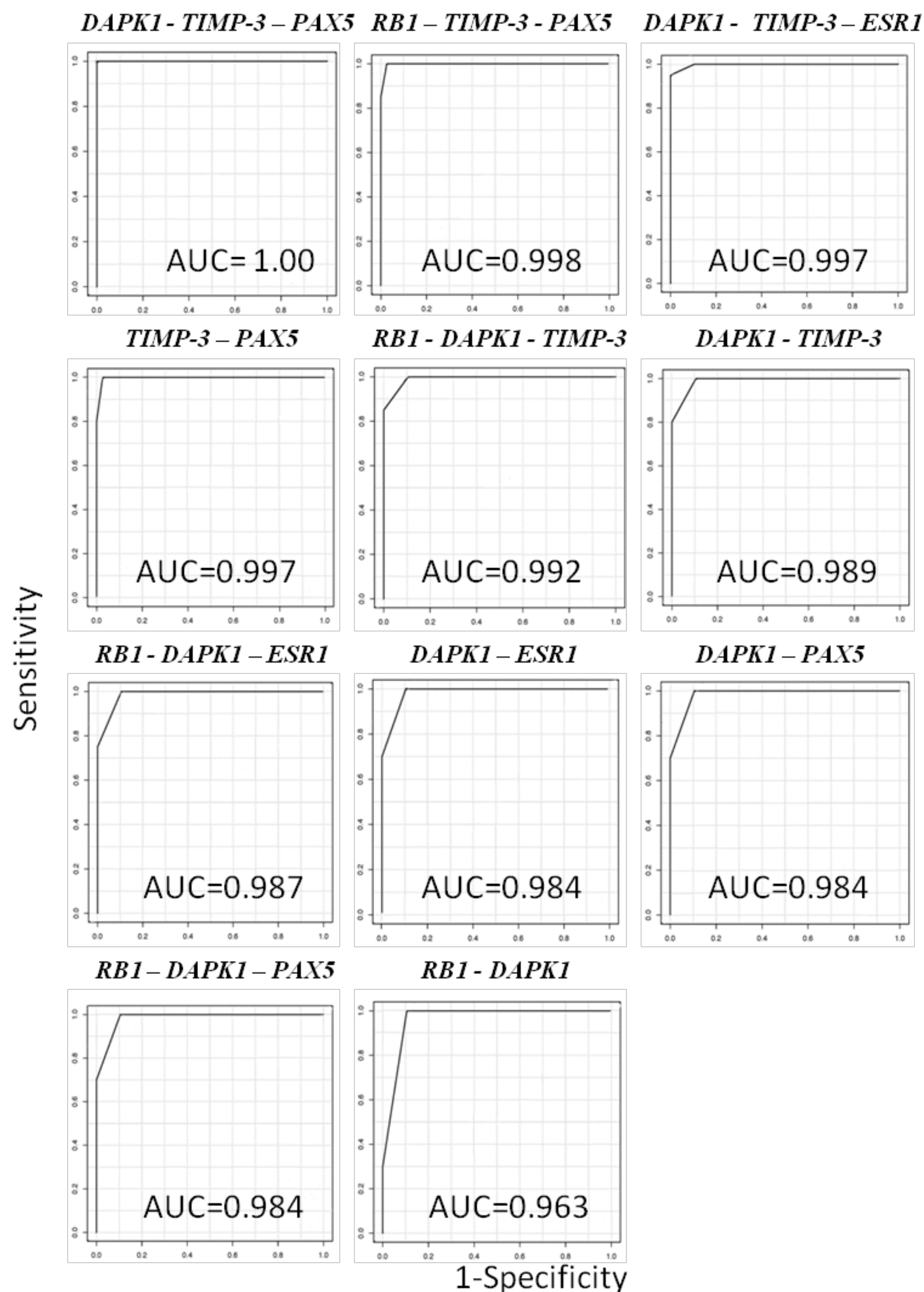


Figure 6: Receiver operating characteristic (ROC) curves were generated to define the genes that most significantly distinguished PanNETs in cluster 3 from PanNETs in clusters 1 and 2. Sensitivity, the true positive rates, is shown along the y-axis, while specificity, or the false positive rate, is shown along the x-axis. The calculated AUC is given for each plot in rank order beginning with highest AUC.

Table 8. Clinicopathologic features of different cluster tumor groups

	No	Sex	Age		Genetic		Syndrome						Diamerer (cm)		Mit.	Ki67	Grading			Staging				Follow-up			
		F/M	mean	range	MEN1	Sporadic	NF	INS	GLUC	ZES	VIP	ACTH	mean	range			G1	G2	G3	I	II	III	IV	AFD	AWD	DOD	DOC
Cluster 1	13	7/6	45	32-65	1	12	5	7	0	0	1	0	3.3	0.3-11	<1	2.9%	8	5	0	1	5	2	0	11	1	1	0
Cluster 2	25	14/11	50	18-79	1	24	10	12	2	1	0	0	4.8	1.4-18	2.28	4.5%	12	13	0	5	7	7	1	17	4	2	2
Cluster 3	20	8/12	50	30-76	0	20	13	4	0	1	1	1	5.5	1.5-11	2.4	3.75%	8	12	0	1	8	3	6	9	1	9	1

F: female; M: male; NF: nonfunctioning; INS: insulinoma; GLUC: glucagonoma; ZES: Zollinger-Ellison Syndrome; VIP: VIPoma; ACTH: Cushing's syndrome; Mit: mitoses X 10HPF; AFD: alive free of disease; AWD: alive with disease; DOD: died of disease; DOC: died of other cause.

DNA hypermethylation clusters and LINE-1 hypomethylation

We used the quantitative bisulfite pyrosequencing to determine the methylation status of LINE-1 repetitive sequences in the 58 PanNETs compared to eight samples of normal pancreas (Figure 7). In normal samples little variation in LINE-1 methylation was observed. By contrast, PanNETs exhibited a high variability in LINE-1 methylation ranging from 44.5% to 70%. Although overall lower levels were observed in tumors compared with normal samples (average 60.2 % ± 4.7% *versus* average 64.3 % ± 0.9 %, respectively), differential LINE-1 hypomethylation levels were observed among the three hypermethylation clusters of PanNETs as exemplified in Figures 2 and 3. Cluster 1 was a relatively homogeneous group showing no significant LINE-1 hypomethylation compared to normal pancreas. By contrast, both cluster 2 and 3 showed a high variability in LINE-1 methylation although important differences were found between the two groups. Cluster 2 exhibited the lowest levels of LINE-1 methylation both comparing these tumors with normal pancreas (p=0.009) and with PanNETs in cluster 1 and 3 (p=0.02). Cluster 3 showed on average a modest degree of LINE-1 hypomethylation. Most tumors in this group showed an increase in LINE-1 methylation compared to normal pancreas and only four cases displayed low levels of LINE-1 methylation similarly to those observed in cluster 2 (Figure 2A). Notably, regardless of DNA hypermethylation clusters, decreased levels of LINE-1 methylation were significantly correlated with advanced stage (61.7% ± 3.6 in I and II stages *versus* 57.4% ± 5.5 in III and IV stages; p=0.002) and poor prognosis (p<0.0001) (Figure 7B). To determine whether LINE-1 methylation status was an independent prognostic factor all variables which were significant in univariate analyses, i.e. grading (p=0.01), Ki67 proliferative index (p=0.02), ENETS Stage (p=0.0002) and DNA hypermethylation clusters (p=0.004) were included in the multivariate analysis, which was conducted using the Cox proportional hazards model. The analysis revealed that low LINE-1 methylation status was an independent prognostic factor of poor prognosis (p 0.006), instead DNA hypermethylation clusters showed a borderline significance (p=0.08). No other independent prognostic factor was found (Table 9).

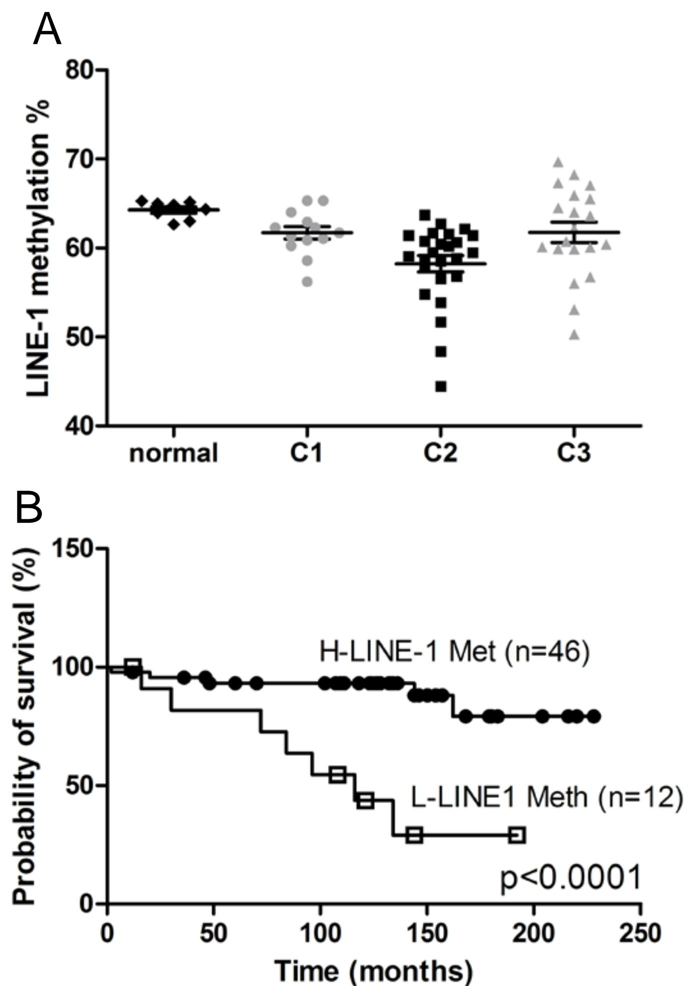


Figure 7: A) Quantitative LINE-1 methylation analysis in normal pancreas and in the three different clusters of PanNETs (C1: cluster 1; C2: cluster2; C3: cluster 3). Scatter plot comparison show significant lower levels of LINE-1 methylation in cluster 2 compared to normal pancreas (p=0.009) to cluster 1 and 3 (p=0.02). B) Kaplan Meier surve indicating that decreased levels of LINE-1 methylation are significantly correlated with poor prognosis. Patients were stratified into high (H-LINE-1 Meth.) and low (L-LINE-1 Meth.) groups using 58% as LINE-1 percentage.

Table 9. Multivariate analysis for the prognostic significance of clinico-pathologic factors and DNA methylation in PanNETs

Variable	HR	95% CI	p-value
LINE-1 methylation	0.18	0.05 - 0.61	0.006
Methylation clusters	3.00	0.8 - 10.7	0.08
Ki67 index	1.05	0.9 - 1.2	0.34
ENETS stage	1.5	0.4 - 5.6	0.50
Grade	1.2	0.3 – 3.9	0.80

Legend: CI, confidence interval; HR, hazard ratio

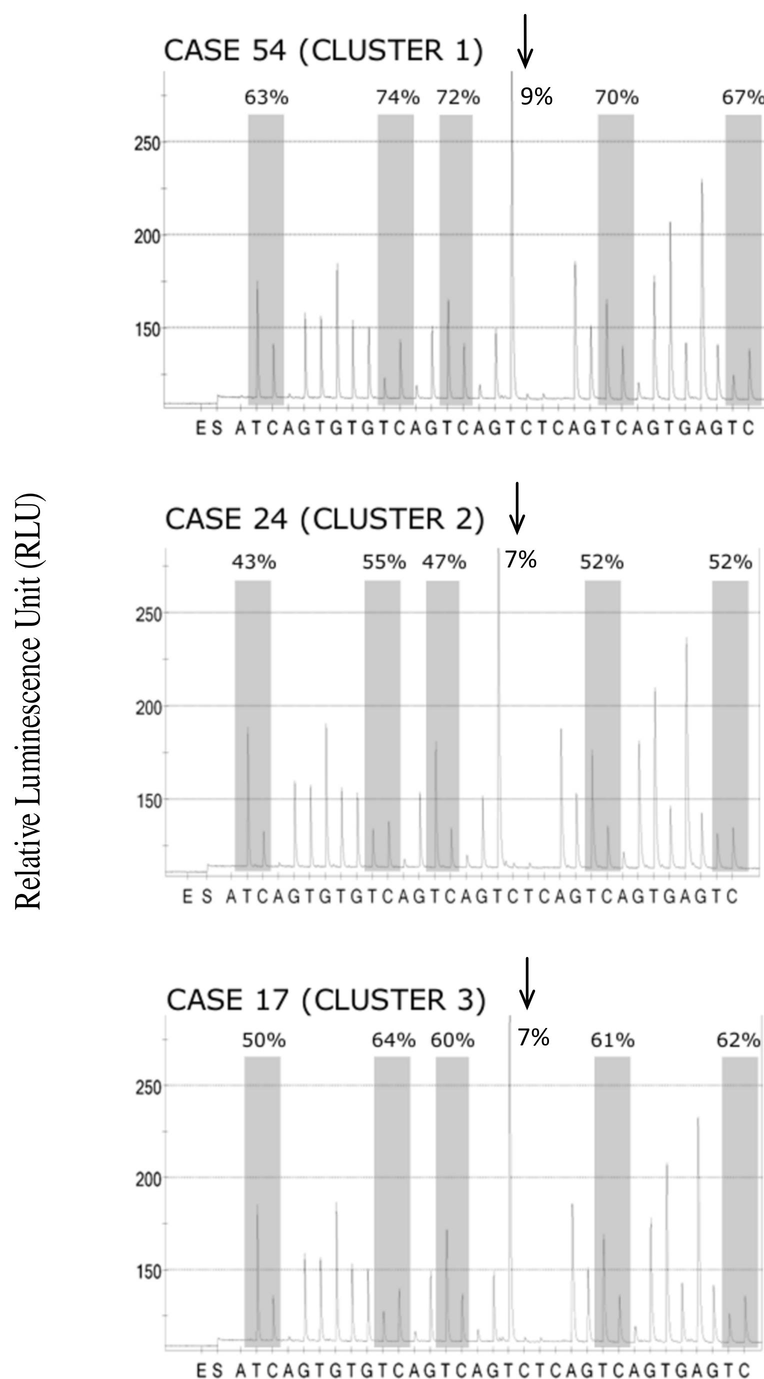


Figure 8: Examples of pyrograms for LINE-1 quantitative methylation analysis in three representative cases from the three methylation clusters. At each CpG site the proportion of C (%) after bisulfate conversion is reported. The overall LINE-1 methylation level was calculated as the average of the proportions of C (%) at the five CpG sites. Black arrows point the internal control cytosine residues that check for the adequacy of bisulfate treatment in the pyrosequencing analysis.

DNA hypermethylation clusters and CNAs

CNA analysis at 53 different chromosome loci was possible in 47 PanNETs. An average of 14 (27%; range 2% to 51%) probes per sample showed abnormal patterns and an overview of the distribution of CNAs is shown in Table 10. A significant higher frequency of gains was observed compared with losses (mean number of 14 gains versus 1 loss per case, $p \leq 0.001$). TIMP-3 was the most frequently affected gene, followed by *CDKN1B, VHL, PTCH1, CHFR, CADM1, CFTR, STK11, THBS1, p14 CDKN2A, CDKN2B* and *PAX6*.

Table 10. Copy number Alteration (CNA) profiles at 53 different chromosome loci of 47 PanNETs

Locus	Gene	CNA %
4q26-q27	*IL-2*	0
12q22-q24.2	*PAH*	0
19q13.41	*KLK3*	0
13q12-q13	*BRCA2*	0
10p12.1-p11.	*CREM*	0
10p13	*CELF2*	2
18q21.3	*BCL2*	4
3p21	*CTNNB1*	6
2q33-q34	*CASP8*	6
16q22.1	*CDH1*	6
7q21-q22	*CDK6*	6
3p24	*RARB*	6
14q24.3	*MLH3*	9
6q25.2-q27	*PARK2*	9
16q23.3	*CDH13*	9
16p13.3	*TSC2*	9
11p13	*CD44*	11
17q21.31	*BRCA1*	13
3p22.3	*MLH1*	13
16p11.2	*PYCARD*	13
11p13	*WT1*	13
5q21-q22	*APC*	15
12p13.2	*TNFRSF1A*	17
3q21.1	*CASR*	19
6q24-q27	*ESR1*	19
10q23	*PTEN*	19
2p16	*MSH6*	21
9p13.2	*PAX5*	23
1p36.3	*TP73*	23
3p14.2	*FHIT*	23
17p13.1	*TP53*	23
11q22-q23	*ATM*	26
17p12	*PMP22*	26
3p21.3	*RASSF1*	26
11q13.2	*GSTP1*	28
13q14.2	*RB1*	28
20q13.33	*GATA5*	34
10q26	*MGMT*	34
17p13.3	*HIC1*	34
9p21	*CDKN2A p16*	38
9q34.1	*DAPK1*	47
11p13	*PAX6*	51
9p21	*CDKN2B*	55
9p21	*CDKN2A p14*	57
15q15	*THBS1*	57
19p13.3	*STK11*	60
7q31-q32	*CFTR*	60
11q23.2	*CADM1*	64
12q24.33	*CHFR*	66
9q22.1-q31	*PTCH1*	70
3p25.3	*VHL*	72
12p13.1-p12	*CDKN1B*	81
22q12.3	*TIMP-3*	87

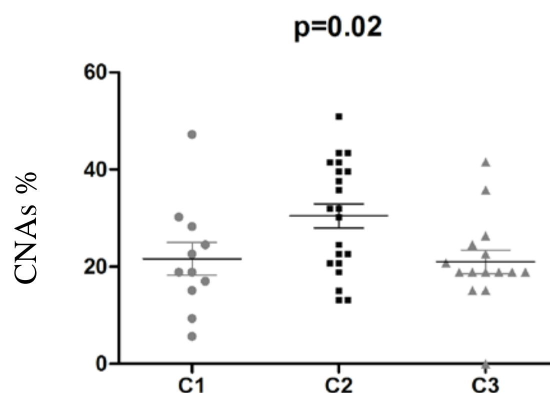
Nr.	CNA %	LINE-1 %	Meth. Cluster
47	51	61	2
48	49	61	1
20	47	62	2
30	47	52	2
1	43	60	3
22	43	48	2
24	43	55	2
49	43	60	2
39	40	59	2
34	38	62	2
38	38	59	2
46	38	66	3
25	34	61	2
27	34	60	2
51	34	57	2
14	30	60	3
33	30	62	1
56	30	65	1
8	26	67	3
57	26	62	1
58	26	64	1
2	25	64	3
32	25	54	2
36	25	64	2
45	25	57	2
4	23	60	3
10	23	53	3
16	23	57	3
18	23	61	3
37	23	61	2
5	21	65	3
11	21	62	3
26	21	58	2
28	21	61	2
52	21	65	1
59	21	63	1
55	19	56	1
3	17	67	3
6	17	60	3
13	17	60	3
29	17	59	2
23	15	61	1
40	15	45	2
35	13	61	2
50	9	60	1
53	6	59	1
17	2	50	3

Legend: analytic description of presence (black cells) or absence of CNA (white cells). Genes are sorted from right to left according to CNA frequency. For each case LINE-1 Methylation percentage and hypermethylation cluster are shown.

We evaluated the CNA percentages in the three hypermethylation clusters and found that cluster 2 PanNETs exhibited significant higher levels of CNAs with respect to cluster 1 and 3 tumors (Figure 9A; p=0.02). In particular, CNAs at six specific loci were more frequently observed in cluster 2 than in cluster 1 and 3 and included *RB1* (p=0.0002), *CADM1* (p=0.0002), *PAX6* (p=0.005), *PTCH1* (p=0.01), *CHFR* (p=0.02) and *THBS1* (p=0.04) (Figure 9B).

High CNA percentages were positively associated with tumor size (p=0.02), G2 grading (p=0.02) and Ki67 Index (p=0.04). No other association with clinico-pathologic features was observed.

A)



B)

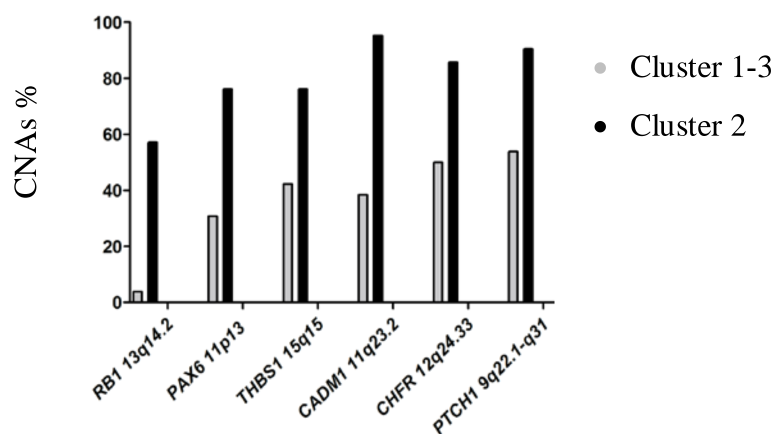


Figure 9: Copy number alterations (CNA) analysis in PanNETs. A) Scatter plots show significant higher CNA percentage in cluster 2 (C2) compared with cluster 1 and 3 (C1 and C3, respectively). B) The histogram shows the six chromosomal regions at wich significantly higher CNA frequencies were observed in cluster 2 compared to cluster 1 and 3.

DISCUSSION

This is the first work addressing DNA methylation profiling of PanNETs and its correlation with CNAs. For this purpose, we chose to study a well-characterized series of PanNETs previously examined [13], in order to correlate their clinicopathologic profiles and follow up data with specific patterns of both DNA aberrant methylation and CNAs.

Firstly, our study demonstrates that the simultaneous methylation analysis of a large number of tumour suppressor genes with a broad spectrum of cellular functions and frequently affected by *de novo* methylation in cancer is a powerful method for classification of PanNETs and allows the identification of specific methylation profiles related to different clinical behaviour. In particular, we found that a subgroup of PanNETs was characterized by a concerted hypermethylation at multiple loci, reminiscent of the CpG island methylator phenotype (CIMP) described in colorectal cancers [118]. Even if this phenotype was associated with worse prognosis in our series ($p=0.02$), unsupervised hierarchical clustering analysis provided the best performance in subtype classification of PanNETs, resulting in a clear separation into three prognostically and biologically different groups. Cluster 1 showed almost complete absence of gene methylation while PanNETs in cluster 2 showed high frequency of hypermethylation in few genes, such as *CASP8, GSTP1* and *RASSF1*. By contrast, cluster 3 displayed a hypermethylated profile compared with the other two groups and showed significant higher frequencies of methylation in a large number of genes, namely *DAPK1, TIMP-3, PAX5, HIC1, CADM1, PYCARD, ESR1, VHL, RARB, WT1* ($p<0.001$). Especially in combination, these markers were able to distinguish reliably this subset of PanNETs from Cluster 1 and Cluster 2 tumors. In particular, combining

DNA hypermethylation of *DAPK1, TIMP-3* and *PAX5* resulted in both sensitivity and specificity of up to 100% in the detection of this specific profile.

PanNETs in cluster 3 exhibited a more aggressive clinical behaviour than tumours in cluster 1 and 2 because they were associated with advanced stage (p=0.04) and poor prognosis (p=0.004). Moreover, cluster 3 included mainly nonfunctioning PanNETs and fewer insulinomas compared with cluster 1 and 2 (p=0.08). Overall these results strongly suggested a role of *de novo* DNA methylation in the progression of PanNETs through simultaneous involvement of multiple genes governing cell differentiation, apoptosis and invasion. In line with our results a positive association of gene hypermethylation with adverse clinical behaviour in PanNETs has been reported in a small number of studies analysing single or few candidate genes [114],[38, 79, 107]. Moreover, recent data support the hypothesis that the impairment of epigenetic pathways is involved in the pathogenesis of these tumours. Recently, whole-exome sequencing of PanNETs demonstrated that the most frequently mutated genes, i.e. *MEN1, DAXX* and *ATRX*, specify proteins implicated in chromatin remodelling suggesting that a primary aberration of epigenetic mechanisms may be a major event in PanNETs [34]. Consistent with this concept are reports showing that specific epigenetic changes, including hypermethylation of *RASSF1A* gene and DNA methylation changes of the *IGF2* Differentially Methylated Region 2, are early and common events in PanNETs [127, 128]. Here, we confirm this observation reporting hypermethylation of *RASSF1A*, *CASP8* and *GSTP1* in most of the tumours examined and we suggest that aberrant DNA methylation may be crucial both to development and to progression of PanNETs.

Besides gene-specific methylation, global CpG methylation has also been characterized in the same tumour set providing a link between DNA methylation profiles and CNAs in PanNETs.

Recent studies showed global hypomethylation in pancreatic and intestinal neuroendocrine tumours compared with normal tissue using LINE-1 as a surrogate for genome-wide methylation [119, 120]. Although several studies reported that DNA hypomethylation correlates with chromosomal instability in a variety of cancers [50, 51, 129, 130], at present it is poorly understood whether these two alterations are linked in PanNETs. A second important and unsolved question is whether genome-wide hypomethylation and gene specific hypermethylation are two independent events or if they are mechanistically linked in cancer. Attempts to answer this question resulted in contradictory findings, with some groups supporting [62, 131, 132] and other refuting [133, 134] a link between the two alterations.

In our study we found a group of highly hypomethylated PanNETs that largely overlap with the cluster 2 tumours exhibiting low levels of gene hypermethylation. Notably, these cases showed the highest degree of CNAs with six chromosome regions more frequently involved in this group compared with the other two clusters. By contrast, in average a modest degree of LINE-1 hypomethylation was observed in cluster 1 and 3 tumors, most of which showed low levels of CNAs.

These findings confirm previous data reported for other cancer sites [135-138] and demonstrate that a significant fraction of PanNETs may arise through a possible mechanism linking DNA hypomethylation to subsequent chromosomal alterations, due the propensity of undermethylated DNA to recombine with a higher frequency than normal [139, 140]. Moreover, our results suggest that PanNETs may arise from two

main distinct progression pathways: one through a progressive accumulation of epigenetic demethylation errors resulting in a variety of chromosomal rearrangements and the other one under pressure to *de novo* methylation of both CpG island promoter and repetitive elements and the other. However, overlap in both processes sometimes occurs, as observed in four PanNETs in cluster 3 (8% of cases), indicating that a cross-talk between DNA demethylation and *de novo* methylation may be hypothesized [132].

Regardless of the underlying mechanisms, our results demonstrate that LINE-1 hypomethylation may increase the malignant potential of PanNETs and is strongly associated with shorter survival. Interestingly, multivariate analysis including well-known clinico-pathologic factors for PanNETs, showed that LINE-1 hypomethylation was the only independent significant predictor of outcome. Although this association needs to be validated in additional tumor cohorts as well as in large scale clinical studies, our results demonstrate that DNA hypomethylation may be considered as an additional powerful marker for prognostication of PanNETs.

In conclusion, our study shows that the combination of global DNA demethylation and gene hypermethylation analyses allows to define biologically and prognostically distinct subsets of PanNETs. Both alterations can be found in these tumors and each one can promote tumorigenesis by independent processes. Further delineating the nature and timing of epigenetic hits, which are in principle reversible, is potentially highly relevant for epigenetic therapy of PanNETs, and perhaps for tumor prevention.

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