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Epigenetic alterations in neuroendocrine tumors: methylation of *RAS-association domain family 1, isoform A* and *p16* genes are associated with metastasis

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Well-differentiated neuroendocrine tumors including pancreatic endocrine tumors and carcinoid tumors are uncommon neoplasms that have site-specific differences in clinicopathological features, clinical course and genetic alterations. The epigenetic alterations in these tumors are not well characterized. We therefore compared methylation of the *RAS-association domain family 1, isoform A* (*RASSF1A*), *p14*, *p16* and *O*^{6}*-methyl-guanine methyltransferase* genes in neuroendocrine tumors from 47 patients including 16 pancreatic, 15 nonileal and 16 ileal neuroendocrine tumors. Methylation of the *RASSF1A* gene was present in 57% of tumors, *p14* in 49%, *p16* in 26% and *O*^{6}*-methyl-guanine methyltransferase* in 13% of tumors. Ileal neuroendocrine tumors lacked methylation of *O*^{6}*-methyl-guanine methyltransferase* gene ($P=0.04$). *RASSF1A* methylation was associated with histopathologic type of tumors ($P=0.03$) and lymph node metastasis ($P=0.004$), and *p16* methylation with older patient age ($P=0.002$) and liver metastasis ($P=0.04$). Two or more genes were methylated in 53% of tumors, one gene was methylated in 30% of tumors, and all four genes were unmethylated in 17% of tumors. Methylation of one or more gene was associated with older age of patients ($P=0.01$), and methylation of two or more genes was associated with liver metastasis ($P=0.044$). Our study shows that in neuroendocrine tumors epigenetic alterations vary by tumor subsite and clinicopathologic features, including age of onset, histopatholoic type and metastasis status.
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Well-differentiated neuroendocrine tumors including pancreatic endocrine tumors and carcinoid tumors of the lung and gastrointestinal tract are uncommon,[1,2] with an age-adjusted annual incidence of 2.5–4.5 per 100 000.[3,4] These indolent malignancies have site-specific differences among well-differentiated neuroendocrine tumors from lung, gastrointestinal tract, and pancreas including clinicopathological features, behavior and genetic alterations.

The molecular mechanisms of neuroendocrine tumorigenesis are poorly understood but have been the focus of many recent reports.[5–11] We have previously shown methylation of *p14* (*ARF*), *p16* (*INK4a*) and *O*^{6}*-methyl-guanine methyltransferase* (*MGMT*) genes in well-differentiated neuroendocrine tumors from pancreas and gastrointestinal tract.[11]

A chromosome 9p21 locus encodes two cell cycle inhibitors, *p16* and *p14*, that are transcribed from two separate promoters and first exons (1α and 1β, respectively) joined through the same splice acceptor site to exon 2 coding sequences but in different reading frames.[12,13] p16 inhibition of cyclin-dependent kinase 4 (CDK4) causes retinoblastoma protein to stay in its active form and arrest the cell cycle at G1–S transition.[14,15] p14 also inhibits the cell cycle by blocking MDM2 inhibition of p53 activity.[16] Inactivation of the *p16* and *p14* genes has been reported for a variety of tumors.[15,17–19] Both *p16* and *p14* can be inactivated through gene mutation, heterozygous or homozygous deletion, and methylation of the CpG island in the promoter region. In

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pancreatic and gastrointestinal well-differentiated neuroendocrine tumors, methylation of the promoter region is the major mechanism for inactivation of these two tumor suppressor genes.[8]

MGMT is a DNA repair gene that is responsible for removal of mutagenic adducts from the O^6 position of guanine.[20] In cells lacking MGMT activity, O^6-alkyl-guanine mispairs with thymine during DNA replication and results in guanine–cytosine transition mutation. Methylation of CpG islands in the promoter regions of the *MGMT* gene can cause loss of transcription of the gene, and promoter methylation has been reported in many tumor types,[20] including pancreatic well-differentiated neuroendocrine tumors.[7]

RAS-association domain family 1 has seven different isoforms that are produced by alternative splicing and transcription from two different promoters with CpG islands.[21] RAS-association domain family 1, isoform A (*RASSF1A*) gene is a tumor suppressor gene in the RAS pathway that can regulate proliferation, induce apoptosis, and bind to and stabilize microtubules. *RASSF1A* gene is frequently methylated in a variety of tumors,[21] including pancreatic, pulmonary and gastrointestinal well-differentiated neuroendocrine tumors.[7,22–24]

In the present study, we studied well-differentiated neuroendocrine tumors from the pancreas, lung and gastrointestinal tract for methylation of *RASSF1A*, *p14*, *p16* and *MGMT* genes, and associated the epigenetic alterations with clinicopathologic features including metastasis status. We found associations with tumor site, histopatholoic type, patient age and metastasis that have implications for the pathogenesis of the tumors and clinical characteristics.

Materials and methods

Characteristics of Specimens and Patients

Frozen tumor and non-neoplastic tissue were obtained from surgical specimens of patients undergoing resections for well-differentiated neuroendocrine tumors in the frozen section laboratory of the Department of Pathology, MD Anderson Cancer Center. The MD Anderson Cancer Center Surveillance Committee (institutional review board) approved this study. The patient records and histopathological findings were reviewed. The tumors were classified as benign well-differentiated neuroendocrine tumors, well-differentiated neuroendocrine tumors of uncertain malignant potential, and well-differentiated neuroendocrine carcinomas using established criteria.[25] There were three benign well-differentiated neuroendocrine tumors, nine well-differentiated neuroendocrine tumors of uncertain malignant potential, and 35 well-differentiated neuroendocrine carcinomas (Figure 1). Of these well-differentiated neuroendocrine tumors, 16 were from pancreas (pancreatic endocrine tumors), and 31 were from lung and gastrointestinal tract (carcinoid tumors). Of the tumors, 16 were in the ileum. The nonileal tumors included five pulmonary, three gastric, two duodenal, one appendiceal, three cecal and one rectal tumor. The functional status of tumors was ascertained by serum measurements of hormones and/or clinical syndrome due to hormonal hypersecretion. There was one gastrinoma and one insulinoma. Two patients with pancreatic neuroendocrine tumors had multiple endocrine neoplasia type 1 (MEN1), and two patients with gastric well-differentiated neuroendocrine tumors had chronic atrophic gastritis of the body and fundus associated with Pernicious anemia.

DNA Extraction

DNA from both tumor and non-neoplastic tissue was extracted from microdissected fresh-frozen specimens using a commercial kit (Qiagen DNA extraction kit, Qiagen Inc., Valencia, CA, USA), after a hematoxylin- and eosin-stained slide from a frozen block was reviewed. The tumor cell cellularity was at least 90% in all samples examined. The non-neoplastic tissue was from the pancreatic or lung parenchyma, or normal mucosa from the primary site of tumor and had a minor component of neuroendocrine cells or islets of Langerhans. The tumor tissue was obtained from the liver metastases in one patient with pancreatic and five patients with ileal well-differentiated neuroendocrine carcinomas and the non-neoplastic tissue from the primary site was unavailable from these patients (Figure 1).

Methylation of *RASSF1A*, *p14*, *p16*, and *MGMT* Genes

Methylation of the *RASSF1A*, *p14*, *p16*, and *MGMT* genes was evaluated by bisulfite treatment of DNA followed by methylation-specific polymerase chain reaction (MSP), as previously described.[6,20,26–28] In brief, 2 μg of microdissected genomic DNA was denatured with 2 M NaOH at 37°C for 10 min, followed by incubation with 3 M sodium bisulfite, pH 5.0, at 50°C for 16 h in darkness. After treatment, DNA was purified using the DNA Cleanup Kit (Promega Corporation, Madison, WI, USA) as recommended by the manufacturer, incubated with 3 M NaOH at room temperature for 5 min, precipitated with 10 M ammonium acetate and 100% ethanol, washed with 70% ethanol, and finally resuspended in 20 μl of distilled water. *RASSAF1* gene methylation was determined by PCR amplification of unmethylated alleles by using primer pair: 5′-TTTGGTTGGAGTGTGTT AATGTG-3′ and 5′-CAAACCCCACAAACTAAA AACAA-3′, and methylated alleles by using primer pair: 5′-GTGTTAACGCGTTGCGTATC-3′ and 5′-AAC CCCGCGAACTAAAAACGA-3′.[28] PCR was carried

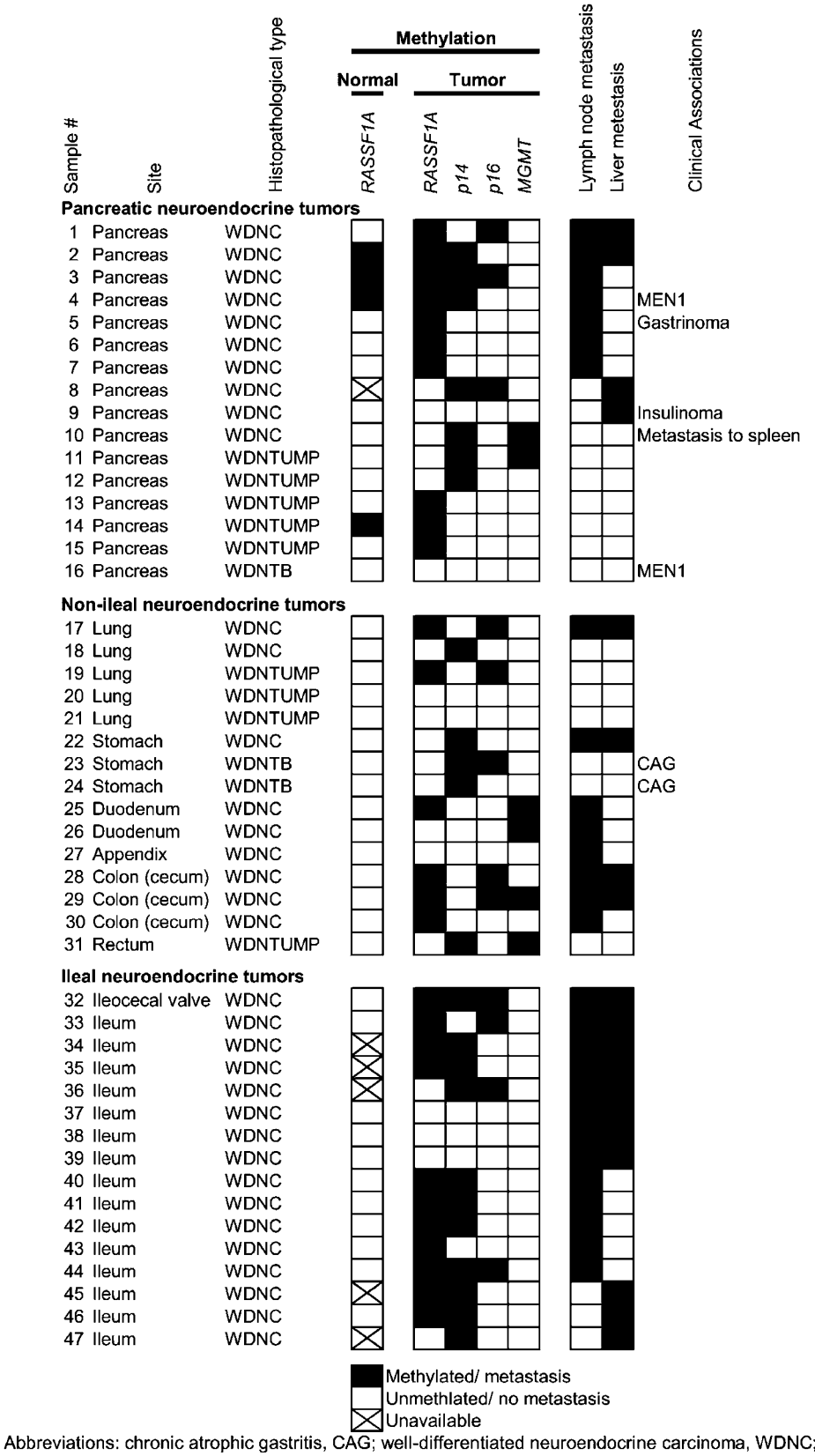

Figure 1 Methylation status of *RASSF1A*, *p14*, *p16* and *MGMT* genes in pancreatic, nonileal and ileal neuroendocrine tumors, histopatholoic type, and lymph node and liver metastasis status.

out using the following conditions: denaturation at 95°C for 15 min, 35 cycles (95°C for 30 s, 60°C for 30 s, 72°C for 45 s) and extension at 72°C for 10 min. The primers and PCR conditions for the *p14*, *p16* and *MGMT* genes are as previously reported.[20,26,27] The colon cancer cell line RKO (American Type Culture Collection, Manasas, VA, USA) and water were used as positive and negative controls, respectively. PCR products from methylated and unmethylated reactions were electrophoresed on 6% acrylamide gels and visualized by ethidium bromide staining (examples in Figure 2). Samples with low level of methylation (less than 5% methylated alleles compared to unmethylated alleles were designated as unmethylated based on previous studies.[6] The methylation status of *p14*, *p16* and *MGMT* genes of 12 of the tumors has been reported previously.[6]

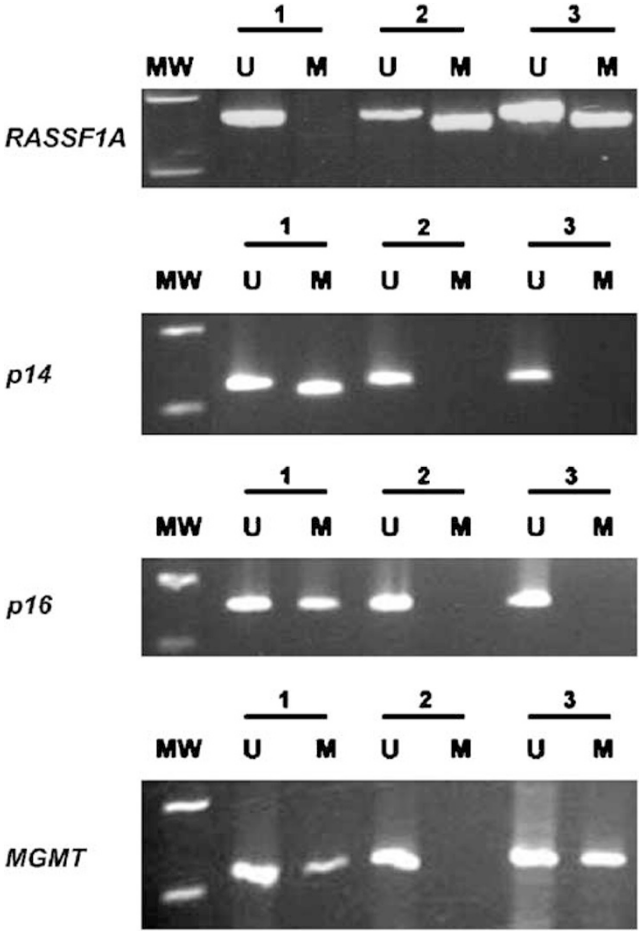

Figure 2 Methylation-specific PCR for *RASSF1A*, *p16*, *p14* and *MGMT* genes using unmethylated (U) and methylated (M) primers in three representative samples. Sample numbers and molecular weight (MW) is on the top of the lanes. Sample 1 is unmethylated and samples 2 and 3 are methylated for *RASSF1A* gene, samples 1 is methylated and samples 2 and 3 are unmethylated for *p14*, sample 1 is methylated and samples 2 and 3 are unmethylated for *p16*; and samples 1 and 3 are methylated and samples 2 is unmethylated for *MGMT*.

Statistical Analysis

All statistical analysis was performed using SPSS (SPSS Inc., Chicago, IL, USA). Comparisons of categorical variables were made using χ^2 and Fisher's exact test. Continuous data, including age at diagnosis and tumor size, were evaluated by Student's *T*-test and one-way ANOVA.

Results

Clinicopathologic Features

The clinicopathological features of the 16 patients with pancreatic well-differentiated neuroendocrine tumors, 15 with nonileal well-differentiated neuroendocrine tumors and 16 with ileal well-differentiated neuroendocrine tumors have been previously reported.[11] Liver metastasis was present in 69% of patients with ileal neuroendocrine tumors compared to 27% with nonileal neuroendocrine tumors and 25% with pancreatic neuroendocrine tumors ($P=0.02$). Age, gender, size of tumor, and vital status were not statistically different among the three groups.

Methylation of *RASSF1A*, *p14*, *p16*, and *MGMT* Genes

Methylation of the *RASSF1A* gene was present in non-neoplastic pancreatic parenchyma of 26% (4/15) patients with pancreatic neuroendocrine tumors, but was unmethylated in non-neoplastic lung parenchyma from five patients, and three gastric, two duodenal, eight ileal and four colorectal (including one from the patient with appendiceal neuroendocrine tumor) non-neoplastic mucosal samples from patients with nonileal and ileal neuroendocrine tumors. Methylation of *p14*, *p16* and *MGMT* genes is infrequent in non-neoplastic pancreas or gastrointestinal mucosa, as reported in our previous study.[6]

Methylation of the *RASSF1A* gene was present in 57% (27/47) of tumors, of the *p14* gene in 49% (23/47), of the *p16* gene in 26% (12/47), and of the *MGMT* gene in 13% (6/47) of tumors (Figure 1 and Table 1). Pancreatic, nonileal and ileal neuroendocrine tumors had methylation of *RASSF1A* gene in 63, 40 and 69% of tumors, respectively (not significant), *p14* gene in 44, 33 and 69% (not significant), *p16* gene in 19, 33 and 25% (not significant), and *MGMT* gene in 13, 27 and 0% ($P=0.04$).

Epigenetic Alterations and Clinicopathologic Features

The mean age of patients whose non-neoplastic pancreatic parenchyma had *RASSF1A* gene methylation was 54.2 ± 15.4 years, compared to 55.8 ± 17.2 years for those whose non-neoplastic pancreatic parenchyma was unmethylated (not significant), and 58.7 ± 13.1 years for those whose non-neoplastic

Table 1 Frequency of methylation of *RASSAF1A*, *p14*, *p16* and *MGMT* genes in pancreatic, nonileal and ileal neuroendocrine tumors

Methylation	*Pancreatic neuroendocrine tumors* (n = *16) % (no.)*	*Nonileal neuroendocrine tumors* (n = *15) % (no.)*	*Ileal neuroendocrine tumors* (n = *16) % (no.)*	P-*value*[a]
RASSF1A	63 (10)	40 (6)	69 (11)	NS[b]
p14	44 (7)	33 (5)	69 (11)	NS
p16	19 (3)	33 (5)	25 (4)	NS
MGMT	13 (2)	27 (4)	0 (0)	0.04

[a]Comparison among all three groups.
[b]NS, not significant.

pancreatic or lung parenchyma, or mucosa was unmethylated (not significant).

Methylation status of the *RASSF1A*, *p14*, *p16* and *MGMT* genes and clinicopathologic features are compared in Table 2. CpG island methylation at *RASSF1A* was associated with histopathologic type of tumor and lymph node metastasis, and *p16* methylation was associated with patient age and liver metastasis status. *RASSF1A* methylation was present in 66% of well-differentiated neuroendocrine carcinomas, 56% of well-differentiated neuroendocrine tumors of uncertain malignant potential but in 0% of benign well-differentiated neuroendocrine tumors ($P = 0.03$). Lymph node metastasis was present in 78% of patients whose tumors had *RASSF1A* methylation compared to 35% in patients whose tumors were unmethylated at *p16* ($P = 0.004$). The mean age of patients whose tumors had *p16* methylation was 68.3 ± 8.7 years, compared to 54.8 ± 13.0 years for those without *p16* methylation ($P = 0.002$). Liver metastasis was present in 67% of patients whose tumors had *p16* methylation compared to 31% in patients whose tumors were unmethylated at *p16* ($P = 0.04$).

The number of methylated genes and clinicopathologic features are compared in Table 3. Two or more genes were methylated in 53% (25/47) of tumors, one gene was methylated in 30% (14/47) of tumors, and all four genes were unmethylated in 17% (8/47) of tumors (Figure 2). The mean age of patients whose tumors had methylation of two or more gene was 61.4 ± 12.7 years, compared to 60.6 ± 10.7 years for those with methylation of one gene, and 45.7 ± 14.9 years for those with no methylated gene ($P = 0.01$). Liver metastasis was present in 52% of patients whose tumors had methylation of two or more genes, compared to 14% in patients whose tumors had methylation of one gene and 50% in those patients who had no methylation of any of the genes ($P = 0.044$).

Some of these associations were also present when tumor subsites were considered (Tables 2 and 3). Patients whose pancreatic well-differentiated neuroendocrine tumors were methylated at *RASSF1A* gene had more frequent lymph node metastasis ($P = 0.002$), and whose tumors had methylation of one or more genes were older compared to those whose tumors were unmethylated at all four genes ($P = 0.01$). Patients whose nonileal well-differentiated neuroendocrine tumors were methylated at *RASSF1A* gene had more frequent lymph node metastasis ($P = 0.049$), and whose tumors had *p16* gene methylation had more frequent liver metastasis ($P = 0.04$). The patients whose ileal well-differentiated neuroendocrine tumors had *p16* methylation were older compared to those without *p16* methylation ($P = 0.03$).

Discussion

In the current study, we found *RASSF1A* gene to be frequently methylated in all three sites of well-differentiated neuroendocrine tumors: 63% in pancreatic tumors, 40% in nonileal tumors, and 69% in ileal tumors. It was the most frequently methylated gene in our study. Similarly, high frequency of *RASSF1A* gene methylation has been reported in pancreatic and gastrointestinal well-differentiated neuroendocrine tumors,[7,23,24,29] but a slightly lower frequency in pulmonary well-differentiated neuroendocrine tumors.[22] The *RAS-association domain family 1* gene has several isoforms including *RASSF1A* and *RASSF1C* that are transcribed from two different CpG island promoters.[29–31] The hypermethylation of *RASSF1A* promoter is frequent in many human cancers, and there is an inverse correlation between *RASSF1A* silencing by methylation and *K-RAS* activation.[21,29–31] Previous studies have shown that well-differentiated neuroendocrine tumors lack *K-RAS* or *BRAF* mutations,[11,32,33] but have methylation of *RASSF1A* gene in pancreatic, pulmonary and gastrointestinal well-differentiated neuroendocrine tumors.[7,22–24] This suggests that RAS pathway is involved in well-differentiated neuroendocrine tumors mostly by gene silencing of *RASSF1A* gene by methylation.

In our study, methylation of *p16* was found in 19% of pancreatic tumors, 33% of nonileal tumors and 25% of ileal tumors as we reported previously.[6] Similarly, methylation of *p16* gene was reported in more than 50% of gastrinomas and nonfunctional pancreatic well-differentiated neuroendocrine in previous studies[34] and in 40% of pancreatic well-differentiated neuroendocrine tumors in another

Table 2 Methylation of *RASSF1A*, *p14*, *p16* and *MGMT* genes in pancreatic, nonileal and ileal neuroendocrine tumors and clinicopathologic associations

Clinicopathologic features	RASSF1A *gene % (no.)*		p14 *gene % (no.)*		p16 *gene % (no.)*		MGMT *gene % (no.)*	
	Methylated (n = *27*)	*Unmethylated* (n = *20*)	*Methylated* (n = *23*)	*Unmethylated* (n = *24*)	*Methylated* (n = *12*)	*Unmethylated* (n = *35*)	*Methylated* (n = *6*)	*Unmethylated* (n = *41*)
Age (mean ± s.d.) years	60.0 ± 12.1	54.4 ± 14.2	58.9 ± 12.0	57.6 ± 14.8	68.3 ± 8.7[a]	54.8 ± 13.0[a]	59.0 ± 10.9	58.1 ± 13.8
Gender								
Female	41 (11)	65 (13)	52 (12)	50 (12)	42 (5)	54 (19)	50 (3)	51 (21)
Male	59 (16)	35 (7)	48 (11)	50 (12)	58 (7)	46 (16)	50 (3)	49 (20)
Size of tumor (mean ± s.d.) (cm)	3.1 ± 2.5	3.3 ± 5.1	3.6 ± 4.7	3.3 ± 2.4	3.2 ± 2.3	3.6 ± 4.1	4.7 ± 2.7	3.3 ± 3.8
Histpathological type								
WDNTB	0 (0)[b]	15 (3)[b]	9 (2)	4 (1)	8 (1)	17 (2)	0 (0)	7 (3)
WTNTUMP	15 (4)[b]	25 (5)[b]	13 (3)	25 (6)	8 (1)	23 (8)	33 (2)	17 (7)
WDNC	85 (23)[b]	60 (12)[b]	78 (18)	71 (17)	83 (10)	71 (25)	67 (4)	76 (31)
Lymph node metastasis								
Present	78 (21)[c]	35 (7)[c]	52 (12)	67 (16)	75 (9)	54 (19)	50 (3)	61 (25)
Absent	22 (6)[c]	65 (13)[c]	48 (11)	33 (8)	25 (3)	46 (16)	50 (3)	39 (16)
Liver metastasis								
Present	41 (11)	40 (8)	43 (10)	38 (9)	67 (8)[d]	31 (11)[d]	7 (1)	44 (18)
Absent	59 (16)	60 (12)	57 (13)	62 (15)	33 (4)[d]	69 (24)[d]	83 (5)	56 (23)
Vital status								
Alive	89 (24)	95 (19)	96 (22)	87 (21)	83 (10)	94 (33)	100 (6)	90 (37)
Dead	11 (3)	5 (1)	4 (1)	13 (3)	17 (2)	6 (2)	0 (0)	10 (4)

WDNC, well-differentiated neuroendocrine carcinoma; WDNTB, well-differentiated neuroendocrine tumor, benign; WDNTUMP, well-differentiated neuroendocrine tumor, uncertain malignant potential.

[a] $P = 0.002$. The mean age of patients with ileal neuroendocrine tumors methylated at *p16* gene was 68.0 ± 2.9 years *vs* 53.3 ± 4.6 years for unmethylated tumors ($P = 0.03$).

[b] $P = 0.03$.

[c] $P = 0.004$. Lymph node metastasis was present in 70% (7/10) of pancreatic neuroendocrine tumors methylated at *RASSF1A* gene *vs* 0% (0/6) of unmethylated tumors ($P = 0.002$), and in 83% (5/6) of nonileal neuroendocrine tumors methylated at *RASSF1A* gene *vs* 22% (2/9) of unmethylated tumors ($P = 0.049$).

[d] $P = 0.04$. Liver metastasis was present in 60% (3/5) of nonileal neuroendocrine tumors methylated at *p16* gene *vs* 10% (1/10) of unmethylated tumors ($P = 0.04$).

Table 3 Number of methylated genes in pancreatic, nonileal and ileal neuroendocrine tumors and clinicopathologic associations

Clinicopathologic features	*Methylated genes % (no.)*			P-*value*
	None (n = *8*)	*One* (n = *14*)	*Two or more* (n = *25*)	
Age (mean ± s.d.) (years)	45.7 ± 14.9	60.6 ± 10.7	61.4 ± 12.7	0.01[a]
Gender				
Female	63 (5)	57 (8)	36 (9)	NS
Male	37 (3)	43 (6)	64 (16)	
Size of primary tumor (mean ± s.d.) (cm)	2.0 ± 1.7	4.4 ± 6.2	2.9 ± 2.0	NS
Histopathological type				
WDNTB	13 (1)	7 (1)	4 (1)	NS
WDNTUMP	25 (2)	29 (4)	12 (3)	
WDNC	62 (5)	64 (9)	84 (21)	
Lymph node metastasis				
Present	50 (4)	50 (7)	68 (17)	NS
Absent	50 (4)	50 (7)	32 (8)	
Liver metastasis				
Present	50 (4)	14 (2)	52 (13)	0.044
Absent	50 (4)	86 (12)	48 (12)	
Vital status				
Alive	100 (100)	86 (12)	92 (14)	NS
Dead	0 (0)	14 (2)	8 (2)	

WDNC, well-differentiated neuroendocrine carcinoma; WDNTB, well-differentiated neuroendocrine tumor, benign; WDNTUMP, well-differentiated neuroendocrine tumor, uncertain malignant potential.

[a]The mean age of patients with pancreatic neuroendocrine tumors methylated in two or more genes was 59.6 ± 3.2 years compared to 60.7 ± 5.7 years for tumors methylated in one gene and 27.5 ± 8.5 years for tumors unmethylated in all four genes ($P = 0.01$).

study.[7] In contrast, *p16* methylation was infrequent in pulmonary neuroendocrine tumors.[22]

p14 methylation was most prevalent in gastrointestinal tumors compared to other types of human tumors.[26] In our study, we found frequent methylation of *p14* in well-differentiated neuroendocrine tumors. In contrast, we and others had previously reported a low frequency of *p14* methylation in pancreatic well-differentiated neuroendocrine tumors.[6,7]

In our study, *MGMT* methylation was not present in ileal well-differentiated neuroendocrine tumors and was infrequent in pancreatic and nonileal well-differentiated neuroendocrine tumors. We have previously shown that complete loss of chromosome 18 is frequently present in ileal well-differentiated neuroendocrine tumors but not in nonileal or pancreatic well-differentiated neuroendocrine tumors.[11] These findings suggest that ileal well-differentiated neuroendocrine tumors have differences in genetic and epigenetic alterations compared to well-differentiated neuroendocrine tumors from other sites. The *MGMT* gene is responsible for removing alkylation of DNA at the O^6 position of guanine. Methylation of CpG islands in the promoter region of *MGMT* can cause gene silencing.[20] Frequent *MGMT* methylation is reported in brain tumors, colorectal cancers, lung cancers, head and neck cancers and high-grade lymphomas but is uncommon in pancreatic, renal, bladder, endometrial and breast carcinoma.[20]

CpG island methylator phenotype has been reported in cancers from a variety of sites and types including colon, stomach and pancreas.[35–37] In our study, methylation of *p16* gene and methylation of two or more genes were associated with liver metastasis, and methylation of *RASSF1A* gene with histopathologic type of tumor and lymph node metastasis. In another study, similar associations were reported between methylation of *p16* gene and lymph node metastasis, and between methylation of multiple genes and lymph node or liver metastasis in patients with pancreatic neuroendocrine tumors.[7]

In our study, *p16* gene methylation was more common in tumors from older patients. Age-related increase in methylation has been reported in normal tissue and cancer.[38–40] In our study, *RASSF1A* gene methylation was present in four samples of non-neoplastic pancreatic parenchyma. However, in our study islets of Langherhans were a minor component in the samples of non-neoplastic pancreatic parenchyma, and it is possible that *RASSF1A* gene methylation may be present in the exocrine cells. Similarly, a previous study has reported *RASSF1A* gene methylation of non-neoplastic parenchyma.[23]

Age-related methylation of *RASSF1A* gene has been reported in a variety of tissues including pancreas[41] but no association was present between age and *RASSF1A* gene methylation of non-neoplastic or tumor samples in our study.

In summary, our study shows that methylation profiles differ by tumor subsite, patient age and metastatic status. The findings in our study further support the concept that these three groups of tumor are fundamentally different and the tumorigenic process evolves along different pathways.

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