

Comparison of genetic alterations in neuroendocrine tumors: frequent loss of chromosome 18 in ileal carcinoid tumors

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Carcinoid tumors and pancreatic endocrine tumors are uncommon neuroendocrine neoplasms, and their genetic alterations are not well characterized. These tumors have site-specific differences in neuroendocrine characteristics, clinical course and genetic alterations. We compared clinicopathological features and loss of heterozygosity of chromosomes 11q, 16q and 18, and *BRAF* gene mutations in 47 patients with neuroendocrine tumors including 16 with pancreatic endocrine tumors, 15 with nonileal carcinoid tumors and 16 with ileal carcinoid tumors. Patients with carcinoid tumors had more frequent history of alcohol consumption compared to patients with pancreatic endocrine tumors ($P=0.02$), and patients with ileal carcinoid tumors more frequently had liver metastasis compared to patients with nonileal carcinoid tumors and pancreatic endocrine tumors ($P=0.02$). Allelic loss of chromosome 11q was present in 21% of tumors, chromosome 16q in 13%, and chromosome 18 in 30%. These alterations differed with the anatomical subsite of tumor: allelic loss of chromosome 18 was present in 69% of ileal carcinoid tumors, 13% of nonileal carcinoid tumors and 6% of pancreatic endocrine tumors ($P=0.001$). In contrast to pancreatic endocrine tumors and nonileal carcinoid tumors, all 11 ileal tumors with loss of chromosome 18 had complete loss of both chromosomal arms. No *BRAF* mutations were identified. Complete allelic loss of chromosome 18 was associated with smaller tumor size ($P=0.02$). Our study indicates that genetic alterations vary by tumor subsite and clinicopathologic features, and ileal carcinoid tumors have distinctive clinicopathologic and genetic profiles.

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Pancreatic endocrine tumors and carcinoid tumors are both rare, indolent neuroendocrine neoplasms with an age-adjusted annual incidence of 2.5–4.5 per 100 000.^{1,2} Neuroendocrine tumors are divided by their embryological site of origin into foregut carcinoid tumors, comprising tumors from the lung, stomach, duodenum and pancreas; midgut carcinoid tumors, comprising tumors from the jejunum, ileum, appendix and right colon; and hindgut carcinoid tumors, comprising tumors from the left colon and rectum. Tumors originating from the midgut are most common, with the majority located in the ileum.^{1,2} However, there is heterogeneity

among the neuroendocrine tumors of various subsites, including clinicopathologic features, behavior and genetic alterations. For example, the majority of appendiceal carcinoid tumors have benign clinical behavior, but the majority of ileal carcinoid tumors have metastasis at presentation.

The molecular pathogenesis of pancreatic endocrine tumor and carcinoid tumor is poorly understood.³ Multiple endocrine neoplasia type-1 (MEN1) is an autosomal dominantly inherited disorder characterized by the development of multiple endocrine tumors including pancreatic endocrine tumors.⁴ MEN1 results from germline mutations of *MEN1*, a 10-exon gene located on chromosome 11q13 that encodes for menin, a 610-amino-acid protein.^{3,5–8} Recent studies suggest menin regulates the transcription of multiple differentiation-regulating genes in association with a histone methyltransferase complex.⁹ Mutations of the *MEN1* gene and allelic loss of chromosome 11q13 are reported in

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sporadic carcinoid tumors and sporadic pancreatic endocrine tumors.¹⁰

Recent advances have been made in characterization of molecular events underlying the tumorigenesis of carcinoid tumors. Allelic loss of chromosomes 11q, 16q and 18q is reported in typical midgut carcinoids.^{3,11–14} Allelic loss of chromosome 18q is frequent in colorectal carcinomas.¹⁵ *DPC4* (*Smad4*) gene, present on chromosome 18q, has mutations in about 50% of pancreatic¹⁶ and in about 20% of colorectal carcinomas.¹⁷

The RAS-RAF-MEK (mitogen-activated protein/extracellular signal-regulated kinase kinase)–ERK (extracellular signal-regulated kinase)–MAP (mitogen-activated protein) kinase pathway mediates cellular responses to growth signals. *BRAF* mutations have been found in a variety of human cancers including colorectal carcinomas.¹⁸ Mutations in *BRAF* gene occur in two regions of the BRAF kinase domain, that is, the activation segment that protects the substrate binding site, and less commonly, the G loop that mediates binding of ATP. Previous studies have shown that carcinoid tumors or pancreatic endocrine tumors lack alterations of *KRAS* and *p53* genes,^{5,19} but *BRAF* mutations have not been reported.

In the present study, we studied pancreatic endocrine tumors and carcinoid tumors for allelic loss of chromosomes 11q, 16q and 18, and *BRAF* gene mutations, and associated the genetic alterations with clinicopathologic features.

Materials and methods

Characteristics of Specimens and Patients

Frozen tumor and non-neoplastic tissue were obtained from surgical specimens of patients undergoing resections for pancreatic endocrine tumors or carcinoid tumors in the frozen section laboratory of the Department of Pathology, The University of Texas 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 histopathology was reviewed and the tumors were classified as pancreatic endocrine tumors and carcinoid tumors using established criteria.^{20,21} There were 16 pancreatic endocrine tumors, 29 carcinoid tumors, and two pulmonary atypical carcinoid tumors (Figure 1). In total, 16 of the carcinoid tumors were in the ileum. The nonileal carcinoid tumors included five pulmonary, three gastric, two duodenal, one appendiceal, three cecal and one rectal tumor. Immunohistochemistry was performed by using standard techniques including antigen retrieval as described previously,²² using mouse monoclonal antibodies to chromogranin A (1:4000 dilution, Chemicon International, Temecula, CA, USA) and synaptophysin (1:4000 dilution, Chemicon International, Temecula, CA,

USA), and polyclonal antibodies to gastrin (prediluted, DakoCytomation, Inc., Carpinteria, CA, USA), glucagon (1:2000 dilution, DiaSorin, Inc., Stillwater, MN, USA), insulin (1:100 dilution, DakoCytomation, Inc.), pancreatic polypeptide (1:600 dilution, DakoCytomation, Inc.), somatostatin (1:500 dilution, DakoCytomation, Inc.) and vasoactive intestinal peptide (1:75 dilution, Bachem Biosciences, Inc., King Prussia, PA, USA). The functional status of tumors was ascertained by serum measurements of hormones and/or clinical syndrome due to hormonal hypersecretion.

DNA Extraction

DNA from both tumor and normal tissue were microdissected and extracted from fresh-frozen specimens using a commercial kit (Qiagen DNA extraction kit, Qiagen, Inc., Valencia, CA, USA), after reviewing a hematoxylin and eosin-stained slide from a frozen block. The neoplastic cellularity was at least 70%. Tumor samples were taken from the primary site of tumor in all but four tumors (three ileal carcinoid tumors and a cecal carcinoid tumor) that were harvested from the liver metastasis.

Loss of Heterozygosity of Chromosomes 11q, 16q and 18

Loss of heterozygosity of chromosomes 11q, 16q and 18 was determined using 5, 5 and 27 dinucleotide microsatellite markers, respectively (Figure 1). Loss of heterozygosity was determined by PCR amplification using fluorescent dye-labeled (6-FAM, NED or VIC) and unlabeled primers (Applied Biosystems, Foster City, CA, USA or Invitrogen, Carlsbad, CA, USA). PCR was performed in 15 μ l reaction volumes containing 20 ng of DNA, 9 μ l ABI Prism[®] True Allele[™] PCR Premix (Applied Biosystems), and 5 pmol of each primer. The cycling conditions were denaturation at 95°C for 12 min, 10 cycles (94°C for 15 s, 55°C for 15 s, 72°C for 30 s), 32 cycles (89°C for 15 s, 55°C for 15 s, 72°C for 30 s), and extension at 72°C for 10 min. A 0.25 μ l aliquot of each PCR product was combined with 12 μ l of formamide and 0.5 μ l of GENESCAN[®] 400HD [ROX] size standard (Applied Biosystems). The samples were then subjected to capillary electrophoresis on an Applied Biosystems 3730 DNA Analyzer. Loss of a marker was considered to be present when the assay showed absence or decrease in intensity by more than 50% of one of two alleles from a tumor sample as compared with the paired control non-neoplastic tissue (examples in Figure 2). Loss of heterozygosity of chromosomal arms was defined by allelic loss of one or more polymorphic (informative) microsatellite markers present on that chromosomal arm. Loss of chromosome 11q using five markers, 16q using five markers, and 18q using eight markers has been previously reported for 12 tumors,²² but all

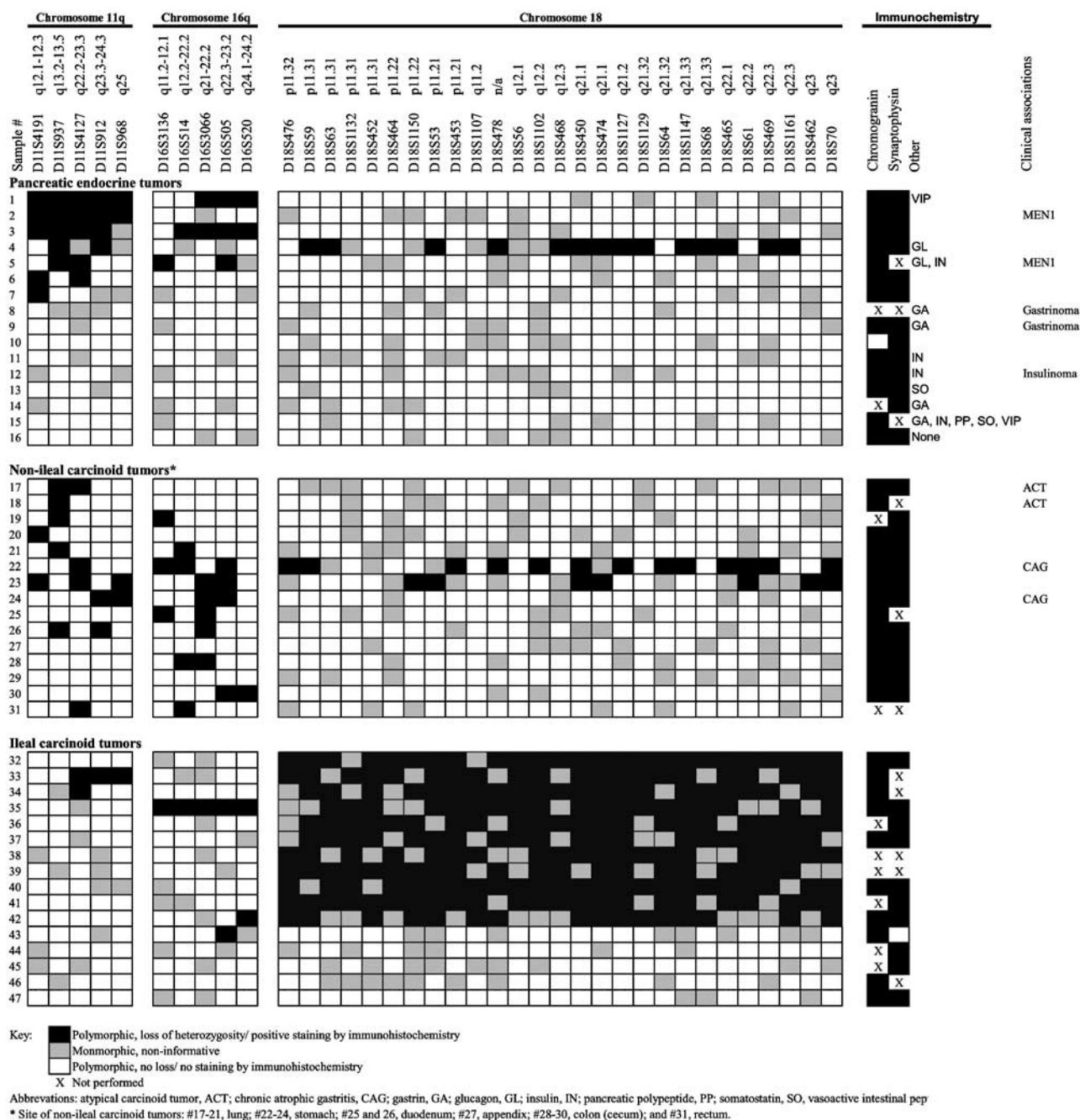


Figure 1 Immunohistochemistry profile, clinical associations, and loss of chromosomes 11q, 16q and 18 in pancreatic endocrine tumors, and nonileal and ileal carcinoid tumors.

chromosome 18 markers were repeated using microdissected DNA. Previously characterized colon carcinoma samples with loss of chromosome 18q were used as positive control and nontumor DNA from the same patient was used as negative control.

Sequencing of *BRAF* Gene

Exons 11 and 15 of the *BRAF* gene were amplified and sequenced as previously described.²³ Exons 11

and 15 were amplified by genomic PCR using intronic primers and a commercial DNA sequencing kit according to the manufacturer's instructions (BigDye Terminator version 1.1 Cycle Sequencing Kit, Applied Biosystems, Foster City, CA, USA). The PCR products were analyzed with an Applied Biosystems 3730 automated sequencer using forward and reverse primers. A previously characterized colon carcinoma sample with a *BRAF* mutation was used as positive control and PCR amplification buffer without DNA was used as negative control.

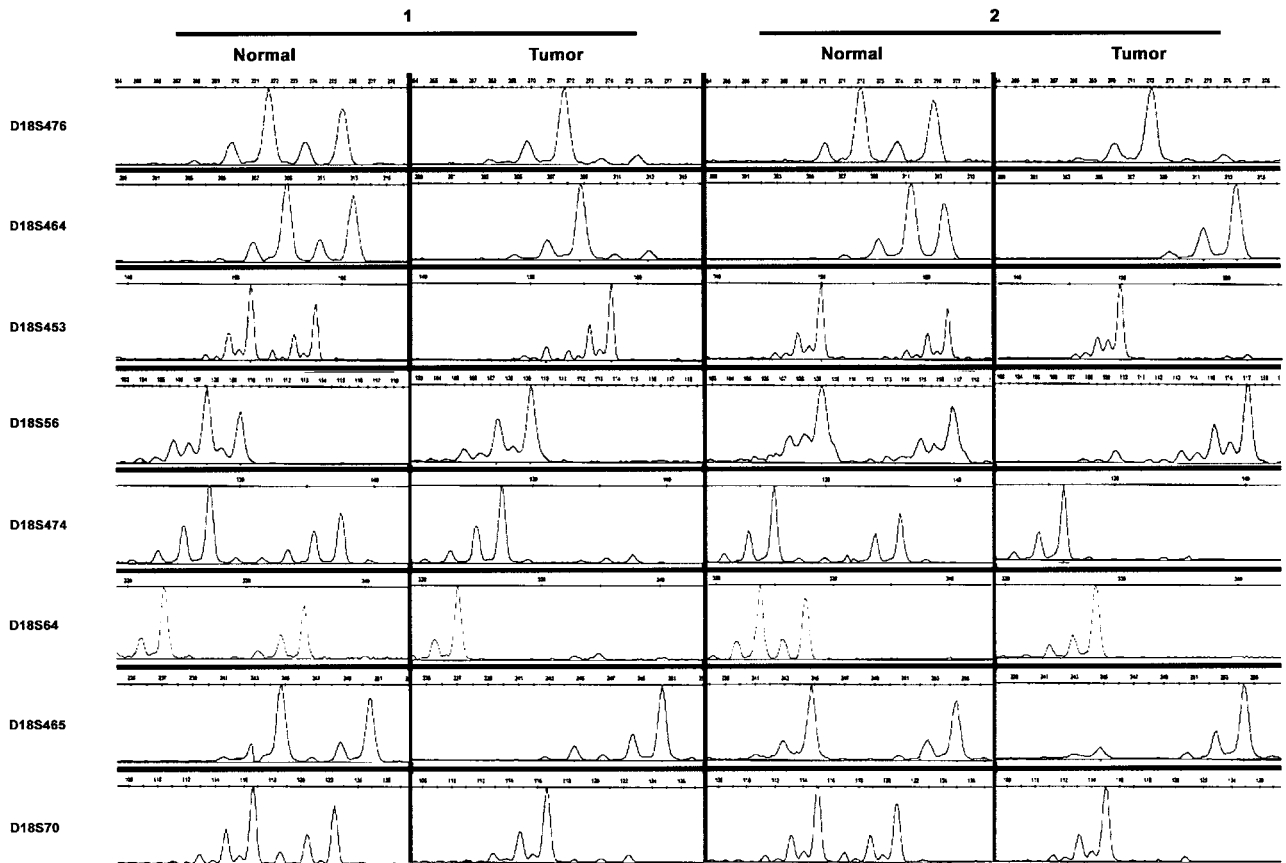


Figure 2 Chromosome 18 loss by PCR amplification using fluorescent primers in two representative neuroendocrine tumors for each marker. PCR was performed using non-neoplastic DNA and tumor DNA. The markers are indicated on the left. Allelic loss is present in the neuroendocrine tumor samples in all panels.

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 of diagnosis and tumor size, were evaluated by Student’s *t*-test and one-way ANOVA.

Results

Clinicopathologic Features

The clinicopathologic features of the 16 patients with pancreatic endocrine tumors, 15 with nonileal carcinoid tumors and 16 with ileal carcinoid tumors are compared in Table 1. History of alcohol consumption was present in 60% of patients with nonileal carcinoid tumors and 44% of patients with ileal carcinoid tumors compared to 13% of patients with pancreatic endocrine tumors ($P=0.02$). Liver metastasis was present in 69% of patients with ileal carcinoid tumors compared to 27% with nonileal carcinoid tumors and 25% with pancreatic endocrine tumors ($P=0.02$). Age, gender, size of tumor,

history of smoking, and vital status were not statistically different among the three groups.

Expression for chromogranin or synaptophysin was present in all 44 tumors evaluated by immunohistochemical staining. Staining for chromogranin was present in 97% (37/38) of tumors and for synaptophysin in 97% (36/37) of tumors. A total of 11 pancreatic endocrine tumors were evaluated by immunohistochemical staining for pancreatic hormones. Three tumors had staining for gastrin, two for insulin, one each for glucagon, somatostatin, vasoactive intestinal peptide, one for insulin and glucagon, one for glucagon, insulin, pancreatic polypeptide, somatostatin and vasoactive intestinal peptide, and one tumor had no staining for any pancreatic hormones. There were two gastrinomas and one insulinoma ascertained by serum measurements of hormones (Figure 1).

Allelic Loss of Chromosomes 11q, 16q and 18

Allelic loss of chromosome 11q was present in 21% (10/47) of tumors, chromosome 16q in 13% (6/47), and chromosome 18 in 30% (14/47) (Figure 1 and Table 2). Allelic loss of chromosome 11q was

Table 1 Clinicopathologic features of patients with pancreatic endocrine, and nonileal and ileal carcinoid tumors

<i>Clinicopathologic features</i>	<i>Pancreatic endocrine tumors (n = 16)</i>	<i>Nonileal carcinoid tumors (n = 15)</i>	<i>Ileal carcinoid tumors (n = 16)</i>	<i>P-value^a</i>
Age (mean ± s.d.) (years)	56.1 ± 15.9	59.5 ± 11.0	59.0 ± 13.2	NS ^b
<i>Gender</i>				
Female	56 (9)	53 (8)	44 (7)	NS
Male	44 (7)	47 (7)	56 (9)	
<i>History of smoking</i>				
Current or former	56 (9)	67 (10)	38 (6)	NS
Never	44 (7)	33 (5)	62 (10)	
<i>History of alcohol consumption</i>				
Current or former	13 (2)	60 (9)	44 (7)	0.02 ^c
Never	87 (14)	40 (6)	56 (9)	
<i>Size of primary tumor (mean ± s.d.) (cm)</i>	4.1 ± 2.7	3.3 ± 5.9	2.1 ± 1.0	NS
<i>Lymph node metastasis</i>				
Present	44 (7)	53 (8)	87 (12)	NS
Absent	56 (9)	47 (7)	13 (4)	
<i>Liver metastasis</i>				
Present	25 (4)	27 (4)	69 (11)	0.02 ^d
Absent	75 (12)	73 (11)	31 (5)	
<i>Vital status</i>				
Alive	87 (14)	87 (13)	100 (16)	NS
Dead	13 (2)	13 (2)	0 (0)	

Values are expressed as percent and numbers in parentheses.

^aComparison among all three groups.

^bNot significant, NS.

^cPancreatic endocrine tumors vs carcinoid tumors, *P* = 0.01 for ethanol consumption.

^dNonileal carcinoid tumors vs ileal carcinoid tumors, *P* = 0.02 for liver metastasis.

Table 2 Summary of loss of heterozygosity of chromosomes 11q, 16q and 18, and *BRAF* mutations in pancreatic endocrine tumors, nonileal carcinoid tumors and ileal carcinoid tumors

<i>Alteration</i>	<i>Pancreatic endocrine tumors (n = 16)</i>	<i>Non-ileal carcinoid tumors (n = 15)</i>	<i>Ileal carcinoid tumors (n = 16)</i>	<i>P-value^a</i>
Loss of chromosome 11q	38 (6)	13 (2)	13 (2)	NS ^b
Loss of chromosome 16q	19 (3)	0 (0)	19 (3)	NS
Loss of chromosome 18, any loss	6 (1)	13 (2)	69 (11)	0.001 ^c
Loss of chromosome 18, complete loss	0 (0)	0 (0)	69 (11)	0.001 ^c
<i>BRAF</i> mutations	0 (0)	0 (0)	0 (0)	NS

Values are expressed as percent and numbers in parentheses.

^aComparison among all three groups.

^bNot significant, NS.

^cPancreatic endocrine tumors vs carcinoid tumors, *P* = 0.01 for any loss of chromosome 18, and *P* = 0.006 for complete loss of chromosome 18.

present in 38% of pancreatic endocrine tumors, 13% of nonileal carcinoid tumors, and 13% of ileal carcinoid tumors (not significant). Allelic loss of chromosome 16q was present in 19% of pancreatic endocrine tumors, 0% of nonileal carcinoid tumors, and 19% of nonileal carcinoid tumors (not significant). By contrast, allelic loss of chromosome 18 was

present in 69% of ileal carcinoid tumors compared to only 13% in nonileal carcinoid tumors and 6% in pancreatic endocrine tumors (*P* = 0.001). In all 11 ileal carcinoid tumors with loss of chromosome 18, all polymorphic markers on the short arm (p) and long arm (q) of chromosome 18 showed loss, compared to none of nonileal carcinoid tumors or

pancreatic endocrine tumors ($P=0.001$, Figure 1). Of note, nonileal carcinoid tumors were comprised of small number of tumors from multiple sites.

BRAF Mutations

No *BRAF* gene mutations were present in any carcinoid tumor or pancreatic endocrine tumor.

Genetic Alterations and Clinicopathologic Features

Associations of chromosome 18 loss status and clinicopathologic features are compared in Table 3. The size of tumors was associated with allelic loss of chromosome 18: the mean size of tumors with complete loss of chromosome 18 was 2.2 ± 0.7 cm compared to 8.7 ± 12.8 cm for tumors with partial loss of chromosome 18 and 3.4 ± 2.4 cm for tumors with no loss of chromosome 18 ($P=0.02$). There was no association between the genetic alterations and immunohistochemical staining for hormones.

Discussion

In our study, we found loss of chromosome 11q in pancreatic endocrine tumors and occasional loss in

ileal carcinoid tumors and one atypical carcinoid tumor of lung. The long arm of chromosome 11 is a gene-rich region but contains only a few known or putative tumor suppressor genes,²⁴ 11q13 includes *MEN1*, *succinate-ubiquinone oxireductase subunit D* and *PGL2* genes, and 11q22–q23 includes *PGL1* and *PPP2R1B* (*serine/threonine protein phosphatase subunit locus*) genes. Allelic loss of more distal chromosomal regions located at 11q25 was identified in breast and ovarian cancer.²⁴ In previous reports, allelic loss of 11q was identified in pancreatic endocrine tumors, and ileal and lung carcinoid tumors but was not found in any appendiceal or rectal carcinoid tumors.^{14,25–27} Another study reported a high frequency of allelic loss at 11q13 locus (62%) in midgut carcinoid tumors, but a low frequency of *succinate-ubiquinone oxireductase subunit D* gene mutations, suggesting that other tumor suppressor genes may be targets for allelic loss at this region.²⁸

In our study, pancreatic endocrine tumors and carcinoid tumors demonstrated occasional loss of chromosome 16q with loss of loci at 16q11.2–q24.2. This chromosomal region harbors two putative tumor suppressor genes: *CTCF* (*CCCTC-binding factor*) gene, a transcriptional repressor of *c-myc*; and the *E-cadherin/CDH1* gene involved in cell-adhesion. Reduced expression of E-cadherin by

Table 3 Chromosome 18 loss in pancreatic endocrine tumors, nonileal and ileal carcinoid tumors and clinicopathologic associations

Clinicopathologic features	Chromosome 18 status			P-value ^a
	No loss (n = 33)	Partial loss (n = 3)	Complete loss (n = 11)	
Age (mean \pm s.d.) (years)	56.1 \pm 13.3	65.3 \pm 12.9	62.6 \pm 13.1	NS ^b
<i>Gender</i>				
Female	55 (18)	33 (1)	45 (5)	NS
Male	45 (15)	67 (2)	55 (6)	
<i>History of smoking</i>				
Current or former	58 (19)	67 (2)	36 (4)	NS
Never	42 (14)	33 (1)	64 (7)	
<i>History of alcohol consumption</i>				
Current or former	33 (11)	67 (2)	45 (5)	NS
Never	67 (22)	33 (1)	55 (6)	
<i>Size of primary tumor (mean \pm s.d.) (cm)</i>	3.4 \pm 2.4	8.7 \pm 12.8	2.2 \pm 0.7	0.02
<i>Lymph node metastasis</i>				
Present	55 (18)	33 (1)	73 (8)	NS
Absent	45 (15)	67 (2)	27 (3)	
<i>Liver metastasis</i>				
Present	36 (12)	33 (1)	55 (6)	NS
Absent	64 (21)	67 (2)	45 (5)	
<i>Vital status</i>				
Alive	91 (30)	67 (2)	100 (11)	NS
Dead	9 (3)	33 (1)	0 (0)	

Values are expressed as percent and numbers in parentheses.

^aComparison among all three groups.

^bNot significant, NS.

immunohistochemical analyses has been correlated with malignant behavior among patients with gastrointestinal carcinoid tumors.²⁹ Homozygous loss of function of *CTCF* gene by tumor-specific rearrangements has been identified in breast carcinomas.^{30,31} Interestingly, reported allelic loss of 16q21–qter in metastatic midgut carcinoid tumors but not in primary carcinoid tumors suggests that putative tumor suppressor genes mapped at this locus may be involved in tumor progression.¹⁴

In our study, we identified frequent complete loss of chromosome 18 in ileal carcinoid tumors compared to pancreatic endocrine tumors and nonileal carcinoid tumors. Chromosome 18 loss in midgut carcinoid tumors has been previously reported.^{3,13,14,32} Interestingly, a higher frequency of allelic loss of 18q was reported for classical midgut carcinoids^{14,32} but not for lung carcinoid tumors.^{13,27} In our study, the high frequency of loss of the entire chromosome 18 in ileal carcinoid tumors suggest that there are multiple tumor suppressor genes in chromosome 18 involving both the short and the long arms that play an important role in the tumorigenesis of ileal carcinoid tumors. Among the tumor suppressor genes harbored on chromosome 18q, the most extensively studied are the *DCC* gene localized at 18q21.2 and the *DPC4* (*Smad4*) gene localized at 18q21.1. Inactivation of chromosome 18q is identified not only in colorectal carcinoma,³³ but also in pancreatic,³⁴ gastric,³⁵ small intestinal,³⁶ appendiceal,³⁷ breast,³⁸ prostatic³⁹ and squamous cell carcinomas.^{40,41} *DPC4* (*Smad4*) gene inactivation is present in pancreatic,³⁴ small intestinal³⁶ and appendiceal carcinomas,³⁷ but is uncommon in other malignancies.^{42,43} Data from the present study and the previous studies show that chromosome 18q loss is infrequent in pancreatic endocrine tumors⁴⁴ but common in midgut carcinoid tumors³² and goblet cell carcinoid tumors of appendix.²² However, these tumors lack *DPC4* gene mutations or loss of *DPC4* protein expression suggesting that *DPC4* gene is not the target of this chromosomal loss. Loss of heterozygosity of chromosome 18q was a frequent event associated with poor prognosis in pancreatic adenocarcinomas, and restoration of chromosome 18 reduced the tumorigenicity.⁴⁵ Allelic loss at 18q12.3–q23 was reportedly common in squamous cell carcinoma but not in solar keratoses.⁴⁰ Also, loss of chromosome 18p may be associated with adverse clinical outcome in patients with high-risk breast cancer.⁴⁶ Other putative tumor suppressor genes localized immediately telomeric to *DCC* and *DPC4* at microsatellite locus 18q21.3 are serpins (Serine Proteinase Inhibitors) some of which (maspin) reportedly can suppress tumor growth and spread in breast carcinoma.⁴⁷ *Loss of cables*, a novel cyclin-dependent kinase interacting protein, which maps to human chromosome 18q11 has been associated with ovarian cancer.⁴⁸

In our study, no *BRAF* mutation was present in pancreatic endocrine tumors or carcinoid tumors.

Previous studies have shown that carcinoid tumors or pancreatic endocrine tumors lack *KRAS* mutations,^{5,19} but have methylation of *RASSF1A* gene in pancreatic endocrine tumors⁴⁹ and lung carcinoid tumors.⁵⁰

Our study demonstrated that alcohol consumption was more common in carcinoid tumors and compared to pancreatic endocrine tumors, and ileal carcinoid tumors had frequent loss of the entire chromosome 18. However, a limitation of our study is that the nonileal carcinoid tumors are comprised of small number of tumors from multiple sites. Several molecular differences among pancreatic endocrine tumors, and ileal and nonileal carcinoid tumors have been previously reported.³ Although ileal and nonileal carcinoid tumor and pancreatic endocrine tumor share a number of similarities, it is important that these groups of tumors continue to be analyzed separately rather than considered in combined series. Not only are these three general groups of tumors functionally different, but they also may differ in biologic behavior in terms of metastatic spread and aggressiveness.

In conclusion, the loss of chromosomes 11q, 16q and 18 identified in the majority of these tumors suggests that putative or yet unknown tumor suppressor genes may have a major role in the pathogenesis of ileal and nonileal carcinoid tumors and pancreatic endocrine tumors. The findings in our study further support the concept that these three groups of tumor are fundamentally different and the tumorigenic process evolves along at least two different pathways.

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