Comparative Genomic Hybridization Identifies Loss of 18q22-qter as an Early and Specific Event in Tumorigenesis of Midgut Carcinoids

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Carcinoid tumors are rare neuroendocrine tumors occurring in the lung or in the digestive tract where they are further subclassified as foregut, midgut, or hindgut carcinoids. To gain a better understanding of the genetic basis of the different types of carcinoid tumors, we have characterized numerical imbalances in a series of midgut carcinoids, and compared the results to previous findings in carcinoids from the lung. Numerical imbalances were revealed in 16 of the 18 tumors, and the most commonly detected aberrations were losses of 18q22-qter (67%), 11q22-q23 (33%), and 16q21-qter (22%), and gain of 4p14-qter (22%). The total number of alterations found in the metastases was significantly higher than in the primary tumors, indicating the accumulation of acquired genetic changes in the tumor progression. Losses of 18q and 11q were present both in primary tumors and metastases, whereas loss of 16q and gain of 4 were only detected in metastases. Furthermore, the pattern of comparative genomic hybridization alterations varied depending on the total number of detected alterations. Taken together, the findings would suggest a progression of numerical imbalances, in which loss of 18q and 11q represent early events, and loss of 16q and gain of 4p are late events in the tumor progression of midgut carcinoids. When compared to previously published comparative genomic hybridization abnormalities in lung carcinoids, loss of 11q was found to occur in both tumor types, whereas loss of 18q and 16q and gain of 4 were not revealed in lung carcinoids. The results indicate that inactivation of a putative tumor suppressor gene in 18q22-qter represents a frequent and early event that is specific for the development of midgut carcinoids. (*Am J Pathol* 2001, 158:1803–1808)

Carcinoid tumors are rare neuroendocrine tumors, with an estimated incidence of 1 to 2 cases per 100,000 inhabitants based on cancer registries.¹ In a Swedish study, the incidence was 8.4, when calculated on the basis of both surgical specimens and autopsies.² The tumors most commonly occur in the lung or in the digestive tract and are further divided into foregut, midgut, or hindgut carcinoids according to their embryological origin. Foregut carcinoids usually occur in the lungs; midgut carcinoids are located in the small intestine, appendix, and proximal large bowel; and hindgut carcinoids develop in the distal colon and rectum. Out of the three types of carcinoids, the midgut forms are the most commonly encountered, with the majority of tumors located in the distal ileum or the appendix. Carcinoids located in the appendix typically have a benign biological behavior, in contrast to the more aggressive ileal carcinoids. The term midgut carcinoid usually refers to carcinoids located in the ileum. These patients frequently develop liver metastases, which in turn will give rise to the carcinoid syndrome characterized by symptoms of cutaneous flushing, bronchial constriction, episodic diarrhea, and right-sided cardiac valvular fibrosis.³

The genetic mechanisms involved in the tumorigenesis of carcinoid tumors are poorly understood. The vast majority of cases are sporadic, although familial cancer syndromes associated with an increased risk of carcinoid tumors are also seen, mainly in multiple endocrine neoplasia type 1 (MEN 1). Most of the carcinoids associated with MEN 1 have been reported to be of foregut origin.⁴ The gene responsible for MEN 1, *MEN1* at 11q13, has recently been cloned and its involvement in the tumorigenesis of sporadic MEN 1-associated tumors has been characterized.^{5,6} In carcinoids from the lung frequent somatic deletions of the *MEN1* region have been demonstrated using loss of heterozygosity (LOH) and comparative genomic hybridization (CGH) analyses,^{7–12} and in a significant proportion of the cases inactivating *MEN1* mutations have also been detected.⁸ Loss of 11q has also been characterized in a limited number of carcinoids,

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Table 1. Clinical Characteristics of the 18 Midgut Carcinoids in the Study

Case no.	Lab i.d.	Age	Sex	Histopathological diagnosis	Staining for Grimelius and/or Chromogranin A	Staining for Masson	Size* (cm)	Metastases		Analyzed
								Igl	Liver	
1	2192	63	M	Midgutcarcinoid	Positive	Positive	1.5	Yes		Primary
2	2289	64	F	Midgutcarcinoid	Positive	Positive	1.5	Yes	Yes	Primary
3	576	69	M	Midgutcarcinoid	Positive	Positive	4–5		Yes	Metastasis
4	1302	71	M	Midgutcarcinoid	Positive	Positive	0.8	Yes		Primary
5	1845	77	M	Midgutcarcinoid		Positive	1	Yes		Primary
6	1454	46	F	Midgutcarcinoid	Positive	Positive	3.5	Yes	Yes	Primary
7	1130	69	M	Midgutcarcinoid	Positive	Positive	1		Yes	Primary
8	1916	71	M	Midgutcarcinoid		Positive	2.5		Yes	Primary
9	1320	75	F	Midgutcarcinoid	Positive	Positive	1.2			Primary
10	1386	39	M	Midgutcarcinoid	Positive	Positive	1.3	Yes	Yes	Primary
11	738	44	F	Midgutcarcinoid	Positive	Positive	1.8	Yes	Yes	Primary
12	1400	50	M	Midgutcarcinoid	Positive	Positive	1.5	Yes	Yes	Primary
13	690	73	F	Midgutcarcinoid	Positive	Positive	2.5	Yes	Yes	Metastasis
14	350	57	F	Midgutcarcinoid	Positive	Positive	3–4		Yes	Primary
15	1281	65	F	Midgutcarcinoid	Positive	Positive	5	Yes	Yes	Metastasis
16	119	74	M	Midgutcarcinoid	Positive	Positive	2	Yes	Yes	Primary
17	38	53	F	Midgutcarcinoid	Positive	Positive	1.2		Yes	Metastasis
18	88	59	F	Midgutcarcinoid	Positive	Positive	1.7	Yes	Yes	Metastasis

*The size given refers to that of the primary tumor.

which were of ileal, duodenal, and gastric origin,^{7,13} and on 18q in gastrointestinal carcinoids.¹²

Other frequently detected genetic alterations in carcinoid tumors include losses of 3p, 5q, 9p, 10q, and 13q in lung carcinoids.^{10,11} The *TP53* gene, which is frequently mutated in most human tumors, including gastrointestinal tumors,¹⁴ is only rarely mutated in carcinoids,^{15,16} indicating that *TP53* is not important in the tumorigenesis.

To gain a better understanding of the genetic basis of the different types of carcinoid tumors, we have characterized numerical imbalances in a series of midgut carcinoids using CGH, and compared the results to previous findings in carcinoids from the lung.

Materials and Methods

Tumor Specimens

The study includes 18 tumor specimens from 18 patients operated on for midgut carcinoids at the Karolinska Hospital between 1986 and 1997. The samples were snap-frozen in liquid nitrogen after surgery and stored at -70°C until analysis. The histopathological diagnosis and classification were based on the tumor location, the tumor growth pattern, on signs of secretory granules as determined by immunohistochemical analysis of Chromogranin A, and/or a positive reaction with the Grimelius silver technique, and finally on a positive reaction for Masson's silver staining as a marker of serotonin production.³ In addition, the size of the primary tumor, and the presence of metastases to the lymph nodes and the liver were recorded for each patient (Table 1).

Nine of the 18 tumor specimens were from female patients and nine were from males, and the median age at the initial surgery of all patients was 62 years (range, 39 to 77 years). By histopathological investigation all frozen tumor samples included in this study were shown to contain a minimum of 80% tumor cells. Informed con-

sent was obtained from all patients, and the study was approved by the Ethical Committee of the Karolinska Hospital.

CGH

High molecular weight DNA was isolated from frozen tumor tissues according to standard procedures and used for CGH analyses. CGH was performed essentially as previously described.¹⁷ Briefly, tumor DNA samples were labeled with fluorescein isothiocyanate-dUTP (DuPont, Boston, MA) by nick translation, and normal reference DNA was labeled with Texas Red-dUTP (Vysis Inc., Downers Grove, IL). Tumor and reference DNA were then mixed with unlabeled Cot-1 DNA (Life Technologies, Inc., Gaithersburg, MD), denatured, and applied onto slides with denatured metaphases of normal lymphocytes (Vysis Inc.). After hybridization at 37°C for 48 hours, the slides were washed in $0.4\times$ standard saline citrate/ 0.3% Nonidet P-40 at 74°C for 2 minutes and in $2\times$ standard saline citrate/ 0.1% Nonidet P-40 at room temperature for 1 minute. After air drying, the slides were counterstained with 4,6-diamidino-2-phenylindole (Vysis Inc.). Two control experiments were performed. In the first, normal male and normal female DNAs were labeled and hybridized to normal metaphases. For the second experiment, DNA from a previously characterized breast cancer cell line (MPE 600, Vysis Inc.) and DNA from a normal female were labeled and hybridized to normal male metaphases.

Ten three-color digital images (4,6-diamidino-2-phenylindole, fluorescein isothiocyanate, and Texas Red fluorescence) were collected from the hybridizations using a Zeiss Axioplan 2 (Carl Zeiss Jena GmbH, Jena, Germany) epifluorescence microscope and Sensys (Photometrics, Tucson, AZ) charge-coupled device camera interfaced to a IPLab Spectrum 10 workstation (Signal Analytics Corporation, Vienna, VA). Green-to-red (g/r) fluorescence ratios <0.8 were considered as losses

Figure 1. Summary of DNA copy number alterations detected by CGH in 18 midgut carcinoids. Each line represents one alteration detected in one tumor, with losses illustrated to the left and gains to the right of the ideograms.

whereas ratios >1.2 were scored as gains of genetic material. Heterochromatic regions, the short arm of the acrocentric chromosomes, and chromosome Y were not included in the evaluation.

LOH Studies

Matched pairs of blood and tumor DNA samples from nine patients (cases 2, 3, 4, 6, 8, 9, 11, 15, and 18) were genotyped for six polymorphic microsatellite markers including D18S1270, D18S541, D18S858, D16S2624, D16S539, and D16S422. The markers were labeled with fluorescent dyes (ie, HEX, TET, FAM). Fifty ng of DNA was amplified in a 10- μ l reaction solution containing 1.0 μ l 10 $\times$ buffer (Finnzyme, Oy), 1 mmol/L $MgCl_2$ (Perkin Elmer, Emeryville, CA), 0.5 μ mol/L primer pairs, 0.1 mmol/L dNTPs, and 0.2 μ l DNA polymerase (Dynazyme, Finnzyme, Oy). Amplifications were performed using a 10-minute initial denaturation at 95°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C or 60°C, and 30 seconds at 72°C, and a final 5-minute extension at 72°C. The fluorescently labeled PCR products were electrophoresed using 5% polyacrylamide gels (Long Ranger; FMC BioProducts, ME) on an ABI 377 apparatus (Applied Biosystems, Perkin Elmer) and the results were analyzed by Genescan Analysis 3.1 software package (Applied Biosystems, Perkin Elmer). LOH was confirmed when the ratio of allele intensity of the tumor DNA to normal DNA was 50% or less.

Statistical Analyses

Individual chromosome copy number changes of midgut carcinoids and bronchial carcinoids¹⁰ were compared using the Fisher's exact test, and correlations between CGH aberrations and clinical and histopathological fea-

tures were analyzed using the Mann-Whitney *U* test in the StatView 4.5 software. Probabilities of <0.05 were accepted as significant.

Results

In this study 18 midgut carcinoids were screened for numerical imbalances using CGH. The diagnosis was verified in each case by histopathological evaluation and positive Masson staining indicating serotonin production. In addition 16 of the tumors were positive for immunohistochemical analyses of Chromogranin A and/or histochemical Grimelius silver staining (Table 1). Furthermore, 16 of the patients demonstrated one or more of the symptoms that are typical for a carcinoid syndrome, ie, flush, episodic diarrhea/abdominal pain, cardiac failure, and asthma-like symptoms.

DNA samples from all 18 tumor samples were successfully analyzed by CGH. The chromosomal regions with increased and decreased DNA sequence copy numbers are illustrated in Figure 1 and detailed for each tumor in Table 2. The mean number of CGH alterations per tumor was 2.1 ± 1.7 , mean $\pm$ SD (range, 0 to 6). Numerical imbalances were detected in 16 of the 18 tumors, and losses were more common than gains. The most commonly seen losses were detected at 18q22-qter (67%), 11q22-q23 (33%), and 16q21-qter (22%), whereas gains most frequently involved 4p14-qter (22%). High-level amplifications were not detected.

The CGH findings for chromosomes 16q and 18q were confirmed through LOH analyses of nine blood-tumor pairs by typing of three microsatellites on distal 18q and three microsatellites on distal 16q. In all nine cases the findings by LOH were completely in agreement with the CGH results. Cases 3, 4, 11, 15, and 18 showed LOH for

Table 2. The Most Frequent CGH Alterations in Relation to the Total Number of Alterations in the 18 Midgut Carcinoids

Case no.	No. of changes	Losses and gains involving chromosomal regions					
		Loss 18q22-qter	Loss 11q22-q23	Loss 16q21-qter	Gain 4p14-qter	Other losses	Other gains
1	0						
2	0						
3	1	18					
4	1	18q					
5	1	18q22-qter					
6	1	18q21-qter					
7	1						Xp11-qter
8	1						Xp11-q24
9	1		11q22-q23				
10	2	18					19p
11	2	18q12-qter	11q14-qter				
12	2	18q	11q14-qter				
13	2	18q12-qter		16q21-qter			
14	3	18q12-qter			4p15.1-qter	21q22	5pter-q22
15	4	18	11q22-qter	16q21-qter	4p14-qter		
16	4	18				9p21-pter, 13q21-qter	16p
17	5		11q14-qter	16q	4	14q21-q22	20
18	6	18	11q21-qter	16q21-qter	4		19p, 20

all informative markers on 18q and also loss by CGH, whereas in cases 2, 6, 8, and 9 no losses were seen either by LOH or by CGH. Furthermore cases 15 and 18 demonstrated LOH with all informative 16q markers and also loss of the same region by CGH, whereas cases 2, 3, 4, 6, 8, 9, and 11 had retained heterozygosity and no CGH losses for 16q.

The four most frequently detected CGH alterations were compared to the total number of alterations in the individual tumors, and whether they were detected in primary tumor specimens or in metastases. These comparisons revealed several significant correlations. The pattern of CGH alterations in the individual tumors were found to vary depending on the total number of detected alterations (Table 2). Loss of 18q22-qter and loss of 11q22-q23 were both detected as single aberrations in four and one cases, respectively. However, loss of 16q21-qter and gain of 4p14-qter were only detected in tumors having a total of two to six alterations (Table 2). Furthermore, the total number of alterations found in the metastases were significantly higher than in the primary tumors ($P = 0.036$, Mann-Whitney). Losses of 18q22-qter and 11q22-q23 were detected both in primary tumors and metastases without significantly different frequencies (Table 3). However, significantly different frequencies were seen for loss of 16q21-qter that were only seen in metastases ($P = 0.0016$, Fisher's exact test), and gain of 4p14-qter that was present mainly in metastases ($P = 0.044$, Fisher's exact test) (Table 3). Taken together, the

findings would suggest a progression of numerical imbalances, in which loss of 18q22-qter and 11q22-q23 represent early events in the tumorigenesis, and loss of 16q21-qter and gain of 4p14-qter are late events associated with metastasizing of midgut carcinoids.

The four most frequently detected CGH alterations were also compared to previously published findings in the same regions in foregut carcinoids from the lung (Figure 2).^{10,12} Both tumor types demonstrated frequent loss of 11q22-q23, in frequencies that did not vary significantly between the two types of tumors. However, loss of 18q22-qter, which was seen in the majority of midgut

Table 3. The Most Frequent CGH Alterations Detected in Primary Tumors as Compared to Metastases of Midgut Carcinoids in This Study

Alteration	Primary tumors	Metastases	<i>P</i> value*
Loss 18q22-qter	8/13	4/5	n.s.
Loss 11q22-q23	3/13	3/5	n.s.
Loss 16q21-qter	0/13	4/5	0.0016
Gain 4p14-qter	1/13	3/5	0.0441

*n.s., not significant.

Figure 2. Comparison between the most frequent copy number imbalances detected by CGH in midgut carcinoids in the present study and in lung carcinoids as previously published.^{10,12} Each line represents one alteration detected in one tumor, with losses illustrated to the left and gains to the right of the ideograms.

carcinoids (12 of 18), were not reported in any of the lung carcinoids ($P < 0.0001$, Fisher's exact test). Similarly, loss of 16q21-qter as well as gain of 4p14-pter, detected solely in metastases from midgut carcinoids, was not detected in lung carcinoids ($P = 0.011$ and $P = 0.043$, respectively, Fisher's exact test) (Figure 2).

Discussion

Acquired genetic changes are the main causes of malignant transformation of somatic cells. In a variety of malignant tumors, identification and classification of these aberrations have proved to be of great value for diagnostic, prognostic, and treatment purposes. In the present study four recurrent numerical chromosomal abnormalities were identified by CGH in 16 of the 18 cases analyzed. In two cases no CGH alterations were detected, which could be because of balanced aberrations not affecting DNA sequence copy number or aberrations beyond the resolution capacity of CGH.

The four recurrent numerical imbalances detected included losses of 18q22-qter, 11q22-q23, and 16q21-qter, and gains of 4p14-qter. Chromosome 18q, especially the region of 18q12.3-q21.3, is frequently deleted in many types of tumors,¹⁸ such as pancreatic and colorectal tumors.^{19,20} Three tumor suppressor genes located in the 18q12.3-q21.3 region, *MADH2/SMAD2* at 18q12.3, *MADH4/SMAD4* in 18q21.1, and *DCC* in 18q21.2, have been evaluated as targets for the frequent deletions, but specific alterations of these genes have only been rarely described.²¹⁻²³ The minimal region of overlapping deletions detected in midgut carcinoids was mapped to 18q22-qter, which is located immediately distal to the region mentioned above. This would suggest the existence of a yet uncharacterized tumor suppressor gene locus whose inactivation represents a frequent and early event in the development of midgut carcinoids. Interestingly, no 18q losses were detected in two independent CGH studies of lung carcinoids,^{10,12} whereas this was the most common alteration in midgut carcinoids, indicating that the gene in 18q is specifically involved in the early development of midgut carcinoids but not in carcinoids of the lung.

Loss of 11q22-q23 occurs frequently in both hematological malignancies and in many types of solid tumors, such as breast, lung, cervical, and ovarian carcinoma and melanoma.¹⁸ The long arm of chromosome 11 is gene-rich and harbors multiple tumor suppressor genes especially within the region of q22-q23, which thus represent candidate genes for carcinogenesis of midgut carcinoids. The *PPP2R1B* gene (the serine/threonine protein phosphatase subunit locus), has been shown to be mutated in lung and colon cancer,²⁴ and recently, the *SDHD* (succinate-ubiquinone oxidoreductase subunit D) gene in 11q23 was demonstrated to be responsible for hereditary paraganglioma.²⁵ The role of the *ATM* (ataxia telangiectasia mutated) gene in carcinogenesis is still not clear, but it is assumed to function as a tumor suppressor gene, and has been thoroughly studied in breast cancer.²⁶ Other possible candidate genes include *CHK1*, a

protein kinase required for the DNA damage checkpoint,²⁷ *LOH11CR2A*,²⁸ and *DDX10*, a putative RNA helicase,²⁹ and *ALL-1* (disrupted in acute leukemias)³⁰ that all reside within 11q23.

Homozygous inactivation of the *MEN1* tumor suppressor gene in 11q13 by mutation of one allele and loss of the second allele through a gross deletion, has been observed in a third of sporadic lung carcinoids.⁸ Interestingly, loss of 11q in lung carcinoids was never restricted to the *MEN1* gene locus in 11q13, but always extended distally to the 11q22 region (Figure 2). On the other hand, loss of 11q22-q23 has been seen in lung carcinoids without loss of 11q13.^{7,10} This could be interpreted as indicating that both *MEN1* and a more distally located gene on 11q are involved in lung carcinoids. Furthermore, because the minimal regions of loss are the same in midgut and lung carcinoids (Figure 2), it is likely that one and the same gene in 11q22-q23 is involved in both types of tumors.

Carcinogenesis can be regarded as a multistep process, which is associated with the accumulation of genetic alterations,³¹⁻³³ and this view may also be applied to the development of midgut carcinoids. Consequently, more CGH alterations were seen in the metastatic lesions than in the primary tumors analyzed, and in addition the pattern of CGH alterations varied depending on the total number of detected alterations. Taken together, the findings were suggestive of a progression of numerical imbalances, in which loss of 18q and 11q represent early events, and loss of 16q and gain of 4p are late events associated with the tumor progression. Identification of the exact molecular alterations reflected by these numerical imbalances is likely to bring along an improved understanding of carcinogenesis of midgut carcinoids. Furthermore, these will provide starting points for the development of markers for diagnostic purposes.

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