

Analysis of sporadic neuroendocrine tumours of the enteropancreatic system by comparative genomic hybridisation

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Abstract

Background—Chromosomal instability is observed in a wide spectrum of human cancer syndromes. However, to date, little is known of the characteristic genetic changes in sporadic neuroendocrine tumours of the gastroenteropancreatic system.

Aims and method—We have studied copy number aberrations (CNAs) in 26 sporadic neuroendocrine tumours of the enteropancreatic system (12 foregut and 14 midgut tumours) by comparative genomic hybridisation (CGH), allowing simultaneous evaluation of the entire tumour genome.

Results—Nearly all tumours (25/26; that is, 96%) showed chromosomal imbalances, including full chromosomal aneuploidies, losses and gains of chromosome arms, interstitial deletions, and amplifications. Whereas gains of chromosomes 4, 5, and 19 were found in both foregut and midgut tumours, gains of chromosomes 20q (58%), 19 (50%), as well as 17p (50%), and partial losses of chromosomes 1p (42%), 2q (42%), 3p, 4q, and 6q (25% each) were frequently observed only in foregut tumours. In contrast, midgut tumours displayed less CNAs. Gains were detected for chromosomes 17q and 19p (57%). Most frequent losses affected chromosomes 18 (43%) and 9p (21%).

Conclusions—The results of our CGH analyses revealed new distinct candidate regions in the human genome associated with sporadic neuroendocrine tumours. Some of the genetic alterations were shared by foregut and midgut tumours while others discriminated between the two groups. Thus our results allude to the involvement of identical as well as discriminative genetic loci in tumorigenesis and progression of neuroendocrine neoplasms of the foregut and midgut. Based on these findings potential new candidate genes will be discussed.

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little is known of the genetic alterations in neuroendocrine tumours (NET) of the enteropancreatic system. NETs are rare tumours and occur with an incidence of 1–2/100 000, either sporadically or in association with a rare familial tumour syndrome, designated multiple endocrine neoplasia type 1 (MEN1). The syndrome is characterised by tumours of the parathyroid glands and the endocrine pancreas or duodenum, as well as pituitary tumours. However, tumours in MEN1 patients may also occur at other locations—almost exclusively—attributable to the foregut, including lung, stomach, and thymus.

According to their location, NETs can be classified into one of three groups: tumours of the foregut (lung, stomach, pancreas, and duodenum), midgut (jejunum, caecum, and ileum), and hindgut (colon and rectum).¹ Approximately 50% of NETs present with a clinical syndrome of either hormone or peptide hypersecretion; these tumours are termed functional NETs. However, even in the absence of clinical functionality these tumours synthesise and store secretory products and vesicles, as demonstrated by immunohistochemical staining for the secretory marker molecules synaptophysin and chromogranin A. This in turn indicates that the clinically observed disturbances of hormone, peptide, or neurotransmitter synthesis, storage, and secretion may translate into distinct alterations of genes controlling these pathways.² Malignant behaviour varies widely and depends on size and location of the primary invasive pattern with respect to regional lymph nodes and distant metastasis, cellular atypia of the tumour cells, mitosis, and immunohistochemical staining for the proliferation associated marker Ki67.^{3–4} Thus depending on the location of the primary tumour, the five year survival rate varies from 65% for jejunal and ileal NETs to 34% for pancreatic NETs.⁵

The *MEN1* gene, a putative tumour suppressor gene located at 11q13, was isolated by positional cloning and a large number of mutations have been reported.^{6,7} *MEN1* encodes a protein which interacts with JUN-D, a transcription factor of the AP1 family, thereby repressing JUN-D mediated transcription activation.⁸ The interaction with JUN-D is abolished by missense mutations detected in the

Abbreviations used in this paper: CGH, comparative genomic hybridisation; CNA, copy number aberration; DAPI, 4',6-diamino-2-phenylindole dihydrochloride; NET, neuroendocrine tumours; MEN1, multiple endocrine neoplasia type 1.

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Malignancies of various tissues and cell types are caused and accompanied by genetic instability, including losses and gains of entire chromosomes or particular fragments. To date,

MEN1 gene of patients. Whereas *MEN1* mutations have been identified in more than 90% of all familial cases, *MEN1* mutations are only observed in up to 30% of all sporadic NETs of the foregut.⁹ By contrast, *MEN1* mutations are rarely found in midgut and hindgut tumours. This suggests that other, as yet unidentified, genes must be involved in the tumorigenesis of the foregut as well as the midgut tumours.

To identify additional loci in the human genome which may be implicated in the tumorigenesis of NETs, we have carried out a comparative genomic hybridisation (CGH) survey in 26 tumours (12 foregut and 14 midgut). CGH allows identification of chromosomal regions showing (i) increased copy number (gain or amplification), which may indicate involvement of proto-oncogenes; or (ii) decrease in copy number (deletion of chromosomal material) in regions containing putative tumour suppressor genes.¹⁰

Distinct chromosomal imbalances were identified in NETs of the foregut and midgut. Based on these findings more detailed molecular analysis of the respective chromosomal intervals are currently under investigation. Candidate genes, potentially involved in the tumorigenesis of NETs, will be presented and discussed.

Patients and methods

CLINICAL DATA

Tissue samples from 26 patients with histopathologically proved tumour disease were studied. Mean age of the patients was 57.2 years (range 15–72) and there were 10 females and 16 males. Patient data are summarised in table 1. Patients were suffering from neuroendocrine tumours of the gastrointestinal system

(25) or bronchial tract (1). Neuroendocrine tumours were classified as foregut (pancreas nine, duodenum two, bronchial one) or midgut (jejunum one, ileum nine, ileocecal valve three, caecum one) depending on the anatomical location of the primary tumour, according to Williams and Sandler.⁹ This classification was complemented by a tumour biological approach considering immunohistological staining for the neuroendocrine marker molecules synaptophysin, chromogranin A, neurone specific enolase, and the proliferation associated marker Ki67, in addition to location, metastases, and size of the primary tumour.³ Metastases had occurred in 21 of 26 neuroendocrine tumours (liver 20, lymph nodes six, ovary one, lung one, pleura one). Hormone or peptide hypersecretion causing a clinical syndrome (that is, functionality) was observed in 19 of 26 neuroendocrine tumours; functional syndromes consisted of the carcinoid syndrome (13), Whipple's triad with insulinoma (three), and Zollinger-Ellison's syndrome and secretory diarrhoea with gastrinoma (three). Tissue samples were collected before patients received any treatment other than surgery.

TISSUE SAMPLES

All fresh tumour samples, obtained during surgery, were immediately snap frozen. An aliquot was stored at -70°C , and the remaining samples were submitted for histological analysis. All tissue samples were microdissected for tumour tissue under direct light microscopic guidance. Reference DNA for CGH analysis was either extracted from peripheral blood from a karyotypically normal male or from the tumour patient investigated (see table 1).

Table 1 Clinical, histopathological data, and comparative genomic hybridisation (CGH) findings of the 26 sporadic neuroendocrine gastroenteropancreatic tumours and six adenocarcinomas

Case ^a	Sex/age	Histology	Primary	Metastases	Functionality	CGH gains	CGH losses
1	M/43	NET	Foregut (pancreas , ^b gastrinoma)	Liver	+	15; 16p; 17p; 20q	None
2	M/34	NET	Foregut (pancreas , insulinoma)	No	+	4; 5; 11; 12; 15; 18p; 18q; 19; 20; 21	9q; 22
3	F/15	NET	Foregut (duodenum/jejunum, gastrinoma)	Liver , lymphnodes	+	None	3p; 20p
4	M/52	NET	Foregut (pancreas , gastrinoma)	Liver	+	2q; 12; 14	3; 5q
5	F/69	NET	Foregut (pancreas)	Liver	+	4; 5; 7; 9; 12; 13; 14; 16p; 17; 18; 19; 20; 21q; 22	1p; 2p; 2q; 3; 6q; 8q
6	F/67	NET	Foregut (pancreas , insulinoma)	No	+	3p; 5q; 7; 10p; 15q; 17p; 19; 20q	2q; 4q; 12q; 13q
7	M/53	NET	Foregut (pancreas , insulinoma)	No	+	8q; 9p; 9q; 10q; 13q; 15q; 21q	1p; 1q; 2q; 6q
8	M/65	NET	Foregut (bronchial system)	Liver , pleura	+	4; 7; 9; 16; 19; 20q; 22q	1p; 2q; 11p; 11q; 12p; 13q; 14q
9	M/49	NET	Foregut (pancreas)	Liver , lymphnodes	-	4; 5; 7; 9p; 12; 13; 14; 17; 18; 19; 20	1p
10	F/65	NET	Foregut (duodenum)	No	-	10p; 11q; 16p; 17p; 17q; 19; 20q; 22	18q
11	M/60	NET	Foregut (pancreas)	Liver, lung	-	2p; 10q; 20p	1p; 1q; 4q; 6q; 11; 18
12	F/37	NET	Foregut (pancreas)	Liver	-	1q; 8q; 11p; 11q; 12p; 12q; 14q; 17p; 17q; 18q; 20p	2q; 4q; 6p; 7q; 10p
13	F/64	NET	Midgut (ileum)	Liver, ovary	+	4; 5; 7; 9q; 10q; 14q; 17p; 17q; 19	11q
14	M/72	NET	Midgut (ileum)	Liver , lymphnodes	+	4; 5; 7; 14; 17q; 20;	2q; 3q; 9; 13q; 18
15	M/68	NET	Midgut (ileum)	Liver, lymphnodes	+	4; 8q; 10p; 10q; 11q; 16p; 17q; 19; 20;	1p; 2p; 2q; 3p; 6q; 9p;
16	M/62	NET	Midgut (ileocecal valve)	Liver, lymphnodes	+	None	11p; 18q
17	M/59	NET	Midgut (caecum)	Lymph nodes	+	4; 10q; 14; 17q; 19p; 19q; 20	18p
18	F/72	NET	Midgut (ileocecal valve)	Liver	+	4; 5; 17q; 20q	None
19	F/54	NET	Midgut (ileum)	Liver	+	4; 5; 13q; 14q; 20q	8p; 16p; 17; 19; 22
20	F/57	NET	Midgut (ileum)	Liver	+	4q; 5p; 5q; 10; 12q; 17p; 19q	8p; 11q; 12p; 13; 18
21	M/55	NET	Midgut (ileum)	Liver	+	17q; 19; 22q	None
22	M/65	NET	Midgut (ileum)	Liver	+	19; 22q	3; 9; 18
23	F/59	NET	Midgut (ileocecal valve)	Liver	+	17q; 19p	18
24	M/69	NET	Midgut (jejunum)	Liver	-	17q; 19p	None
25	M/58	NET	Midgut (ileum)	No	-	None	None
26	M/65	NET	Midgut (ileum)	Liver	-	1p; 16p; 19; 22	None

^aCGH analysis was performed with reference DNA of peripheral blood lymphocytes of patients in bold type.

^bCGH analysis was performed on tissue samples highlighted in bold.

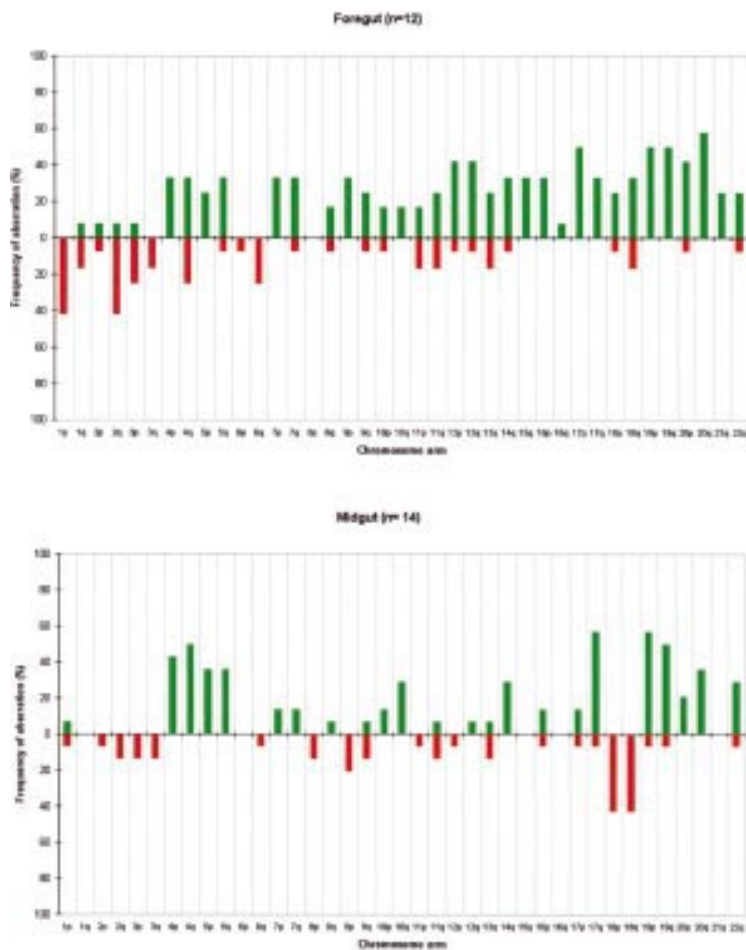


Figure 1 Copy number abnormalities in relation to chromosome arm affected for each tumour group investigated. Gains of chromosomal material are marked by a vertical bar above baseline (0 value) and losses below the baseline.

COMPARATIVE GENOMIC HYBRIDISATION

The CGH technique was performed as described previously¹¹ with slight modifications. Briefly, high molecular weight genomic DNA was prepared from EDTA blood (reference DNA) and tumour samples (test DNA) according to standard protocols. High molecular weight DNA was labelled by nick translation using direct SpectrumGreen (test DNA) and SpectrumOrange (reference DNA) conjugated dUTP (Vysis). DNase and DNA polymerase I concentrations were chosen to produce labelled probe fragments of 500–1500 base pairs, as observed on a non-denaturing 1.3% agarose gel.

Metaphase spreads were prepared from PHA stimulated peripheral blood lymphocytes from a karyotypically normal healthy male. Slides were dehydrated in 70%, 80%, and 95% ethanol, denatured for two minutes in 70% formamide/2× SSC, pH 7, at 70–72°C, followed by further dehydration in ice cold ethanol. For each hybridisation, 400 ng of labelled test DNA, 400 ng of reference DNA, and 50 µg of Cot-1 DNA were mixed and ethanol precipitated. DNA was resuspended in 14 µl of hybridisation mix containing 50% formamide, 2× SSC, and 10% dextran sulphate, denatured at 70°C for five minutes and applied after 45 minutes preannealing to denatured

metaphase spreads. Slides were incubated at 37°C in a moist chamber for 2–3 days. Post-hybridisation washes were performed as described previously.¹² The slides were counterstained with 4',6-diamino-2-phenylindole dihydrochloride (DAPI) and mounted with antifading solution (Vectashield, Vector Laboratories, Inc. Burlingame, California, USA).

DIGITAL IMAGE ANALYSIS

CGHs were analysed using an epifluorescence microscope (Axiophot, Zeiss, Germany) fitted with different single band pass filter sets for DAPI (blue), Spectrum Green (green), and Spectrum Orange (red) fluorescence. The microscope was equipped with an integrated high sensitivity monochrome charge coupled device camera (Hamamatsu) for image acquisition. Image analysis and karyotyping was performed with an ISIS digital image analysis system (Metasystems, Altusheim, Germany). Three images (red, green, and blue) were captured from 5–10 metaphases for each tumour sample. Selection and analysis of metaphase spreads were carried out as described previously.¹³ The metaphases were karyotyped and analysed to detect chromosomal aneuploidies on each chromosome. To determine the quality of our CGH approach and to test the diagnostic thresholds, experiments with positive and negative controls were done. As a positive control for CGH, DNA of a bladder carcinoma cell line (EJ30) with well known aberrations was used.¹⁴ Normal DNAs of probands investigated previously by conventional cytogenetics were used as negative controls showing no ratio deviations crossing our diagnostic thresholds of 0.85 and 1.17 used for the identification of chromosomal under representations (deletions) and over representations (duplications).¹⁵ The sex chromosomes were excluded from CGH analysis because hybridisation of test DNAs from different genders would give false signals reflecting the differences in the number of the X chromosomes in the sample.

Results

DNA extracted from microdissected shock frozen tumour specimens derived from neuroendocrine tumours of the foregut and midgut was analysed by CGH. Similar to other tumour entities, chromosomal instability was found in all three groups. Foregut and midgut NETs showed common as well as distinct copy number aberrations (CNAs).

FOREGUT TUMOURS

CNAs were detected in all 12 foregut tumours (mean 13.5 affected chromosome arms/tumour; range 2–30) (table 1). Gains (72.8% of aberrations) were more frequent than losses (27.2%). The most common chromosomal region for CNAs, found in 58% of foregut tumours, was gain of 20q followed by gains of chromosome 19 (50%) and chromosome 17p (50%) (figs 1, 2). Common chromosome arms for the most frequent partial losses were 1p (42%) (common region 1p13–31), 2q (42%) (common region 2q22–32), 3p, 4q, and 6q (25% each).

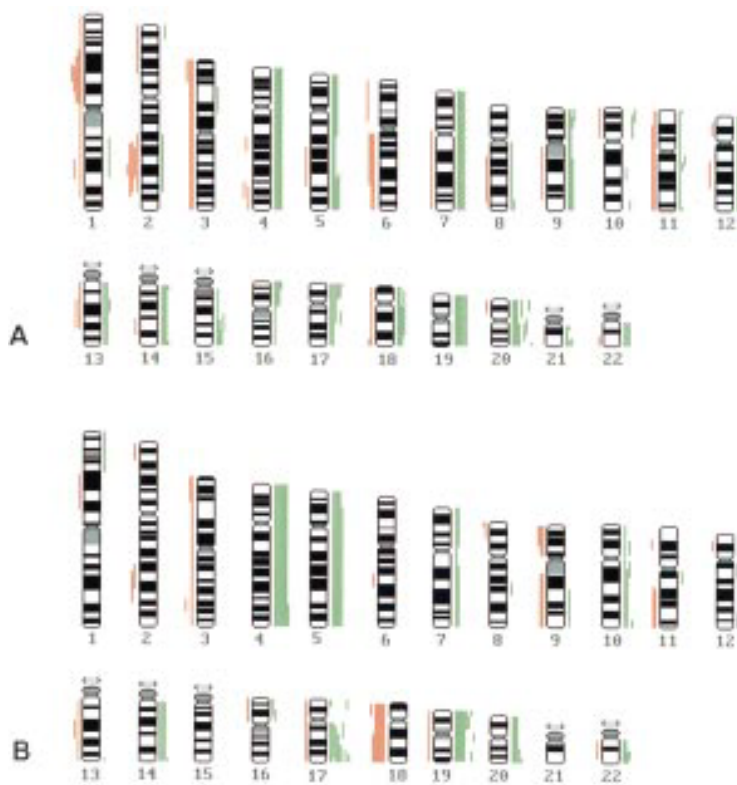


Figure 2 Summary of gains and losses of DNA sequence copy number detected in foregut tumours (A) and midgut tumours (B). Vertical lines on the left side (red) of each chromosome ideogram represent loss of genetic material in a given tumour at the marked chromosomal segment whereas vertical lines on the right side (green) correspond to gains of genetic material.

Gains of chromosome 4 were associated with gains of chromosome 19 in 36% of foregut tumours (case Nos 2, 5, 8, 9) (table 1). Deletions in chromosomes 2q (case Nos 5, 6, 7, 12) (table 1) and 6q (case Nos 5, 7, 11)

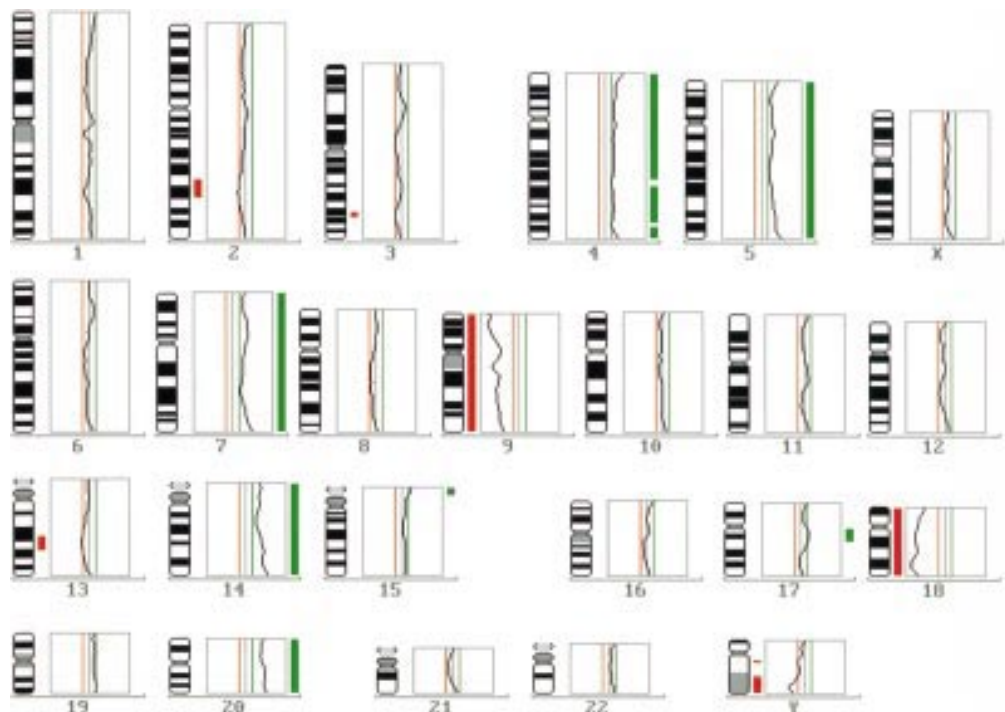


Figure 3 Average ratio profile of tumour 14 (midgut tumour, see table 1). Ratio profiles along the individual chromosomes are shown on the right side of each ideogram. Left (red), middle (grey), and right (green) vertical lanes represent the lower, middle, and upper threshold of the normal range (see text for details).

(table 1) were detected in primary tumours of the pancreas as also were gains of material in chromosome 15q (common region 15q22-*qter*; case Nos 1, 2, 6, 7) (table 1).

MIDGUT TUMOURS

CNAs were found in 13 of 14 midgut tumours (mean 8.7 affected chromosome arms/tumour; range 1–21) (table 1). Gains (67.2% of aberrations) were more frequent than losses (32.8%).

Chromosomal gains were most frequent in 17q and 19p (57%) followed by 19q and 4q (50% each). Additional regions of increased copy number were 4p (43%), chromosome 5, and 20q (36% each). Forty three per cent of midgut tumours showed losses of chromosome arms 18p or 18q (in five cases loss of the whole chromosome was detected) (see figs 2, 3) whereas 21% of midgut tumours showed partial or full loss of chromosome arm 9p. Four of six cases with gain of whole chromosome 4 also showed gain of chromosome 5 (case Nos 13, 14, 18, 19) (table 1). Four cases with gain of chromosome 4 showed full or partial trisomy for chromosome 14 (case Nos 13, 14, 17, 19) (table 1, fig 3).

COMPARISON OF TUMOUR SUBTYPES

CGH analysis of 12 foregut and 14 midgut tumours revealed identical but also different chromosomal abnormalities in both subgroups (for comparison of chromosomal subregions see fig 2). Tumours of the foregut showed a higher number of CNAs (13.5 affected chromosome arms/tumour) compared with the midgut tumour subgroup (mean value of 8.7 affected chromosome arms/tumour). There were no gross differences in gains of chromosomes 4, 5, and 19 in both subgroups (see fig 1). In contrast, gain of chromosome arm 17q

was found in 57% of midgut tumours but in only 27% of foregut tumours. Similarly, the gain of 20q was detected in 58% of foregut tumours versus 36% in midgut tumours.

The most characteristic differences between foregut and midgut tumours were observed for chromosomes 1, 2, and 18. The frequently observed loss in 1p and 2q in foregut tumours (42%) was hardly detected in midgut tumours. By contrast, loss of chromosome 18 was common in midgut tumours (43%) but less frequent in foregut tumours (18q, 18%). All midgut tumours with full monosomy 18 showed functionality.

Discussion

We have analysed, using comparative genomic hybridisation, 26 cases of sporadic neuroendocrine gastrointestinal tumours (NET) derived from the foregut (n=12) and midgut (n=14). Our study was designed to assess similarities and differences in the genetic constitution of these two tumour subclasses and to map novel candidate regions within the human genome which are involved in tumorigenesis and progression. Copy number aberrations (CNAs) were more frequent and more complex in foregut tumours (13.5 affected chromosome arms/tumour) compared with neoplasms of the midgut (8.7 affected chromosome arms/tumour) (fig 3). Both groups showed gains of chromosomes 4, 5, and 19 while other CNAs were found in either group only. Most prominent in the group of foregut tumours was the gain of chromosomal material from 20q (58%) and chromosome 19 (50%) and deletions in 1p and 2q (42% each). Tumours of the midgut were characterised by gains of 17q and 19p (57%) and losses of chromosome 18 (43%). Some CNAs were combined in a characteristic manner. In 36% of foregut tumours, gain of chromosome 4 was associated with gain of chromosome 19. Similarly, in midgut tumours, four of six cases with gain of chromosome 4 also showed gain of chromosome 5 and four cases with gain of chromosome 4 showed full or partial trisomy for chromosome 14. CNAs were also reported in other CGH analyses of sporadic neuroendocrine tumours of the digestive system, including gains of chromosome arms 5q, 7pq, 12q, 14q, 17pq, and 20q and losses of 3pq, 6pq, 11pq, and 16p.^{16, 17} Some were also observed in our group of tumour samples (gains of 5q, 7pq, 12pq, 14q, 17pq, and 20q). However, losses of 1p13-p31 and 2q22-q32 in more than 40% of foregut tumours and loss of chromosome arms 18p and/or 18q in 42% of midgut tumours have not been described previously.

To date, little is known of the involvement of particular genes. Mutations in *MEN1*, a putative tumour suppressor gene located on chromosome 11q13, were identified in 15–20% of sporadic NETs of the foregut and midgut.^{18, 19} In the present study, losses of chromosome 11 or deletions in 11q were observed in four of 26 NETs (case Nos 8, 11, 13, and 20; see table 1). *MEN1* mutation screening was performed in three patients (case Nos 11, 13, and 20) but only one sequence alteration was

identified (patient No 11, missense mutation G42S, for reference see Toliat and colleagues¹⁸). These results suggest an additional tumour suppressor gene on chromosome 11 possibly involved in tumorigenesis of NETs. This is supported by LOH studies which identified deletions distal to *MEN1*.²⁰ Another tumour suppressor gene, designated *CDKN2A* and located on the short arm of chromosome 9 (9p21), is homozygously deleted or transcriptionally silenced in more than 90% of gastrinomas and non-functioning pancreatic neuroendocrine tumours. Homozygous deletions are present in 41% of these tumours; gene silencing by hypermethylation of the promoter region occurs in 58%.²¹ In our samples, deletions in 9p were detected in 20% of midgut tumours and may involve *CDKN2A*. However, deletions in 9p21 may be present more frequently but escape detection due to the low resolution of CGH compared with deletion screening with gene specific markers. In addition, the group of midgut tumours was characterised by loss of chromosome 18 (42%). This chromosome contains the tumour suppressor genes *DPC4/SMAD4* and *DCC*, both located in 18q21. Interestingly, mutations in *DPC4* were found in 50% of non-functioning neuroendocrine tumours of the foregut.²² These results and our CGH analyses indicate that *DPC4* may be a good candidate gene for the development of NETs derived from the midgut. In addition, another gene involved in regulated secretion may be located on 18q as all tumours with a deletion in 18q demonstrated functionality. Thus 18q may be a candidate region for a gene that participates in the regulation of secretion by possibly inhibiting secretion unless the appropriate stimulus is elicited. Once this gene is deleted and/or inactivated by point mutations, regulation of the secretory process might be abolished, thus leading to functionality with a clinical syndrome of hypersecretion.

In contrast with the group of midgut tumours (which was characterised by gains of 17q and 19p, and losses of chromosome 18), neoplasms attributable to the foregut were dominated by gains of 20q and 19 and partial losses of chromosome arms 1p and 2q. Although deletions of the short arm of chromosome 1 (1p32-p33) were frequently observed in an aggressive subtype of endometrial cancer,²³ no prominent tumour suppressor gene was isolated from the corresponding interval. The high frequency of 1p deletions (42%) in foregut tumours suggests an important gene within this chromosomal region, which initiates tumorigenesis or contributes to the progression of foregut NETs. The same holds true for the long arm of chromosome 2 where deletions were detected with the same frequency (42%). This region is also deleted in small cell lung carcinoma.^{24, 25}

Chromosome instability is often found in tumour cells and can result from uncontrolled cell proliferation and defective DNA repair systems or may be due to alterations in genes which are directly involved in chromosome segregation. The latter was shown for *STK15/*

BTAK and *BRCA1*, two genes which are required for normal centrosome number and function.^{26, 27} Gene amplification or overexpression of *STK15*, a centrosome associated kinase, leads to multiple functional centrosomes in a single nucleus. Consequently, chromosomes segregate unequally, resulting in aneuploidy and transformation of cells. The *STK15* gene is located in 20q13, a region which showed gain of chromosomal material in 58% of foregut tumours. Accordingly, *STK15* overexpression or an increased gene dosage may contribute to the genetic instability in neuroendocrine tumours of the foregut.

In conclusion, the CGH data presented here define a set of genomic regions in the human genome that are likely to harbour genes which play an important role in the genesis and progression of neuroendocrine tumours of the enteropancreatic system. Further molecular genetic studies will identify more precisely the relevant regions and hopefully lead to identification of genes and mutations associated with neuroendocrine malignancies.

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