High-resolution genomic profiling reveals gain of chromosome 14 as a predictor of poor outcome in ileal carcinoids

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Abstract

Ileal carcinoids are malignant neuroendocrine tumours of the small intestine. The aim of this study was to obtain a high-resolution genomic profile of ileal carcinoids in order to define genetic changes important for tumour initiation, progression and survival. Forty-three patients with ileal carcinoids were investigated by high-resolution array-based comparative genomic hybridization. The average number of copy number alterations (CNAs) per tumour was 7.1 (range 1–22), with losses being more common than gains (ratio 1.4). The most frequent CNA was loss of chromosome 18 (74%). Other frequent CNAs were gain of chromosome 4, 5, 14 and 20, and loss of 11q22.1–q22.2, 11q22.3–q23.1 and 11q23.3, and loss of 16q12.2–q22.1 and 16q23.2–qter. Two distinct patterns of CNAs were found; the majority of tumours was characterized by loss of chromosome 18 while a subgroup of tumours had intact chromosome 18, but gain of chromosome 14. Survival analysis, using a series of Poisson regressions including recurrent CNAs, demonstrated that gain of chromosome 14 was a strong predictor of poor survival. In conclusion, high-resolution profiling demonstrated two separate patterns of CNAs in ileal carcinoids. The majority of tumours showed loss of chromosome 18, which most likely represents a primary event in the development and pathogenesis of tumours. A different genetic pathway is operative in a subgroup of tumours; this is characterized by gain of chromosome 14 and is strongly associated with poor prognosis. Predictive fluorescence *in situ* hybridization analysis of chromosome 14 status in patients with ileal carcinoids is suggested.

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Introduction

Ileal carcinoids are malignant neuroendocrine tumours of the small intestine. The annual incidence of ileal carcinoids is 0.31 per 100 000 and it is increasing (Modlin *et al.* 2003). They are slow-growing tumours and usually present late with either local manifestations (mass, bleeding and bowel obstruction) or symptoms due to overproduction of hormones (the midgut carcinoid syndrome). The overall 5-year survival rate for ileal carcinoids in the SEERs database is 64%, with a significant difference in survival for localized and distant disease (73.8 and 46.4% respectively; Modlin *et al.* 2003). Significantly better 5-year survival rates have been reported from single

centers after active interventions (Wängberg *et al.* 1996, Sarmiento & Que 2003). The only curative treatment is radical surgery, but for palliation of symptoms the treatment can include cytoreductive surgery, ischaemic treatment of liver metastases or medical therapy with somatostatin analogues sometimes combined with interferon α (Kvols *et al.* 1986, Faiss *et al.* 2003, Kölby *et al.* 2003, Roche *et al.* 2003, Osborne *et al.* 2006). Recently, somatostatin receptor-mediated radiotherapy has been introduced as palliative treatment (Kwekkeboom *et al.* 2005, 2008). Ileal carcinoids are composed of enterochromaffin cells that secrete serotonin and tachykinins, which may give rise to hormonal symptoms. Other tumour markers are

chromogranin A and vesicular monoamine transporter 1 (Stridsberg *et al.* 1995, Jakobsen *et al.* 2001). The aetiology of ileal carcinoids is unknown. The majority of tumours occurs sporadically and familial cases are extremely rare. Our knowledge about the genetic and epigenetic alterations that lead to ileal carcinoid formation is limited and pathogenetic mechanisms have not yet been identified. Several genes have been studied, but no mutations have been identified in *TP53*, *KRAS*, *CTNNB1*, *CDKN2A*, *SMAD2*, *SMAD4* and *BRAF* (Weckström *et al.* 1996, Younes *et al.* 1997, Löllgen *et al.* 2001, Chan *et al.* 2003, Wang *et al.* 2005, Kulke *et al.* 2008). However, epigenetic changes such as genome-wide hypomethylation of LINE-1 and Alu-repetitive sequences and promoter hypermethylation of specific genes, i.e. *RASSF1A*, *CTNNB1*, *LAMB3*, *CDKN2A*, *CDH1*, *LAMC2* and *THBS1*, have been reported in a subset of tumours (Chan *et al.* 2003, Liu *et al.* 2005, Zhang *et al.* 2006, Choi *et al.* 2007).

There have been few cytogenetic studies of ileal carcinoids, mainly due to difficulties in obtaining good-quality metaphase chromosomes (Pfragner *et al.* 1996, Sjögren *et al.* 2000, Van Buren *et al.* 2007). Thus, little is known about structural alterations, e.g. chromosome translocations, in these tumours. Using microsatellite markers, chromosome-based comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) analysis, recurrent copy number alterations (CNAs) have been identified. The most common CNA is loss of chromosome 18 (Terris *et al.* 1998, Zhao *et al.* 2000, Kytölä *et al.* 2001, Löllgen *et al.* 2001, Tönnies *et al.* 2001, Stancu *et al.* 2003, Wang *et al.* 2005, Kim do *et al.* 2008, Kulke *et al.* 2008). Other recurrent CNAs include losses involving chromosomes 9, 11q and/or 16q, and gains involving chromosomes 4, 5, 7, 14 and/or 20. No correlations have so far been identified between CNAs and clinicopathological data, most likely because ileal carcinoids are rare tumours and the fact that previous studies have been based on small numbers and used low-resolution techniques (Kim do *et al.* 2008, Kulke *et al.* 2008).

In order to obtain a high-resolution genomic profile of ileal carcinoids, we performed array-based CGH (aCGH). Samples from a total of 43 patients were analyzed and the results were correlated to clinical and histopathological features. We found multiple and recurrent CNAs in ileal carcinoids, the most frequent of which was loss of chromosome 18. In addition, we found a strong correlation between gain of chromosome 14 and poor survival.

Material and methods

Tumour material

Forty-three patients who underwent surgery for ileal carcinoids at the Sahlgrenska University Hospital, Göteborg, Sweden, from 1991 to 2007 were included. Fresh samples were collected at the time of surgery. Samples from a total of 24 primary tumours and 28 liver metastases were obtained; in nine cases, both primary tumour and liver metastases were available from the same patient. We retrieved complete clinical data for all patients including age at diagnosis, sex, hormonal symptoms, carcinoid heart disease (defined as tricuspid regurgitation by echocardiography), surgery, somatostatin analogues, hepatic arterial embolization and survival (Westberg *et al.* 2001). The histopathological evaluation was performed on haematoxylin–eosin-stained sections. The immunohistochemical analysis included staining for chromogranin A, serotonin, synaptophysin, cytokeratin 8/18 and Ki67. Diagnostic criteria for ileal carcinoids were according to the World Health Organization (WHO) classification (Capella *et al.* 2000). Grading and staging were according to ENETS recommendations (Rindi *et al.* 2007). Staging of tumours was performed by assessing tumour-node-metastasis (TNM). Grading of tumours was based on mitotic count and Ki67 index. Grade 1 tumours (G1) had <2 mitoses/10HPF and ≤2% Ki67, grade 2 tumours (G2) had 2–20 mitoses/10HPF and 3–20% Ki67 and grade 3 tumours (G3) had >20 mitoses/10HPF and >20% Ki67. Samples used for aCGH analysis were verified by histological examination. Only samples with a purity of at least 70% tumour cells were included.

aCGH analysis

Oligonucleotide-based aCGH (G4410B/G4426A/G4426B; Agilent Technologies, Palo Alto, CA, USA) containing ~44 000 probes distributed over all chromosomes were used. Both coding and non-coding sequences were covered, with a median probe spatial resolution of 43 kb. The probes were annotated against NCBI genome build 36 (UCSC hg18, March 2006) and all mapped genes were represented by at least one probe. High molecular weight genomic DNA was extracted from fresh-frozen tumour specimens using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Human genomic DNA (Promega, Madison, WI, USA) pooled from normal males and females was used as reference DNA. Labelling and hybridization of DNA were performed according to the manufacturer's protocol. Briefly, 1.5 µg high-quality DNA was fragmented by restriction digestion with AluI and

RsaI (Promega). Subsequently, the digested DNA was labelled using random primers, exo-Klenow fragment and fluorescence-labelled nucleotides (Genomic DNA Labelling Kit Plus; Agilent Technologies). Tumour DNA was labelled with Cyanine 5 and reference DNA with Cyanine 3 for 2 h at 37 °C. After termination of the reaction and clean-up using a Microcon YM-30 filter (Millipore, Billerica, MA, USA), the incorporation of dye was determined spectrophotometrically. Sex-matched tumour and reference DNAs for each hybridization were pooled and mixed with Cot-1 DNA (Invitrogen, Carlsbad, CA, USA), blocking agent and hybridization solution (Oligo aCGH Hybridization Kit; Agilent Technologies). For each hybridization mixture, 100 µl were pipetted onto the array and a gasket slide was placed on top. Hybridization was carried out for 24 h at 65 °C in a rotation oven (G2545A; Agilent Technologies). After hybridization, the arrays were washed in Oligo aCGH Wash Buffers (Agilent Technologies) and Stabilization and Drying Solution (Agilent Technologies). Arrays were scanned at 5 µm resolution using the Agilent's Microarray Scanner G2565BA (Agilent Technologies), and images were read and processed using Feature Extraction version 9.1 (G2567AA; Agilent Technologies). Data were further analyzed using CGH Analytics version 3.4 software (G4175AA, Agilent Technologies) using the moving average (window size: 1.0) and *z*-score algorithm (threshold: 2.5). A $\log_2$ ratio of ± 0.3 in >5 consecutive probes was considered as gain/loss. A $\log_2$ ratio of >2 was designated as high-level amplification and a $\log_2$ ratio of <-2 was designated as homozygous loss. CNAs identified by the software were confirmed by visual inspection. Recurrent CNAs were controlled for copy number variations using the Database of Genomic Variants (Iafrate *et al.* 2004).

Fluorescence *in situ* hybridization analysis

Fluorescence *in situ* hybridization (FISH) analysis was performed on paraffin sections using a telomere-specific probe for chromosome 14 (TelVysion 14q SpectrumOrange; Abbott Molecular Inc., Des Plaines, IL, USA) according to the manufacturer's protocol. Briefly, 1 µm paraffin sections of formalin-fixed tumour specimens were deparaffinized and pretreated with 0.2 mol/l HCl at room temperature for 20 min and with 1 mol/l sodium thiocyanate for 30 min at 80 °C. Subsequently, the sections were treated with Protease II (Abbott Molecular Inc.) for 30 min at 37 °C, dehydrated in increasing concentrations of ethanol and air-dried. Probe solutions were then applied and coverslips were placed on top and sealed with rubber

cement. Denaturation (5 min at 73 °C) and hybridization (20 h at 37 °C) were carried out in a hybridization chamber (Hybridizer; Dako, Glostrup, Denmark). After washing with 2×SSC containing 0.3% v/v NP-40 for 10 min at 72 °C, sections were counterstained with DAPI I (Abbott Molecular Inc). Fluorescence signals were analyzed using a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA). The numbers of fluorescence signals were evaluated in at least 300 tumour cell nuclei from each of the specimens.

Survival analysis

The overall survival of patients with ileal carcinoids was analyzed using a series of Poisson regressions, including time since diagnosis, age, sex and recurrent CNAs included one by one. A patient was considered to be positive for a recurrent CNA if the CNA was demonstrated in the primary tumour and/or the liver metastasis. This analysis generated significant variables that were further evaluated in a stepwise multivariate analysis. Hormonal symptoms, carcinoid heart disease and Ki67 were included in the multivariate analysis. *P* values <0.05 were considered significant. The programmes used for estimating death hazard functions by Poisson regression and for the calculation of Kaplan–Meier curves were developed by the statistical consultant (Prof. Anders Odén).

Ethics

This study was approved by the Regional Ethical Review Board in Göteborg, Sweden.

Results

The clinical and histopathological data are summarized in Table 1. All cases involved malignant ileal carcinoids composed of enterochromaffin cells immunoreactive against serotonin and chromogranin A. All tumours were well-differentiated endocrine carcinomas; the majority was of G1 (37 out of 43) and few were of G2 (6 out of 43). Most cases involved distant disease (stage IV; 38 out of 43), but a minority involved regional disease (stage IIIB; 4 out of 43) and there was a single case of localized disease (stage IIB; 1 out of 43).

Ileal carcinoids carry multiple and recurrent CNAs

CNAs were identified in all primary tumours and liver metastases; these are presented in Fig. 1 and Table 2. Altogether, 370 CNAs were detected in 52 tumour

Table 1 Clinical and histopathological data for 43 patients with ileal carcinoids analyzed by array-based comparative genomic hybridization

Case	Age/ sex	Size (mm)	Primary tumour			TNM, grade and stage ^a	Residual disease after primary surgery	HAE	SSTR analogs	IFN- α	Carcinoid heart disease ^c	Hormonal symptom	Other malignancies	Follow-up (mo.)	Current status
			No.	Mitotic- count ^a (10 HPF)	Ki67 ^a (%)		Primary surgery ^b								
1	59/M	10	6	<2	≤2	T3(m)N1M0	G1	IIIB	+	—	—	—	—	26	NED
2	58/F	20	1	<2	≤2	T3N1M1	G1	IV	+ ^d	—	—	+	—	88	AWD ^e
3	69/F	15	4	2–20	≤2	T3(m)N1M0	G2	IIIB	+	N	—	+	—	126	AWD
4	71/F	10	1	<2	≤2	T3N1M1	G1	IV	+	L	2	+	—	54	DWD
5	53/F	17	2	<2	≤2	T3(m)N1M1	G1	IV	+	L	—	+	—	97	AWD
6	74/F	25	2	<2	≤2	T3(m)N1M1	G1	IV	+ ^f	L, PE	1	+	—	83	DWD
7	63/F	13	1	<2	≤2	T3N1M1	G1	IV	+	L	2	+	—	82	DWD
8	66/F	13	1	<2	≤2	T3N1M1	G1	IV	+	L	2	+	—	57	DWD
9	63/M	20	5	<2	≤2	T3(m)N1M1	G1	IV	+ ^d	PE	—	+	—	79	AWD
10	71/F	28	2	<2	≤2	T3(m)N0M0	G1	IIIB	+	—	—	—	—	78	AWD ^e
11	49/M	10	15	<2	≤2	T3(m)N1M1	G1	IV	+ ^f	L	—	+	—	5	DWD
12	68/M	30	1	<2	≤2	T3N1M1	G1	IV	+	N, L	—	—	—	22	DWD
13	64/F	30	1	<2	≤2	T4N1M1	G1	IV	+	L	2	+	—	53	DWD
14	42/F	22	1	<2	≤2	T3N1M1	G1	IV	+	L	5	+	—	120	AWD
15	81/F	30	1	<2	≤2	T3N1M1	G1	IV	+	L, PE	—	+	—	35	DWD
16	59/M	3	1	<2	≤2	T1N1M1	G1	IV	+ ⁱ	L	2	—	—	57	AWD
17	53/F	25	2	<2	≤2	T3(m)N1M1	G1	IV	+	N, L, S	—	+	—	80	DWD
18	68/M	60	1	<2	≤2	T3N1M1	G1	IV	+	L, PE	2	+	—	18	DWD
19	71/F	20	1	<2	≤2	T3N1M1	G1	IV	+ ⁱ	L, PE	4	+	—	33	AWD
20	73/M	5	1	<2	≤2	T2N1M1	G1	IV	+	L	—	+	—	6	DWD
21	56/F	28	1	<2	≤2	T3N1M1	G1	IV	+ ^k	L	—	+	—	14	DWD
22	53/M	5	1	<2	≤2	T3N1M1	G1	IV	+	L	2	+	—	63	DWD
23	80/F	60	1	<2	≤2	T3N1M1	G1	IV	+	L	2	+	—	116	AWD
24	79/M	12	1	<2	≤2	T3N1M1	G1	IV	+ ^d	N, L, PE	—	+	—	24	AWD
25	71/M	6	1	<2	≤2	T3N1M1	G1	IV	+	L	—	+	—	111	AWD
26	56/F	38	1	2–20	3–20	T3N1M1	G2	IV	+ ⁱ	L, PE	2	+	—	38	AWD
27	70/M	50	1	<2	≤2	T3N1M1	G1	IV	+	N, L	1	+	—	15	AWD
28	72/M	11	1	<2	≤2	T3N1M1	G1	IV	+	N	—	+	—	57	AWD
29	64/M	25	2	<2	≤2	T3(m)N1M1	G1	IV	+	N, L, S	3	+	—	97	DWD
30	64/F	30	1	<2	≤2	T3N1M1	G1	IV	+ ^{d,k}	—	—	+	—	34	NED
31	61/F	25	1	2–20	3–20	T3N1M1	G2	IV	+	N, PE	—	+	—	15	DWD
32	71/M	9	5	<2	≤2	T3(m)N1M1	G1	IV	+ ^k	—	—	—	—	80	AWD ^e
33	61/F	9	6	<2	≤2	T3(m)N1M1	G1	IV	+	L	2	+	—	18	AWD
34	70/F	25	1	2–20	3–20	T3N1M1	G2	IV	+ ⁱ	L, PE	2	+	—	14	DWD
35	73/F	20	1	2–20	3–20	T3N1M1	G2	IV	+	L	2	—	—	21	DWD
36	75/F	14	2	<2	≤2	T3(m)N1M1	G1	IV	+ ^f	N, L	2	+	—	34	DWD
37	60/M	27	1	<2	≤2	T3N1M1	G1	IV	+ ^{f,k}	—	—	—	—	49	AWD ^e
38	51/M	50	1	2–20	≤2	T3N0M1	G2	IV	+	N, L	—	+	—	54	AWD
39	47/F	6	1	<2	≤2	T1N1M0	G1	IIIB	+	N	2	+	—	258	AWD

Table 1 continued

Case	Primary tumour									Residual disease after primary surgery	HAE	SSTR analogs	IFN- α	Carcinoid heart disease ^c	Hormonal symptom	Other malignancies	Follow-up (mo.)	Current status
	Age/ sex	Size (mm)	No.	Mitotic-count ^a (10 HPF)	Ki67 ^a (%)	TNM, grade and stage ^a												
40	75/F	15	7	<2	≤2	T3(m)N1M1	G1	IV	+ ^{f,k}	L	1	+	+	—	+	—	40	DWD
41	53/M	10	2	<2	≤2	T3(m)N1M0	G1	IIIB	+	—	4	+	—	—	—	—	110	AWD ^e
42	74/M	25	1	<2	≤2	T3N1M1	G1	IV	+	N, L	—	+	+	—	+	+ ^o	16	DWD
43	74/M	15	1	<2	≤2	T3N1M1	G1	IV	+ ^f	N, L	2	+	—	—	+	—	38	DWD

HAE, hepatic artery embolization; F, female; M, male; P, primary tumour; L, liver metastases; N, lymph node metastases; PE, peritoneal metastases; S, skeleton metastases; AWD, alive with disease; DWD, dead with disease; NED, no evidence of disease.

^aAccording to ENETS proposal (see Ref. Rindi *et al.* (2007)).

^bIncludes resection of primary tumour, regional lymph nodes and cholecystectomy.

^cDefined as tricuspid regurgitation by echocardiography (see Ref. Westberg *et al.* (2001)).

^dPrimary surgery and resection of peritoneal metastases.

^eInterval from primary surgery until recurrence: case 2 (42 mo.), case 10 (20 mo.), case 32 (11 mo.), case 37 (5 mo.) and case 41 (47 mo.).

^fPrimary surgery and high central lymph node resection.

^gBreast cancer.

^hMalignant melanoma.

ⁱPrimary surgery and para-aortal lymph node resection.

^jPrimary surgery, sigmoideum and SOE bilateral resection.

^kPrimary surgery and liver resection.

^lPrimary surgery, hysterectomy and SOE resection.

^mPapillary thyroid cancer.

ⁿGastrointestinal stromal tumour and colon cancer.

^oProstate cancer.

Table 2 Copy number alterations identified by array-based comparative genomic hybridization in 43 patients with ileal carcinoid. Altogether, 52 samples were analyzed (24 primary tumours and 28 liver metastases)

Case	P/M	Gains	Losses
1	P		18
2	P		18
3	P		18
4	P		18
5	P		18
6	P		18
7	P	4	18
8	P	20	18
9	P		16p, 18
10	P	2p25.2–p25.1	18, 22
11	P	14	9p, 3p21.31
12	P	17q25.1	16q13-qter, 18
13	P	5pter-p11.21	11q21–q24.2, 16q12.2-qter, 18
14	P	4pter-q13.13, 4q21.21–q21.23, 4q22.1–q22.3, 4q25–q28.3, 4q31.22–q32.2, 5, 20	1p35.1–p34.3, 1p32.2–p31.3, 1p31.2–p21.1, +1p13.2–p13.1, 16q, 18, X
15	P		1q32.1, 1q32.1, 1q32.1–q41, 3p25.1, 3p24.3–p24.2, 3p24.2, 3p24.1, 3p24.1–p22.2, 3p14.1, 3p13, 4p13–q13.2, 9p11.1–q22.1, 11q13.5, 13q12.12–q12.2, 13q12.3, 13q14.2–q14.3, 16q12.1-qter, 18
16	P	20q11.23-qter	18q22.2-qter
	M	12q14.1	2q22.1–q24.2, 11q22.1–q23.3
17	P	6q27	18
	M	6q27	11q14.2–q24.1, 15q26.1, 17q25.3, 18
18	P		18, 22
	M	4, 5, 7, 8, 10, 14, 15, 17p13.1	11q14.1-qter, 13
19	P	3, 4, 5q14.1-qter, 14q11.2–q13.1, 14q23.3–q24.1, 20	
	M	3, 4, 5, 8, 12pter-p13.2, 14 ^a , 20	
20	P	4, 5, 7, 11q12.2, 14 ^a , 16p11.2, 20	16q21-qter
	M	4p, 5, 7, 14, 16q11.2–q12.1, 20	12p13.2–p11.22, 16q12.1-qter
21	P	4, 5, 6, 14, 20	3p21.31
	M	4, 5, 6, 10q11.23–q21.1, 14 ^a , 20	3p21.31, 9, 17q11.2, 22
22	P		2q22.3–q24.3, 7p22.2–p12.1, 18
	M	20q	2q22.3–q24.3, 4p15.33, 7p22.1–p12.1, 9p22.2–p22.1, 9p21.3–p21.2, 9p21.1–p13.1, 9q, 10q22.3, 11q22.3–q23.1, 12p13.31–p13.2, 12q12, 12q21.1, 12q21.31, 13q12.3, 13q34, 18
23	P	5q34.1, 7q36.1, 12q14.1	18
	M	3q11.2–q21.1, 3q21.1–q25.1, 3q26.2–q26.31, 4, 5, 21q22.13-qter	2, 3p14.1–p12.3, 3p12.3–p12.1, 3q21.1, 3q25.1–q26.2, 3q26.31–q27.1, 3q29, 6p24.1–p22.3, 9, 11, 13, 18, 21q22.13

Table 2 continued

Case	P/M	Gains	Losses
24	P	4, 5, 12p12.3–p11.22, 12q21.2-qter, 14q24.3-qter, 16q11.2–q12.2, 20	12p13.2–p12.3, 12q12, 12q14.2–q21.1, 14q23.3, 14q24.1–q24.3, 16q12.2-qter, 18
	M	4, 5, 12p12.3–p11.1, 12q21.2-qter, 14q24.3-qter, 16q11.2–q12.2, 20	12p13.2–p12.3, 12q12, 12q14.2–q21.1, 14q23.3, 14q24.1–q24.3, 16q12.2-qter, 18
25	M		16q12.1–q22.1, 18
26	M	20p	9p, 18
27	M	4	1p35.2–p35.1, 18
28	M	20	3p14.1–p12.3, 3q22.1–q22.3, 13q14.2–q14.3
29	M	7p15.2	11q14.1–q24.2, 12p13.31, 17p13.1, 18
30	M	3p21.31, 11q13.2, 12p13.31	18, 22q11.21, X
31	M	4, 5, 7, 10, 14 ^a , 17q21.2-qter, 20	
32	M		1q25.2–q31.3, 1q32.1–q42.12, 2p13.3, 3q22.2–q22.3, 3q26.2–q26.31, 3q26.32–q26.33, 18
33	M	11q13.2, 17q12-qter, 21, X	9, 11q14.1–q24.1, 18
34	M	4, 5, 7, 12p13.31, 14 ^a , 20, 21	12p13.31
35	M	1q, 4, 5, 7, 10, 12, 14 ^a , 20	1p, 13
36	M	20	1p, 8, 9, 13, 15, 17pter-q22, 18, 19, 21
37	M		3p25.1–p24.2, 3p14.2–p12.3, 3q13.13–q13.2, 3q21.1–q21.2, 3q21.3–q22.1, 3q24–q26.1, 3q26.2–q26.33, 8q12.1, 12p13.2–p13, 18
38	M		3p14.1–p12.3, 3q22.1, 5p15.2, 5q31.1, 5q31.3–q33.1, 5q33.2, 14q13.2, 14q13.3, 14q23.2–q23.3, 14q24.1–q24.3, 18, 22q13.1–q13.2
39	M	11p13, 15q26.3	2pter-p24.1, 3pter-q22.1, 6, 8p12, 9p, 11q14.1-qter, 12p13.2–p12.3, 13, 17pter-p13.1, 22
40	M	1q, 4, 20	1p, 2, 3, 6q, 7q22.1, 9, 11p14.3–p12, 11q22.1–q22.2, 11q23.3–q24.2, 13, 18
41	M	5, 6, 11, 14 ^a , 17, 20, 21, xp21.1	3p26.2–p25.2, 3p14.1–p13, 3q13.13, 3q26.31–q29, 9, 12p13.31, 18
42	M	4, 5, 10, 14, 20	1p36.32, 1p36.22, 1p36.21, 1p36.13–p36.12, 1p36.11–p35.2, 1p34.3–p31.3, 1p31.3–p31.1, 1p31.1–p22.2, 2q21.1–q32.1, 13, 16q12.1
43	M	1p34.1–p33, 2q35, 3q27.3–q28, 4pter-q28.1, 4q31.22-qter, 5q23.3, 6q12, 7p15.2–p14.3, 7q21.11, 7q21.13–q21.3, 7q36.1, 11q22.1–q22.2, 13q32.3–q33, 20, xp11.3–p11.22, xp11.21–p11.1	1p22.3, 4q31.21, 13q13.1–q22.3, 16q23.2-qter, 18

P, primary tumour; M, liver metastasis.

^aConfirmed by FISH analysis.

Figure 1 Genome-wide penetrance plot of CNAs in 24 primary tumours (A) and 28 liver metastases (B). Losses (green) were mainly detected on chromosome 9, 11, 13 and 18, while gains (red) were mainly observed on chromosomes 4, 5, 14 and 20. Loss on chromosome 18 was the most frequent alteration, both in primary tumours and liver metastases.

samples. The average number of CNAs per tumour sample was 7.1 (range 1–22). Losses were more common than gains, with a ratio of 1.4. CNAs were detected most frequently on chromosome arms 18q (71%), 18p (69%), 4p, 20q (40% each), 20p (38%), 4q, 5q (37% each), 14q (35%), 5p (33%), 11q (31%), 12p (29%), 3p and 16q (27% each). Loss or gain of whole chromosomes accounted for 41% of all CNAs. No high-level amplifications were observed. Homozygous loss was observed in one patient. A comparison between primary tumours and liver metastases revealed that the average number of CNAs per tumour was twice as high in liver metastases (9.5, range 2–22) as in primary tumours (4.3, range 1–18). The proportions of losses and gains were similar in primary tumours and liver metastases.

Recurrent CNAs, defined as alterations occurring in three or more patients, accounted for 59% of all alterations. A summary of recurrent CNAs is given in Table 3. Detailed information on all recurrent CNAs is given in Supplementary Table 1, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>. aCGH profiles of individual tumours are given in Fig. 2. The most frequently recurring CNA was loss of an entire chromosome 18, which was observed in 74% of all patients. Loss of chromosome 18 was detected with similar frequency in primary

tumours (79%) and liver metastases (61%). Only one tumour (primary tumour) showed partial loss of 18q22.2-qter. Losses of chromosomes 9 and 13 were found at lower frequency (14 and 16% of patients respectively) and they were only observed in liver metastases.

The most frequently recurring segmental CNAs were found on chromosome 11, and included deletions of three minimal common regions (11q22.1–q22.2, 11q22.3–q23.1 and 11q23.3), all of which were present in 21% of the patients. Candidate tumour-suppressor genes in these regions include radixin (*RDX*), B-cell translocation gene 4 (*BTG4*), protein phosphatase 2, regulatory subunit A, beta isoform (*PPP2R1B*) and transgelin (*TAGLN*). Losses on chromosome 11 were three times more common in liver metastases than in primary tumours. Recurrent CNAs on chromosome 16 included two minimal common regions (16q12.2–q22.1 and 16q23.2–qter), which were lost in 16% of the patients. Candidate tumour-suppressor genes in these segments include *CDH 1*, *3*, *5*, *8*, *11*, *13*, *15*, *TRADD*, *E2F4*, *CTCF*, *OSGIN1*, *WFDC1*, *IRF8*, *FBXO31*, *BANP*, *CBFA2T3*, *ZNF276*, *FANCA* and *GAS8*. Losses on chromosome 16 were twice as common in primary tumours as in liver metastases. Loss of 3p13 was observed in 14% of the patients, and it was four times as common in

Table 3 Recurrent copy number alterations (CNAs) in 43 patients with ileal carcinoid analyzed by array-based comparative genomic hybridization

Chromosome	Minimal common region	Start and stop	Size (Mb)	No. of annotated genes ^a	Frequency of CNAs		
					P ^b	M ^b	Patients ^b
Losses							
1	1p22.3	87123525–87715408	0.6	2	1	2	3
	1p				0	3	3
2	2q22.3–q24.2	147651413–162610374	15	10	1	3	3
3	3p13	72179608–73194811	1.0	5	1	5	6
	3p21.31	48777730–48939935	0.2	4	2	2	3
	3q21.1	124646570–124983177	0.3	3	0	3	3
	3q22.1	132971150–133569797	0.6	13	0	3	3
	3q22.1	134528771–135763122	1.2	2	0	3	3
	3q26.32–q26.33	178193148–180811471	2.6	11	0	4	4
9	9p				1	2	3
	9				0	6	6
11	11q22.1–q22.2	96830209–101803071	5.0	10	2	7	9
	11q22.3–q23.1	108214469–111299004	3.1	20	2	7	9
	11q23.3	115832077–118400328	2.6	45	2	7	9
12	12p13.2	11030657–12766204	1.7	23	1	5	5
13	13q14.2–q14.3	48467994–51626277	3.2	26	1	2	3
	13				0	7	7
16	16q12.1	48563960–48816620	0.3	3	1	2	3
	16q12.2–q22.1	54446243–67347099	13	133	6	3	7
	16q23.2–qter	78736208–88651780	9.9	96	6	3	7
18	18				19	17	32
22	22				2	2	4
Gains							
4	4				5	11	13
5	5				4	11	12
7	7				1	5	5
10	10				0	4	4
14	14				3	9	10
20	20				6	12	14
21	21				0	3	3

^aOnly the numbers of genes in defined chromosome segments are given.^bPrimary tumours: *n*=24; liver metastases: *n*=28; patients: *n*=43.

liver metastases as in primary tumours. One candidate tumour-suppressor gene in this segment is *RYBP*. There were no recurrent gains of specific chromosome segments. There were recurrent gains of whole chromosomes, however, most frequently on chromosomes 20 (33%), 4 (30%), 5 (28%) and 14 (23%). Gains of whole chromosomes were more frequent in metastatic tumours than in primary tumours.

Two groups of ileal carcinoids with distinct patterns of CNAs

Loss of chromosome 18 was the most common CNA in ileal carcinoids. Loss of an entire chromosome 18 was identified in 32 out of 43 patients (74%), while 1 patient had partial loss of chromosome 18. Altogether, loss on chromosome 18 was demonstrated in 33 out of

43 patients (77%). In six patients, loss of chromosome 18 was the only CNA observed (all in primary tumours). Apart from loss of chromosome 18, other CNAs were losses involving chromosomes 3, 9, 11, 13 and/or 16 and gains involving chromosomes 4, 5 and/or 20. In general, tumours with losses on chromosome 18 were characterized by a limited number of CNAs per tumour, with losses being more common than gains (ratio 2.1).

In ten patients, representing 23% of all cases, no CNA involving chromosome 18 was detected. This group of tumours was characterized by a limited number of CNAs per tumour, with losses being less common than gains (ratio 0.5). The most frequent gains in this group involved chromosomes 4, 5, 7, 10, 14 and 20, and these occurred with similar frequency in primary tumours and liver metastases.

Figure 2 Examples of aCGH profiles illustrating recurrent CNAs in ileal carcinoids. (A) Profiles of chromosome 18 demonstrating segmental loss of 18q22.2-qter in one primary tumour (case 16) and loss of an entire chromosome 18 in one primary tumour (case 1) and two liver metastases (cases 29 and 37). (B) Profiles of chromosome 14 demonstrating segmental gains of 14q11.2–q13.1 (case 19, primary), 14q23.3–q24.1 (case 19, primary) and 14q24.3-qter (case 24, liver metastasis) and gain of an entire chromosome 14 in two liver metastases (cases 18 and 20). (C) Profiles of chromosome 11 demonstrating segmental losses with a minimal common region at 11q22.3–q23.1 (cases 39, 33, 16 and 22, liver metastases). The location of candidate tumour-suppressor genes *RDX*, *BTG4* and *PPP2R1B* is indicated.

Survival analysis suggested poor prognosis in patients with ileal carcinoids showing gain of chromosome 14

Survival analysis was performed with respect to all recurrent CNAs detected in ileal carcinoids (Table 3). There was a significant correlation between four variables (gain of 7, 14 and 20, and loss of 18) and overall survival ($P < 0.05$); in this context, three variables (presence of gain of whole chromosomes in general, gain of chromosome 4 and loss of 3p13) were almost significant in the series of Poisson regressions ($P < 0.1$). Following multivariate analysis, there was a significant correlation between 1) loss of 3p13 or 2) gain of chromosome 14 or 3) carcinoid heart disease and survival. Hormonal symptoms and Ki67 did not correlate with survival (see Supplementary Table 2, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>). Patients with loss of 3p13 had a significantly reduced risk of death ($P = 0.028$) with a hazard ratio (HR) of 0.14 (95% CI: 0.02–0.8), while patients with gain of chromosome 14 had a significantly increased risk of death ($P < 0.001$) with an HR of 8.39 (95% CI: 3.04–23.11). Patients with carcinoid heart disease also had a significantly increased risk of death ($P < 0.044$) with an HR of 2.98 (95% CI: 1.03–8.63).

A Kaplan–Meier survival plot for patients with ileal carcinoids showing gain of chromosome 14 is shown in Fig. 3. There was gain of chromosome 14 in 10 out of 43 patients. Gain of chromosome 14 was more common in liver metastases (9 out of 28) than in primary tumours (3 out of 24). One patient with gain of

Figure 3 Kaplan–Meier survival analysis of ileal carcinoids. Patients with gain of chromosome 14 had a significantly worse prognosis than patients with no gain of chromosome 14 (HR 8.39, 95% CI: 3.04–23.11).

Figure 4 FISH analysis of an ileal carcinoid with gain of chromosome 14. FISH analysis was performed on a paraffin section of a primary tumour (case 20), using a telomere-specific probe for chromosome 14, and confirmed the aCGH finding.

chromosome 14 had segmental gains in the primary tumour (14q11.2–q13.1 and 14q23.3–q24.1) and gain of whole chromosome 14 in the liver metastasis. Segmental gain of chromosome 14 (14q24.3–qter) was observed in another patient. Patients with gain of chromosome 14 rarely displayed loss of chromosome 18 (two cases only), but they frequently had gain of chromosomes 4, 5, 7, 10 and 20. Gain of chromosome 7 was found in five out of ten tumours with gain of chromosome 14, but it could not be identified in any of the tumours with loss of chromosome 18.

FISH analysis of ileal carcinoids confirmed gain of chromosome 14

Patients with gain of chromosome 14 identified in the aCGH analysis were analyzed by FISH using a telomeric probe for chromosome 14. The FISH analysis confirmed gain of chromosome 14 in seven out of seven patients analyzed (Fig. 4). Gain of chromosome 14 was not found in 100% of the tumour cells in any of the tumours. The frequency of trisomic cells varied from 36 to 86% in the different tumours.

Discussion

Here, we present for the first time an in-depth analysis of ileal carcinoids, involving high-resolution genomic profiling of tumours from a large number of patients with long follow-up (median 53 months, range 5–258 months). We identified loss of chromosome 18 as the most common CNA; it occurred in 74% of the patients with ileal carcinoids. Loss of chromosome 18 was detected with similar frequency in primary tumours

and liver metastases, suggesting that changes involving chromosome 18 are early events in tumour formation. The question of which genes on chromosome 18 are important for carcinoid tumour formation could not be answered from our data. Only one of our patients displayed segmental loss on chromosome 18 (18q22.2–qter). This is in line with previous studies demonstrating losses of 18q12–qter, 18q21–qter, 18q21.1 or 18q22–qter in ileal carcinoids using chromosome-based CGH or SNP analysis (Zhao *et al.* 2000, Kytölä *et al.* 2001, Kulke *et al.* 2008). Candidate genes at 18q21.1 include *SMAD2* and *SMAD4*, but to date sequencing of these genes has not revealed any mutations (Löllgen *et al.* 2001, Kulke *et al.* 2008).

The most common segmental losses in ileal carcinoids occurred on 11q22.1–q22.2, 11q22.3–q23.1 and 11q23.3. These alterations were three times more frequent in liver metastases than in primary tumours, indicating that these changes are associated with tumour progression. Loss of 11q22–23 has also been described in a number of other solid tumours including carcinoma of the breast and colon, lymphoproliferative diseases and haematological malignancies. *RDX*, *BTG4*, *PPP2R1B* and *TAGLN* are candidate tumour-suppressor genes in these regions. *RDX* is a member of the ezrin–radixin–moesin family of proteins, which act as cytoskeletal adopters and signalling proteins (Hoefflich & Ikura 2004). Expression of *RDX* can be downregulated in adenocarcinomas of the lung (Tokunou *et al.* 2000). *BTG4* is a cell cycle regulator that induces G1 arrest. Loss of *BTG4* is a common event in chronic lymphocytic leukaemia, and epigenetic silencing of *BTG4* by CpG island methylation occurs in colorectal cancer (Auer *et al.* 2005, Toyota *et al.* 2008). *PPP2R1B* regulates RalA GTPase and inhibits cell growth (Sablina *et al.* 2007). Inactivation of *PPP2R1B* by downregulation, or mutation, has been demonstrated in B-cell chronic lymphocytic leukaemia, lung cancer and colon cancer (Wang *et al.* 1998). *TAGLN* is an actin cross-linker protein involved in replicative senescence, and loss of this gene has also been demonstrated in breast and colon cancer (Shields *et al.* 2002, Yang *et al.* 2007). There are no reports of *RDX*, *BTG4*, *PPP2R1B* or *TAGLN* expression in ileal carcinoids, and the role of these tumour-suppressor genes in carcinoid tumour progression remains to be determined.

The second most common segmental loss occurred on chromosome 16, including the minimal common regions 16q12.2–q22.1 and 16q23.2–qter. Loss of these regions has been demonstrated in a large number of solid tumours – including tumours of the breast,

prostate, liver, lung and ovary (Rakha *et al.* 2006). A large number of candidate tumour-suppressor genes are present in these regions, including a group of cadherins (*CDH 1, 3, 5, 8, 11, 13* and *15*). E-cadherin (*CDH1*) is a key regulator of the epithelial–mesenchymal transition, which is activated during tumour invasion and dissemination (Schmalhofer *et al.* 2009). Downregulation of E-cadherin has been demonstrated in carcinoid tumours (Sun *et al.* 2002).

The third most common segmental loss occurred on 3p13, which was four times more common in liver metastases than in primary tumours, indicating that this alteration accumulates during tumour progression. Only one tumour-suppressor gene, *RYBP*, was present in this region. *RYBP* (RING1 and YY1 binding protein, also known as *DEDAF*) functions as a repressor of E4TF1 and induces apoptosis in tumour cells (Danen-van Oorschot *et al.* 2004). The preferential tumour-killing activity of *RYBP* has made it a promising target for cancer therapy (Novak & Phillips 2008).

The pattern of CNAs in ileal carcinoids appears to be specific for this type of tumour and it is different from that previously reported for other well-differentiated neuroendocrine tumours, e.g. pancreatic endocrine tumours and lung carcinoids. In the latter tumours, losses on chromosome 11, including the *MEN1* locus, have been shown to be a primary tumour oncogenic event (Walch *et al.* 1998, Zhao *et al.* 2001). When comparing carcinoid tumours with an intact chromosome 18 and tumours with loss of chromosome 18, two distinct patterns of CNAs appeared. Tumours with intact chromosome 18 had more gains of whole chromosomes, in particular gains of 4, 5, 7, 10, 14 and 20, than tumours with loss of chromosome 18. These findings suggest that carcinoid tumours may arise via two separate genetic pathways, as previously suggested by Kulke *et al.* (2008). Loss of chromosome 18 is of importance in the majority of tumours, while a different pathway may operate in a subset of tumours.

The perhaps most striking observation in the present series of carcinoids was that survival analysis demonstrated a strong correlation between gain of chromosome 14 and poor survival (HR 8.39; 95% CI: 3.04–23.11). This is the first time that a genetic event has been linked to the clinical course of ileal carcinoids. We also confirmed our aCGH finding of gain of chromosome 14 by FISH analysis. In a series of 18 ileal carcinoids, Kulke *et al.* (2008) reported gain of 14q11.2 and suggested that *DAD1* might be a potential oncogene in the tumorigenesis. To identify candidate oncogenes on chromosome 14 that may be responsible for the poor prognosis is difficult in the present series of tumours, because segmental gains

(i.e. 14q11.2–q13.1, 14q23.3–q24.1 and 14q24.3–qter) were only observed in two patients. An interesting observation is that ileal carcinoids with gain of chromosome 14 usually had intact chromosome 18. However, gain of chromosome 14 and loss of chromosome 18 were not mutually exclusive, since two patients had both alterations. Gain of chromosome 14 was observed in both primary and metastatic tumours. We therefore suggest that carcinoid tumours with gain of chromosome 14 follow a different genetic pathway than the majority of ileal carcinoids. The clinical relevance of subgrouping carcinoid tumours according to CNAs should be evaluated in a larger series of patients. It is, however, clear that gain of chromosome 14 is an important indicator of poor prognosis. As a consequence, we suggest that biopsies from patients with malignant ileal carcinoids should be subjected to FISH analysis of chromosome 14, which can readily be performed on paraffin-embedded specimens. Patients with gain of chromosome 14 should be under strict surveillance in order to be able to treat recurrences at an early stage.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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