DNA copy number status is a powerful predictor of poor survival in endocrine pancreatic tumor patients

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Abstract

The clinical behavior of endocrine pancreatic tumors (EPTs) is difficult to predict in the absence of metastases or invasion to adjacent organs. Several markers have been indicated as potential predictors of metastatic disease, such as tumor size $\geq$2 cm, Ki67 proliferative index $\geq$2%, cytokeratin (CK) 19 status, and recently in insulinomas, chromosomal instability (CIN). The goal of this study was to evaluate the value of these markers, and in particular of the CIN, to predict tumor recurrence or progression and tumor-specific death, using a series of 47 insulinomas and 24 non-insulinoma EPTs. From these EPT cases, a genomic profile has been generated and follow-up data have been obtained. The proliferative index has been determined in 68 tumors and a CK19 expression pattern in 50 tumors. Results are statistically analyzed using Kaplan–Meier plots and the log-rank statistic. General CIN, as well as specific chromosomal alterations such as 3p and 6q loss and 12q gain, turned out to be the most powerful indicators for poor tumor-free survival ($P \leq 0.0004$) and tumor-specific death ($P \leq 0.0113$) in insulinomas. The CIN, chromosome 7q gain, and a proliferative index $\geq$2% were reliable in predicting a poor tumor-free survival in non-insulinoma EPTs ($P \leq 0.0181$, whereas CK19 expression was the most optimal predictor of tumor-specific death in these tumors. In conclusion, DNA copy number status is the most sensitive and efficient marker of adverse clinical outcome in insulinomas and of potential interest in non-insulinoma EPTs. As a consequence, this marker should be considered as a prognosticator to improve clinical diagnosis, most practically as a simple multi-target test.

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Introduction

The clinical behavior of endocrine pancreatic tumors (EPTs) is difficult to predict on the basis of their histological features. The presence of metastases is generally accepted to be the only definitive feature of malignancy. Therefore, a reliable classification system is crucial to predict the biological behavior of these tumors. The current WHO classification system is based on expert opinion, but so far its power in large series of individual EPT subtypes with a long-term follow-up remains to be evaluated (Heitz *et al.* 2004). The classification criteria comprise the presence of metastases, gross invasion, tumor size, percentage of mitoses, proliferative index, and vascular invasion. Tumor resection, the absence of liver and lymph node metastases, and the presence of multiple endocrine

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neoplasia type 1 (MEN1) syndrome appear to be related with a better survival rate (Tomassetti *et al.* 2005).

Several studies have reported potential biomarkers that are indicators for malignancy of EPTs, such as α-chain of human chorionic gonadotropin-α (HCG-α), cyclooxigenase-2 (COX2), $p27^{KIP1}$, CD99, cytokeratin 19 (CK19), and p53. HCG-α is expressed by ∼65% of malignant functioning EPTs. However, since it is also expressed in benign tumors, this marker is considered to be of limited value (Heitz *et al.* 1987, Graeme-Cook *et al.* 1990). Up-regulation of COX2 and CK19, and down-regulation of p27 and CD99 were found to be associated with Ki67 positive, proliferating tumor cells (Canavese *et al.* 2001, Guo *et al.* 2001, Ohike & Morohoshi 2001, Rahman *et al.* 2003, Goto *et al.* 2004, Ali *et al.* 2006). Controversy exists with respect to p53 expression as a marker for malignancy in EPTs. Lee (1996) suggested no role for p53, whereas Pavelic *et al.* (1995) identified p53 overexpression in all the three cases of malignant insulinomas. We have recently shown that in insulin-producing EPTs, chromosomal instability (CIN), identified by analysis of DNA copy number changes using comparative genomic hybridization (CGH), is an optimal predictor for malignant progression (Jonkers *et al.* 2005). Ki67, p53, and/or CK19 expression have been found to be associated with malignancy only in a few individual insulinoma cases (Jonkers *et al.* 2006*a*).

CK19 is a potential immunomarker described to predict poor survival in EPTs. By comparing classification criteria and CK19 immunostaining in a series of 101 EPTs, it was found that CK19 was the only significant predictor of poor survival (Deshpande *et al.* 2004). In the study presented here, our aim was to investigate the reliability of DNA copy number alterations, including CIN in comparison with CK19 and other clinical parameters to predict poor survival in EPTs. For this purpose, we have collected follow-up data of 71 EPT patients, including 47 cases of insulinoma.

Materials and methods

Tumor material and patient data

Seventy-one EPTs for which a CGH profile was generated (Speel *et al.* 1999, 2001, Zhao *et al.* 2001, Jonkers *et al.* 2005, 2006*b*) and follow-up data could be obtained, were studied here. They included 47 insulinomas, 6 gastrinomas, 2 glucagonomas, 5 vipomas, and 11 non-functioning tumors. These cases and their corresponding follow-up data were available from the archives of the Departments of Pathology of the University Hospital Zurich, Switzerland, and the University of Torino, Italy, the Department of Medical Sciences, University Hospital Uppsala, Sweden, and the Departments of Pathology of the University Medical Centers of Rotterdam, Utrecht and Nijmegen, The Netherlands (Table 1). Ki67 proliferative index could be determined in 68 tumors and CK19 expression in 50 tumors. The study was done in line with the code 'Proper Secondary Use of Human Tissue' as implemented by the Dutch Federation of Biomedical Scientific Societies. The tumors were classified according to the most recent WHO classification (Heitz *et al.* 2004). All tumors were not associated with the inherited MEN1 syndrome. The mean age of the 71 EPT patients, including 40 females and 31 males, was 50.6 years (range 5–82 years). Follow-up ranged from 0.6 to 21.5 years (mean 7.3 years). The diameter of the insulinomas ranged from 0.5 to 10 cm (mean 2.0 cm). The diameter of the other functioning EPTs ranged from 2.4 to 8 cm (mean 4.4 cm) and the non-functioning EPTs from 1.2 to 10 cm (mean 5.8 cm).

Twenty-eight of the patients had localized disease at diagnosis as defined by: 1) the absence of extrapancreatic spread of the tumor as evidenced by Computed Tomography, magnetic resonance imaging or ultrasound scanning and 2) a tumor size smaller than 2 cm in diameter. Nineteen patients had a tumor with uncertain behavior, defined by the absence of extrapancreatic spread of the tumor, but with a tumor size of at least 2 cm in diameter, angioinvasion or a proliferative index of at least 2%. Twenty-four patients showed metastatic disease at diagnosis.

Detection of CIN by CGH analysis

CGH was used to analyze genome-wide DNA copy number imbalances in EPTs (Speel *et al.* 1999, 2001, Zhao *et al.* 2001, Jonkers *et al.* 2005, 2006*a*,*b*). This approach uses differentially labeled tumor and 'reference' DNA, which are competitively hybridized to normal metaphase chromosomes (conventional CGH) or to mapped genomic clones (array CGH). The ratio of the fluorescence intensities detected is indicative of the relative DNA copy number in tumor versus reference DNA (Fig. 1A; Kallioniemi *et al.* 1992, Davies *et al.* 2005, Pinkel & Albertson 2005). The array CGH analyses were all performed in Maastricht. The conventional CGH analyses of the non-insulinoma EPTs were performed in Zurich with the same resolution as the conventional CGH performed in Maastricht.

Table 1 Tumor diagnosis and follow-up status

Tumor type and nr	Follow-up time (months)	Follow-up status	Diagn.	CIN	Ki67 $\geq$2%	Size $\geq$2 cm	CK19 positive	Meta at diagn.
Insulinoma								
1	216	AW	B	−	−	−	−	−
2	132	AW	B	−	−	−	−	−
3	24	AW	B	−	−	−		−
4	252	AW	B	−	−	−	−	−
5	96	AW	B	−	−	−		−
6	120	AW	B	−	−	−		−
7	132	AW	B	−	−	−	−	−
8	12	AW	B	−	−	−	−	−
9	108	AW	B	−	−	−		−
10	72	AW	B	−	−	−		−
11	96	AW	B	−	−	−		−
12	60	AW	B	−	−	−		−
13	84	AW	B	−	−	−	−	−
14	36	AW	B	−	−	−		−
15	156	AW	B	−	−	−	−	−
16	84	AW	B	−	−	−	−	−
17	144	AW	B	−	−	−	−	−
18	72	AW	B	−	−	−		−
19	36	AW	B	−	−	−	−	−
20	72	AW	B	−	−	−		−
21	168	AW	B	−	−	−		−
22	48	AW	B	−	−	−		−
23	24	AW	B	−	−	−	−	−
24	24	AW	B	+	−	−	−	−
25	48	AW	B	−	−	−		−
26	60	AW	B	−	+	−		−
27	72	AW	B	+	−	−	−	−
28	96	AW	UB	+	+	+		−
29	132	AW	UB	+	−	−		−
30	144	AW	UB	−	−	+		−
31	84	AW	UB	+	−	+		−
32	96	AW	UB	+	−	+	−	−
33	216	AW	UB	+	−	+	−	−
34	12	AW	UB	+	−	+	−	−
35	180	AW	M	+	−	−	−	Meta
36	60	AW	M	+	−	+	−	Meta
37	48	AW	M	+	−	+	−	Meta
38	96	AW	M	−	+	−	−	Meta
39	84	AW	M	−	+	+	−	Meta
40	36	AW	M	+	+	+	−	Meta
41	120	AWD	UB	+	−	+	−	−
42	72	AWD	M	+	+	+	−	Meta
43	120	AWD	M	+	−	+	+	Meta
44	12	DOD	M	+	−	+	+	Meta
45	12	DOD	M	+	−	−	−	Meta
46	12	DOD	M	+	−	+		Meta
47	24	DOD	M	+	+	+	−	Meta
Non-insulinoma EPTs								
Gastrinoma								
1	8	AW	UB	−	−	+	−	−
2	131	AW	UB	−	−	+	−	−
3	258	AW	UB	−	−	+	+	−
4	46	AWD	UB	+	+	+	+	−
5	123	AWD	M	+	−	+	+	Meta
6	18	DOD	M	+	+	+	+	Meta

Table 1 *continued*

Tumor type and nr	Follow-up time (months)	Follow-up status	Diagn.	CIN	Ki67 ≥2%	Size ≥2 cm	CK19 positive	Meta at diagn.
Vipoma								
1	162	AW	M	−	−		−	Meta
2	158	AWD	UB	+	−	+	−	−
3	66	DOC	M	+	+	+	−	Meta
4	99	DOD	UB	+	−	+	−	−
5	58	DOD	M	−	−		+	Meta
Glucagonoma								
1	79	DOD	M	+	+	+	+	Meta
2	12	DOD	M	+				Meta
Non-functioning								
1	99	AW	B	−		−	+	−
2	111	AW	UB	−	−	+	−	−
3	108	AW	UB	−	+	+	−	−
4	170	AWD	UB	+	−	+	−	−
5	63	AWD	UB	+	−	+	+	−
6	33	AWD	UB	+		+		−
7	96	AWD	M	+	+	+	−	Meta
8	102	AWD	M	+	+	+	+	Meta
9	7	DOD	M	−	+	+	+	Meta
10	72	DOD	M	+	−	+	+	Meta
11	28	DOD	M	+	+	+	+	Meta

Diagn., diagnosis; meta, metastases; B, benign; UB, uncertain behavior; M, malignant; AW, alive without disease; AWD, alive with disease; DOC, dead of other cause; DOD, dead of disease. +, marker present; −, marker absent; empty cell, not analyzed.

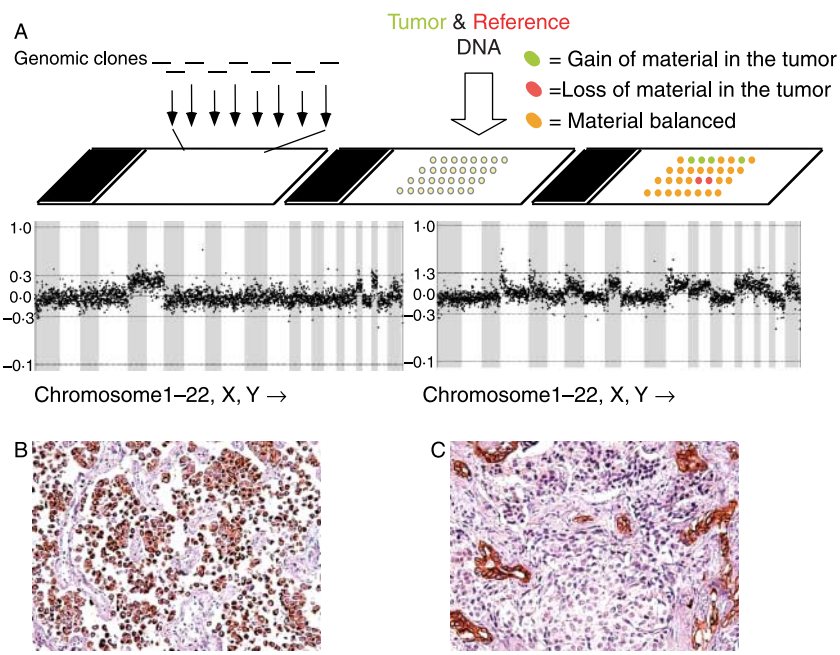

Figure 1 (A) Array CGH procedure with representative examples of array CGH profiles of a benign insulinoma without chromosomal instability (left) and a malignant insulinoma with chromosomal instability (right). Clones are arranged in the order from chromosome 1 to 22, and X, Y on the X-axis. On the Y-axis, the $\log_2$-transformed tumor over reference DNA values are indicated. Immunohistochemistry for cytokeratin (CK) 19 expression, showing a (B) CK19 positive insulinoma and (C) a CK19 negative insulinoma with positive staining in ductal cells.

CIN was defined as the presence of at least eight chromosomal aberrations by conventional CGH or 20 aberrations of at least 10 Mb by array CGH (Fig. 1A; Jonkers *et al.* 2005, 2006*a*). Sixty-three patient samples were analyzed by conventional CGH, 26 by array CGH, and 18 by both methods. In 16 out of these 18 cases, the results matched based on the criteria described above. In the two other cases CIN was detected by conventional CGH, and 18 aberrations of at least 10 Mb were detected by array CGH. These cases were thus considered to have CIN. Also individual chromosomal alterations were evaluated for their predictive value with respect to metastatic disease or poor survival. Of the chromosomal alterations detected by conventional CGH, 97.5% were also detected by array CGH.

CK19 and Ki67 immunohistochemistry

CK19 and Ki67 antigen staining was performed on 4 μm thick paraffin-embedded tissue sections as described previously (Jonkers *et al.* 2005, 2006*a*). Sections were pretreated with 10 mM citrate buffer (pH 6.0) in a microwave oven at 600 W for 15 min, and incubated with a mouse monoclonal antibody directed against Ki67 (MIB1, 1:100 dilution; DAKO, Glostrup, Denmark) or human CK19 (RCK108, 1:200 dilution, MUbio products BV, Maastricht, The Netherlands) respectively. The primary antibodies were detected by the avidin-biotinylated peroxidase complex protocol (ABC Elite kit, Vector laboratories, Burlingame, CA, USA) and peroxidase activity was visualized using diaminobenzidine (DAB)/H_2O_2 (Sigma Chemical Co). The Ki67 proliferative index was expressed as the percentage of tumor cells that were immunopositive. All tumor cases with cytoplasmic staining for RCK108 in $\geq$ 5% of tumor cells were considered CK19 positive (Fig. 1B and C).

Statistical analysis

For the statistical analyses of the clinical data SPSS software was used (SPSS 12.0.1 software, Chicago, IL, USA). The sensitivity and specificity of each factor for predicting the presence of metastatic disease were calculated. The relationship between different parameters was analyzed using the χ^2 or Fisher exact test, as appropriate. The level of significance was defined as $P<0.05$. All factors with statistical significance in a univariate analysis were also included in subsequent multivariate analyses. Survival curves were calculated using the Kaplan–Meier method. The comparison between survival functions for the different factors was assessed with the log-rank statistic.

Tumor-free survival indicated that the patient was still alive, the primary tumor and/or metastases were treated and did not show recurrence and/or progression during follow-up time. When the tumor showed recurrence and/or progression, a patient was designated alive with disease (AWD). Tumor-specific death indicated that the patient died of the disease (DOD) during the follow-up period.

Results

Parameters to predict metastatic disease

Insulinomas

We examined the reliability of different parameters to predict metastatic disease, including tumor size, Ki67 proliferative index, CK19 expression, CIN, and specific chromosomal aberrations. Table 2 shows that CIN turned out to be the most reliable indicator of metastatic disease with a sensitivity of 85%, followed by 7q gain and tumor size. Multivariate analysis showed that a combination of CIN and size or size and 6q loss could increase the sensitivity to 92%. A combination of CIN and Ki67 could even increase this sensitivity to 100%.

Other EPTs

CIN also proved to be the only significant parameter to predict metastatic disease in the non-insulinoma EPTs (Table 2). Multivariate analysis showed an increase in sensitivity when combining CIN with Ki67 or CK19 expression, or when combining 3p loss and 7q or 14q gain as markers. Because this tumor group predominantly comprises tumors with a diameter $\geq$2 cm at presentation, size could not be used as a discriminative predictor for this purpose.

Parameters to predict tumor-free survival and tumor-specific death

Insulinomas

The association between the evaluated parameters and the clinical outcome of insulinomas is presented in Table 3. Tumor recurrence and/or progression or tumor-specific death occurred in a minority of insulinoma patients because of their early presentation usually followed by resection of the tumor. Four insulinoma patients died of disease and three were AWD. Nineteen patients showed CIN including these seven patients. Six out of seven patients with an event had a size larger than 2 cm in diameter. Only two of these patients showed a Ki67 proliferative index of more than 2% or CK19 expression. Kaplan–Meier survival analysis underscored the power of CIN and size as significant markers for poor tumor-free survival

Table 2 Parameters for prediction of metastatic disease in endocrine pancreatic tumors (EPTs)

Insulinomas	Sensitivity[a] (%)	Specificity[b] (%)	OR	95% CI	*P* value
Univariate					
Size ≥2 cm	77	82	15.6	3.3–74.2	0.0005
Ki-67 ≥2%	39	94	10.0	1.6–61.3	0.0189
CK19	17	100			NS
CIN	85	77	17.9	3.3–98.1	0.0005
3p loss	46	91	8.9	1.8–44.3	0.0126
6q loss	54	97	38.5	4.0–372.2	0.0002
7q gain	85	68	11.5	2.2–61.0	0.0039
12q gain	54	85	6.8	1.6–28.7	0.0174
14q gain	62	88	12.0	2.6–55.3	0.0018
Multivariate					
CIN and/or Ki-67 ≥2%	100	74			0.0000
CIN and/or size ≥2 cm	92	74	33.3	3.8–294.3	0.0002
Size ≥2 cm and/or 6q loss	92	82	56.0	6.1–516.8	0.0000
EPTs excluding insulinomas					
Univariate					
Size ≥2 cm	100	13			NS
Ki-67 ≥2%	53	83			NS
CK19	67	71			NS
CIN	81	75	13.0	1.7–99.4	0.0254
3p loss	81	63			NS
6q loss	56	63			NS
7q gain	56	75			NS
12q gain	31	88			NS
14q gain	50	88			NS
Multivariate					
CIN and/or CK19	94	50	15.0	1.3–174.4	0.0506
CIN and/or Ki-67 ≥2%	88	63	11.7	1.5–91.5	0.0390
3p loss and/or 7q/14q gain	94	50	15.0	1.3–174.4	0.0506

OR, odds ratio; 95% CI, 95% confidence interval.
[a]Percent of the patients with metastases with the parameter.
[b]Percent of the patients without metastases without the parameter.

in insulinomas, as shown in Table 4 and Fig 2A and B. The CK19 expression was also shown to be a significant indicator of poor tumor-free survival in insulinomas, although only two out of six insulinomas with an event (one with tumor-specific death and the other with tumor progression) showed a positive CK19 staining. None of the other insulinomas showed expression of CK19.

Also specific chromosomal alterations, including 3p and 6q loss, and 7q, 12q, and 14q gain were strong parameters for tumor recurrence and/or progression or tumor-specific death in these tumors. Fig 2C shows the Kaplan–Meier curve for chromosome 6q loss as the most significant chromosomal marker for poor tumor-free survival. Furthermore, female patients had a significantly better tumor-free and tumor-specific survival when compared with male patients ($P=0.017$ and 0.014 respectively), which is in line with the higher incidence of metastases in male versus female patients (Danforth *et al.* 1984). Multivariate analysis did not improve significance.

Other EPTs

Table 4 shows also the parameters for poor tumor-free survival and tumor-specific death in the non-insulinoma EPT patients. CIN proved to be a reliable marker for poor tumor-free survival in these patients, followed by Ki67 proliferative index and chromosome 7q gain (Fig. 2D and E). CK19 was detected as the most significant marker for tumor-specific death because of its positive staining in six out of seven patients with tumor-specific death (Fig. 2F).

Discussion

In this study, we have examined the value of several proposed indicators of malignancy and clinical outcome in a large group of insulinomas and non-insulinoma EPTs. These parameters include tumor size ≥2 cm, Ki67 proliferative index of ≥2%, CK19 expression, and chromosomal alterations, including CIN. CIN and specific chromosomal alterations turn out to be reliable indicators for metastatic disease and

Table 3 Prognostic parameters and the clinical behavior of endocrine pancreatic tumor (EPT) patients

Type of tumor (*n*)	**AW**	**DOD**	**AWD**	**DOC**
Insulinomas (47)	40	4	3	0
Gastrinomas (6)	3	1	2	0
Glucagonomas (2)	0	2	0	0
Vipomas (5)	1	2	1	1
Non-functioning (11)	3	3	5	0
Parameter (*n*)				
Insulinomas				
CIN				
Present (19)	12	4	3	0
Absent (28)	28	0	0	0
Size				
≥2 cm (16)	10	3	3	0
<2 cm (31)	30	1	0	0
Ki-67				
≥2% (7)	5	1	1	0
<2% (40)	35	3	2	0
CK19				
Positive (2)	0	1	1	0
Negative (26)	22	2	2	0
EPTs excluding insulinomas				
CIN				
Present (15)	0	6	8	1
Absent (9)	7	2	0	0
Size				
≥2 cm (20)	5	6	8	1
<2 cm (1)	1	0	0	0
Ki-67				
≥2% (9)	1	4	3	1
<2% (12)	5	3	4	0
CK19				
Positive (12)	2	6	4	0
Negative (10)	5	1	3	1

AW, alive without disease; DOD, dead of disease; AWD, alive with disease; DOC, dead of other cause.

Table 4 Significant parameters for predicting tumor-specific death (DOD) and poor tumor-free survival (DOD+AWD) in endocrine pancreatic tumor (EPT) patients

Marker	***P* value**	**Marker**	***P* value**
Insulinomas (*n*=47)			
DOD (*n*=4)		DOD+AWD (*n*=7)	
CIN	0.0113	CIN	0.0004
3p loss	0.0000	CK19	0.0011
6q loss	0.0009	Size	0.0017
7q gain	0.0269	3p loss	0.0000
12q gain	0.0003	6q loss	0.0000
14q gain	0.0003	7q gain	0.0013
		12q gain	0.0000
		14q gain	0.0013
EPTs excluding insulinomas (n=24)			
DOD (*n*=8)		DOD+AWD (*n*=16)	
CK19	0.0314	CIN	0.0012
7q gain	0.0497	Ki-67	0.0074
		7q gain	0.0181

DOD, dead of disease; AWD, alive with disease.

poor tumor-free survival in insulinoma and non-insulinoma EPTs, and for tumor-specific death in insulinomas. CK19 expression is not a strong prognostic indicator in insulinomas, but is the most optimal indicator of tumor-specific death in the other EPTs.

From previous studies, it has become clear that malignant progression of EPTs is associated with an accumulation of genetic alterations (Speel *et al.* 1999, 2001, Jonkers *et al.* 2005, 2006*a*). CIN is defined as the presence of at least eight chromosomal aberrations detected by conventional CGH or 20 aberrations of at least 10 Mb detected by array CGH (Jonkers *et al.* 2005, 2006*a*). Although the underlying mechanism leading to CIN is yet unknown, we have shown here that this parameter can reliably predict clinical outcome in insulinomas, and also metastatic disease as already described before (Jonkers *et al.* 2005). Although the sensitivity of CIN to predict tumor-specific death is high, the specificity is rather low. This is with high probability due to a number of patients presenting with tumors of uncertain or malignant behavior showing CIN of which the tumors have been treated successfully. Also several frequently occurring specific chromosomal aberrations, associated with CIN in insulinomas, are highly effective as prognostic indicators. In particular, loss of chromosome 3p and 6q, and gain of 12q prove to be very strong parameters for poor tumor-free survival. This finding underscores previous results by molecular allelotyping providing evidence for association of metastatic progression with chromosome 3p and 6q loss in EPTs (Chung *et al.* 1997, Hessman *et al.* 1999, Barghorn *et al.* 2001*a*,*b*, Rigaud *et al.* 2001, Guo *et al.* 2002). Among the putative candidate genes is *FANCD2* which plays a role in the repair of DNA damage (Jin *et al.* 2003). This gene is located on chromosome 3p25, a critical region of loss in EPTs (Chung *et al.* 1997). One of the putative tumor suppressor genes on chromosome 6q24 is lost on transformation (LOT), a widely expressed zinc finger protein that inhibits cell growth through induction of apoptotic cell death and G1 arrest. It appears to be epigenetically silenced in different types of cancer, including parathyroid adenomas (Pagotto *et al.* 2000, Abdollahi *et al.* 2003).

Large series of individual EPT subtypes other than insulinomas have so far not been studied for the occurrence of CIN. Rigaud *et al.* (2001) examined a group of 16 non-functioning EPTs by flow cytometry and indicated aneuploidy and Ki67 proliferative index to be the prognostic markers for this tumor subtype. In contrast, Chung *et al.* (1998) could not identify a

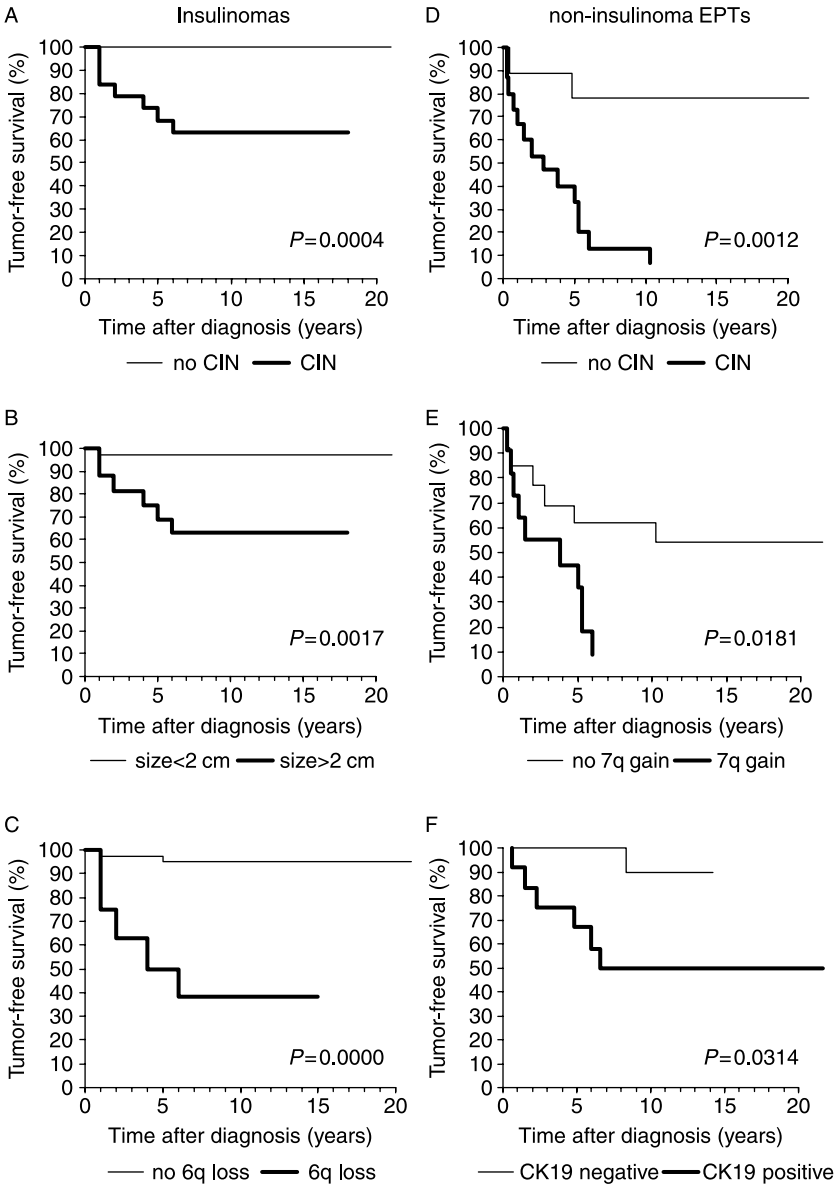

Figure 2 Kaplan–Meier curves correlating tumor-free survival in insulinomas with (A) CIN, (B) tumor size, and (C) chromosome 6q loss; the non-insulinoma EPTs with (D) CIN or (E) chromosome 7q gain, and (F) tumor-specific survival with CK19. Significance scores are indicated.

correlation between frequency of allelic loss and disease stage using genome-wide allelotyping, but this study was hampered by the small groups of individual EPT subtypes. In this study, we have been able to collect follow-up data of 24 non-insulinoma EPTs from which CGH data were available. CIN turned out to be the only significant indicator of metastatic disease and the highest independent predictor of poor tumor-free survival in this group. For the prediction of tumor-specific death, gain of chromosome 7q and CK19 status were the most reliable markers. It will be essential to further substantiate these findings in larger numbers of individual EPT subtypes in subsequent studies. This is of particular importance because genetic studies indicate differences in genetic make-up in the different EPT subtypes, despite the occurrence of CIN in the malignant tumors (Speel *et al.* 1999, Heitz *et al.* 2004).

In a number of cases, the patients with CIN and a poor tumor-free survival or tumor-specific death presented with metastases already at diagnosis. However, one gastrinoma, two vipomas, one

insulinoma, and three non-functioning tumors without detectable metastases at diagnosis presented with tumor recurrence and/or metastatic progression, or DOD during follow-up time (Table 1). Another patient presenting with CIN in an insulinoma of uncertain behavior also developed metastases after 3 years, but this patient was excluded from this study because of the presence of a MEN1 syndrome. This underscores the reliability of CIN in predicting poor clinical outcome. Therefore, a simplified test to predict CIN will be of value in clinical diagnosis of these tumors. Detection of CIN might change patient management, e.g. by intensifying clinical follow-up. In addition, it may help in directing tumor surgery.

As stated above, CK19 expression proved to be the most optimal marker for tumor-specific death in non-insulinoma EPTs. These data are in accordance with the data of Deshpande *et al.* (2004) and a recent study by Schmitt *et al.* (2007), investigating large series of EPTs, including different subtypes. Our data in this study, however, strongly indicate that CK19 expression is a suboptimal marker for poor tumor-free survival in insulinomas. This is probably the consequence of the low percentage of malignant tumors with CK19 immunostaining, as also reported by Ali *et al.* (2006) and in one of our previous studies (Jonkers *et al.* 2006*a*). So far, only one study was unable to correlate CK19 expression with malignancy in EPTs (Albarello *et al.* 2004). A reason for the discrepancy between these studies could be the use of different CK19-directed monoclonal antibodies and/or criteria for evaluation of immunostaining results. We have used the RCK108 clone, which is used by the most other studies and shown to be more reliable for CK19 analysis than the BA17 clone (La Rosa *et al.* 2005).

Of the clinicopathological criteria used in the WHO classification, tumor size proved to be a very simple and reliable clinical marker for metastatic disease and poor tumor-free survival in insulinomas. Although CIN appeared to be a more significant parameter than tumor size to predict tumor outcome, the latter parameter is very useful as a result of the early diagnosis of insulinomas and often successful treatment. The Ki67 proliferative index is often $<2\%$ in insulinomas, and is therefore not a significant marker for poor tumor-free survival in insulinomas. In combination with CIN, however, Ki67 is very useful for predicting metastatic disease in insulinomas and both metastatic disease and poor tumor-free survival in non-insulinoma EPTs. In general, the majority of EPTs are well differentiated according to the most recent WHO classification. In this study, only one insulinoma and one glucagonoma were classified as poorly differentiated with a Ki67 proliferative index of >10 mitoses per 10 high-power fields. The respective patients both DOD, thus underscoring a poor tumor-specific survival for patients with poorly differentiated tumors.

In the non-insulinoma EPTs size cannot be efficiently used to discriminate between benign and malignant tumors, because the tumor diameter is usually larger than 2 cm, as also found in this study. However, size ≥ 2 cm has been described as a predictable marker, because most studies consider EPTs as one group, with the smaller tumors being predominantly benign insulinomas and the larger ones generally comprising the non-insulinoma EPTs with a more malignant behavior (Speel *et al.* 1999, Schindl *et al.* 2000, Ohike & Morohoshi 2005).

In summary, we have identified CIN as well as specific chromosomal alterations as the most reliable indicators of metastatic disease and poor tumor-free survival in all insulinoma and non-insulinoma EPTs, and for tumor-specific death in insulinomas. CK19 expression is the most optimal indicator of tumor-specific death in the non-insulinoma EPTs. Tumor size is particularly powerful as a predictor of metastatic disease in insulinomas. The implementation of these parameters in diagnostic protocols will make the prediction of the clinical behavior of EPTs more accurate.

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