Short Communication

Putative Tumor Suppressor Loci at 6q22 and 6q23-q24 Are Involved in the Malignant Progression of Sporadic Endocrine Pancreatic Tumors

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Our previous comparative genomic hybridization study on sporadic endocrine pancreatic tumors (EPTs) revealed frequent losses on chromosomes 11q, 3p, and 6q. The aim of this study was to evaluate the importance of 6q losses in the oncogenesis of sporadic EPTs and to narrow down the smallest regions of allelic deletion. A multimodal approach combining polymerase chain reaction-based allelotyping, double-target fluorescence *in situ* hybridization, and comparative genomic hybridization was used in a collection of 109 sporadic EPTs from 93 patients. Nine polymorphic microsatellite markers (6q13 to 6q25-q27) were investigated, demonstrating a loss of heterozygosity (LOH) in 62.2% of the patients. A LOH was significantly more common in tumors >2 cm in diameter than below this threshold as well as in malignant than in benign tumors. We were able to narrow down the smallest regions of allelic deletion at 6q22.1 (D6S262) and 6q23-q24 (D6S310-UTRN) with LOH-frequencies of 50.0% and 41.2 to 56.3%, respectively. Several promising tumor suppressor candidates are located in these regions. Additional fluorescence *in situ* hybridization analysis on 46 EPTs using three locus-specific probes (6q21, 6q22, and 6q27) as well as a centromere 6-specific probe revealed complete loss of chromosome 6 especially in metastatic disease. We conclude that the two hot spots found on 6q may harbor putative tumor suppressor genes involved not only in the oncogenesis but maybe also in the malignant

and metastatic progression of sporadic EPTs. (Am J Pathol 2001, 158:1903-1911)

The histopathological classification of endocrine pancreatic tumors (EPTs) is complex and cannot predict the clinical outcome in all cases. Identifying malignancy on morphological grounds alone is only successful if unequivocal signs of invasion and/or metastases are detected.¹⁻³ Furthermore, currently used immunohistochemical and molecular markers are of limited value to predict malignancy of EPTs. In recent years some progress has been made in the elucidation of the molecular pathogenesis of EPTs. Molecular studies of syndromes associated with EPTs, such as the multiple endocrine neoplasia type 1 (MEN 1) and the von Hippel-Lindau syndrome, resulted in the identification of tumor suppressor genes (TSG) on chromosomes 11q13 and 3p25.5, respectively.^{4,5} The major part of EPTs is sporadic in nature and not associated with an inherited syndrome. In these sporadic EPTs only a subset of tumors exhibit MEN 1 gene mutations,⁶⁻⁸ whereas no von Hippel-Lindau syndrome gene mutations have been detected.⁹ Putative TSGs associated with tumor progression and poor clinical outcome were identified on 17p, 3p23-p25, 18q21 (DPC4/Smad4), and chromosome 1.¹⁰⁻¹⁴

Our previous comparative genomic hybridization (CGH) study revealed losses at 6q in a high percentage of sporadic EPTs¹⁵ and provided strong evidence for a significant role of this locus in the malignant progression of EPTs. This was the first time that this locus was described to play a role in EPTs. To further investigate the importance of 6q in the oncogenesis and progression of sporadic EPTs and to narrow down the locus of a putative TSG we analyzed 109 tumor samples from 93 patients for

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allelic deletions on chromosome 6q using nine polymorphic microsatellite markers. Additional double-target fluorescence *in situ* hybridization (FISH) analysis was performed to confirm our allelotyping data. The results were compared with our previous CGH data concerning chromosome 6.

Materials and Methods

Tumor Material and Patient Data

One hundred nine EPT samples from 93 patients (49 females, mean age 55.3 ± 17.3 years; 44 males, mean age 56.8 ± 15.1 years) were selected from the archives of the Departments of Pathology, Universities of Zurich and Basel, Switzerland, and eight EPTs were kindly provided by the Department of Pathology, University of Turin, Italy. All tumors were sporadic in nature, not associated with any known cancer syndrome, namely the MEN1 or von Hippel-Lindau syndrome. The tumors consisted of 87 primaries and 22 metastases. Of 16 patients both primary EPT and metastasis were examined, whereas in six patients only tumor tissue from a metastasis was available. The mean size of the primary tumors was 1.4 ± 0.6 cm (range, 0.5 to 3.0 cm) for benign lesions and 4.9 ± 3.0 cm (range, 1.0 to 13.0 cm) for malignant lesions. Patient 58 presented with a primary tumor of 12.5 cm in diameter. A 3-year clinical follow up of this patient did not reveal any local recurrence or metastasis. Therefore this tumor must be considered as of uncertain malignant behavior although the clinical course so far seems to be benign.

The tumors were classified according to the new World Health Organization-classification of neuroendocrine neoplasms.³ Sixty functioning (ie, hormone secreting) and 33 nonfunctioning EPTs were investigated, with 59 tumors being malignant (Table 1). Fifty-five patients presented with localized disease, defined by the absence of extrapancreatic spread of the tumor, whereas 36 patients revealed advanced disease, ie, invasion of extrapancreatic tissue and/or lymph node and/or organ metastases. In two patients the extension of the tumor could not be determined. The last clinical follow-up was performed in 1999. The maximum duration of follow-up was 15 years (patient 7 with a benign insulinoma of 1.2 cm in diameter).

DNA extraction was performed on tumor and normal tissue from formalin-fixed, paraffin-embedded material of 59 patients, whereas it was frozen tissue in the remaining 34 patients. Although frozen tissue was available of a total of 46 patients (compare FISH analysis on touch preparations) the amount of frozen specimen was too limited for additional DNA extraction in 12 patients in which a lot of additional formalin-fixed, paraffin-embedded material was present. In 19 patients exclusively tumor tissue without matching normal tissue was available.

DNA Extraction

Genomic tumor and normal DNA was isolated from paraffin-embedded material. Tumor and adjacent normal exocrine pancreatic tissue or other normal tissue avail-

Table 1. Distribution of EPT Subtypes and Biological Behavior (*n* = number of patients)

EPT	Benign	Malignant	Total
Functioning	27	33	60
Insulinoma	27	10	37
VIPoma		8	8
Gastrinoma		8	8
Glucagonoma		7	7
Nonfunctioning	7	26	33
Total	34	59	93

able were microdissected from 5 to 10 (10- μ m-thick) unstained tissue sections using a surgical blade and the DNA isolated using proteinase K digestion and phenol/chloroform extraction as previously described.^{8,16,17} Genomic DNA from frozen samples was isolated using the D-5000 Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) as previously described.^{8,16,17}

Microsatellite Analysis

Primer sets specific for nine short tandem repeat polymorphic microsatellite markers (Research Genetics, Huntsville, AL) spanning the long arm of chromosome 6 from centromeric 6q13 to telomeric 6q25-q27 were used (Table 2). The polymerase chain reaction (PCR) was performed according to our published standard protocol⁸ using AmpliTaq Gold DNA polymerase (Perkin Elmer, Norwalk, CT) and a programmable thermal cycler (DNA thermal cycler 9600, Perkin Elmer). Appropriate positive as well as negative controls were run with every PCR performed. Slight modifications of this protocol as well as gel running times for each PCR product are listed in Table 2. After electrophoresis, the DNA was visualized by silver staining and the result analyzed for evidence of allelic deletion. A twofold difference in relative allele intensity ratios between tumor DNA and normal DNA was scored as loss of heterozygosity (LOH).^{8,18} Up to three microsatellite PCRs were performed for each marker if the results of the allelotyping were doubtful.

FISH Analysis

Three fluorescein-labeled locus-specific probes mapping to 6q21 (P1 artificial chromosome (PAC) probe 66H14, kindly provided by Dr. P. Sinclair, Royal Free and University College of Medicine, London, UK), 6q22 (cosmid cCl6-44) and 6q27 (cosmid cCl6-37; both cosmid probes described by Saito and colleagues¹⁹) were used on touch preparations from frozen tumor material. Each probe was labeled with spectrum green-dUTP (Vysis, Downers Grove, IL) by nick translation and hybridized to tumor cells together with cot1 DNA and rhodamin-labeled α repetitive DNA specific for chromosome 6 as previously described.⁸ Detection of the hybridized 6q-specific probe was performed using rabbit anti-fluorescein and swine anti-rabbit Ig fluorescein (DAKO, Glostrup, Denmark). Slides were mounted in Vectashield (Vector, Burlingame, CA) containing 4,6-diamidino-2-phenylindole-

Table 2. Chromosomal Location of the Polymorphic Microsatellite Markers and Variation of the PCR Standard Protocol (1.5 mmol/L MgCl₂ and No Addition of DMSO)

Marker	Locus*	Length [†]	PCR			Urea gel [§]
			Annealing T [‡]	2 mmol/L MgCl ₂	10% DMSO	
D6S239	6q13	162–176	56			135
D6S251	6q14–16.2	144–162	50	X		135
D6S268	6q16.2–21	86–100	50		X	100
D6S262	6q22.1	187–183	54			150
ARG1	6q22.3–23.1	84–104	48			105
D6S448	6q23	169–183	54			150
D6S310	6q23–24	159–183	50		X	150
UTRN	6q24	143–163	50			135
IGF2R-II	6q25–q27	158–166	50		X	135

*www.ncbi.nlm.nih.gov/cgi-bin/Entrez/maps.cgi?org=hum&chr=6

[†]Length of PCR products in bp.

[‡]Temperature in °C.

[§]Running time in min.

antifade (DAPI; Sigma Chemical Co., St. Louis, MO) for nuclear counterstaining.

At least 100 interphases with strong hybridization signals were scored for each tumor. The presence of only one probe signal in >30% of tumor cells was interpreted as an allelic deletion, whereas three or more probe signals in >30% of cells was considered as an aneusomy. This threshold is in accordance with the one used by research groups of the National Cancer Institute, National Institutes of Health, Bethesda, MD.²⁰ Normal pancreatic or connective tissue in the vicinity of the tumors served as internal controls.

CGH Data

Additional CGH data concerning the long arm of chromosome 6 were available from our previous CGH studies of a total of 64 sporadic EPTs.^{15,21} The method used was previously published.¹⁵

Statistics

Contingency table analysis was used to analyze the statistical significance of differences in allelic losses at the different chromosome 6q loci and biological behavior (benign *versus* malignant), disease stage (nonmetastasizing *versus* metastasizing), and tumor size, respectively.

Results

An overview of the results is given in Tables 3 and 4. Appropriate examples are illustrated in Figures 1, 2, and 3.

Allelotyping Differences

Nine highly polymorphic microsatellite markers spanning the long arm of chromosome 6 from 6q13 to 6q25–27 were investigated in tumor samples from 74 patients. The samples consisted of 70 primary EPTs and 20 metastases, so that in 16 patients the primary tumor as well as a metastasis were examined. The microsatellite markers were informative in 27.1 to 94.2% of the patients (Tables

3 and 4). LOH of any of the nine markers was observed in 46 of 74 patients (62.2%). The remaining 28 patients showed a retention of both alleles at all informative loci. The markers D6S262 (21 of 42, 50.0%) and D6S310 (21 of 51, 41.2%) proved to be informative as well as frequently deleted, demonstrating LOH in 50% (21 of 42) and 41.2% (21 of 51) of the patients, respectively. The highest frequency of LOH, however, was detected at marker UTRN (9 of 16, 56.3%) but this was also the least informative of all markers used. The LOH frequency of the other markers ranged from 17.1 to 30.8%. The smallest common regions of allelic deletion were located at 6q22.1 (D6S262) and 6q23–q24 (D6S310–UTRN). Interestingly, identical LOH results were obtained in both the primary as well as the metastasis of the 16 patients except for patient 43 who demonstrated an additional LOH of marker IGF2R-II in the metastasis that was not detected in the primary tumor (Table 3 and Figure 3a). Isolated allelic losses were observed in benign, malignant, as well as metastasizing EPTs with D6S262, D6S310, and IGF2R-II being most frequently affected (Table 3 and Figure 2, a and b).

FISH and CGH

Additional double-target FISH analysis was performed in 46 patients using a red-labeled centromere probe combined with a green-labeled probe specific for 6q21, 6q22, or 6q27 (Table 3). All tumor samples were investigated with the 6q22-specific probe, whereas the other two FISH markers used as additional markers in 15 (6q21) and 11 patients (6q27), respectively. In 17 of these 46 patients no additional normal tissue was available to validate the FISH results by microsatellite analysis. In two other patients only CGH was performed. An allelic loss, ie, loss of the 6q-specific probe with retention of both centromeric probes, was observed in 6 out of 46 patients (13.0%). On the other hand there were 10 patients in which only one copy of each centromeric and 6q-specific probe was detected (21.7%), indicating a complete loss of chromosome 6. This complete loss was confirmed by CGH analysis in eight patients whereas in the remaining two patients (patients 42 and 72) CGH only

Table 3. Genetic Alterations on Chromosome 6q as Detected by Microsatellite, FISH, and CGH Analysis^a

	CLINICAL DATA ^b				MICROSATELLITE MARKERS ^c									FISH ^d			CGH ^e	
	Pat.	Sex	Diag.	Size	239	251	268	262	ARG	448	310	UTR	IGF	6q21	6q22	6q27	6q	
Benign	21		m	Ins	1.5	□	□	□	■	H	H	□	□	□				N
	55	f		Ins	1.2	□	□	□	■	H	□	□	□	□				
	78		m	NF	1.5	-	□	□	■	□	-	H	□	□				
	41	f		Ins	1	-	□	□	□	H	-	■	-	H				N
	91	f		Ins	0.9	-	-	□	□	□	□	■	-	□				
	66	f		Ins	3	□	□	■	□	□	□	□	H	□	□	■		N
	48	f		Ins	1.2	□	H	□	□	□	H	□	□	■	■			N
	12	f		Ins	2	□	H	□	□	□	□	□	H	■	□	□		N
	1	f		Ins	1.5	□	□	□	□	□	□	□	H	■		Tri		N
	58 ^f	f		NF	12.5	-	H	□	□	□	□	□	-	■				
	74		m	Ins	3	□	-	□	-	□	-	-	-	-	□	■		N
	36		m	Ins	1	H	H	□	□	□	□	□	H	□	□	■		N
	13		m	Ins	2	□	□	□	□	□	□	□	H	□		Tri		N
	9		m	Ins	1.2										□	■	□	N
	31	f		Ins	1											Tri		N
51		m	Ins	2.4											Tri		Gain	
63	f		Ins	1.5											Tri	Tet	N	
Malignant without metastasis	6		m	Vip	3.5	H	□	■	■	■	H	■	■	■				Loss
	76	f		NF	4.5	H	□	■	■	■	■	■	H	■				
	89		m	Gas	2.4	-	H	□	■	■	■	■	■	■		□	□	N
	25	f		Glu	2.5	□	□	□	■	H	■	■	■	□	□	NS	□	Loss
	16		m	Ins	3.5	■	■	■	-	H	H	■	□	□	■	■	□	Loss
	67	f		NF	?	H	H	■	H	H	H	■	H	□				
	72		m	NF	3	-	H	H	■	■	-	H	H	□	Mo	Mo		Reg
	77	f		NF	12	-	■	■	-	H	-	H	H	□				
	24	f		Vip	8	-	□	□	-	H	□	■	H	□	□	NS	□	N
	52	f		NF	2	-	□	□	H	H	□	■	H	□				Reg
	90		m	NF	3.5	□	□	□	■	□	□	□	H	□				
	23		m	Ins	1.3	□	-	□	□	H	□	□	H	■				
	35	f		NF	3	■	□	□	□	□	□	-	H	-				
	64	f		NF	2.4	H	□	□	□	H	□	□	H	□		Tri		N
	42		m	NF	4.4										Mo	Mo		Reg
47	f		NF	?										Mo			Loss	
28	f		NF	13										□			Loss	
Malignant with metastasis	59 P+M	f		NF	10	■	■	■	■	H	-	■	■	■				Loss
	83 P+M	f		NF	10	H	-	■	H	■	H	■	H	■		Mo	Mo	Loss
	33 P+M		m	NF	3.7	-	-	■	H	-	■	H	H	■		Mo	Mo	Loss
	2	f		Glu	4.2	H	-	■	■	H	■	■	H	■		Mo		Loss
	39 P	f		Glu	4	-	■	■	■	H	-	-	H	-				
	39 M	f		Glu	M	-	■	■	■	H	■	-	H	■				
	53 P+M		m	Ins	3	-	-	■	-	H	-	H	-	■				
	26	f		Vip	11	-	-	■	-	■	-	-	■	H				
	14		m	Ins	5	-	-	■	■	H	■	H	■	-		Mo		Loss
	44 P+M		m	NF	5	■	H	-	■	H	H	-	H	-				
	43 P		m	NF	5	-	□	-	-	H	-	-	■	□				
	43 M		m	NF	M	-	□	-	-	H	-	-	■	■				
	49 P+M	f		Vip	5	-	-	■	-	-	-	■	-	H		Mo	Mo	Loss
	75 P+M		m	Glu	2.5	-	■	■	■	H	H	-	-	-		Mo		Loss
	92 P+M		m	Ins	3.5	-	■	■	■	-	-	-	■	-	□	□	■	Reg
	82 P+M		m	Vip	2.3	-	H	■	■	H	■	-	H	-				
	79 M	f		Glu	M	-	-	■	-	-	-	-	■	H				
	45 P+M	f		NF	5	H	H	H	■	H	■	■	H	□				
	29		m	Ins	4	H	□	□	□	H	■	H	■	□				
	30 P+M		m	Gas	2.7	-	□	□	H	■	-	□	■	H	□			Reg
	40	f		Ins	1.5	-	-	□	■	-	-	■	-	□				Reg
	37 P+M		m	Vip	6	H	H	□	■	H	H	-	H	-		□		N
	87		m	NF	4	H	H	-	-	H	-	■	H	□				Reg
80		m	NF	5.5	■	□	□	-	H	-	■	H	-					
69	f		Gas	3.9	■	□	□	□	H	□	□	H	□		□		N	
32 P+M		m	NF	1	□	□	□	□	□	□	-	H	-				Gain	
86	f		Ins	9											Mo		Loss	
20 M		m	Gas	M													Loss	
56 M		m	NF	M											□		Loss	

^aOnly patients with genetic alterations in the EPTs ($n = 61$) are included in the table.

^bDiag., diagnosis; P, primary tumor; M, metastasis; f, female; m, male; Ins, insulinoma; NF, nonfunctioning EPT; Vip, VIPoma; Gas, gastrinoma; Glu, glucagonoma.

^c□, Retention of heterozygosity; ■, loss of heterozygosity; H, homozygosity (noninformative loci); -, allelotyping not possible.

^dFISH, Fluorescence *in situ* hybridization; □, disomy (two copies of centromere 6 and 6q-specific probe); ■, loss of one of the 6q-specific targets with retention of both centromere 6 loci; Mo, monosomy; Tri, trisomy; Tet, tetrasomy; NS, FISH result not significant.

^eCGH, Comparative genomic hybridization; N, normal; Gain, chromosomal gain; Loss, chromosomal loss; Reg, regional chromosomal loss.

^fTumor of uncertain malignant behavior (see Material and Methods).

Table 4. Percentage of Loss at Different Microsatellite Loci on Chromosome 6q (*n/n* Cases) and Combined Results of Microsatellite, FISH and CGH Analysis^a

	MICROSATELLITE MARKERS									Microsatellite and/or FISH and/or CGH
	D6S239 6q13	D6S251 6q14-16.2	D6S268 6q16.2-21	D6S262 6q22.1	ARG1 6q22.3-23.1	D6S448 6q23	D6S310 6q23-24	UTRN 6q24	IGF 6q25-27	
Informative cases	64.1 (25/39)	71.9 (41/57)	94.2 (65/69)	80.8 (42/52)	45.5 (30/66)	78.3 (36/46)	87.9 (51/58)	27.1 (16/59)	91.3 (56/62)	
LOH of informative cases	24.0 (6/25)	17.1 (7/41)	29.2 (19/65)	50.0 (21/42)	23.3 (7/30)	25.0 (9/36)	41.2 (21/51)	56.3 (9/16)	26.8 (15/56)	59.1 (55/93)
Benign	0 (0/13) ■	0 (0/17) ■	3.7 (1/27) ■	18.7 (3/16) ■	0 (0/16) ■	0 (0/14) ■	9.1 (2/22) ■	14.3 (1/7) ■	NS ^b	38.2 (13/34) ■
Malignant	50.0 (6/12)	29.2 (7/24)	47.4 (18/38)	69.2 (18/26)	50.0 (7/14)	40.9 (9/22)	65.5 (19/29)	88.9 (8/9)		71.2 (42/59)
No metastases	10.5 (2/19) ■	NS	14.3 (6/42) ■	34.6 (9/26) ■	NS	13.0 (3/23) ■	29.4 (10/34) ■	36.4 (4/11) ■	NS	49.1 (27/55) ■
Metastases	66.7 (4/6)		56.5 (13/23)	75.0 (12/16)		46.2 (6/13)	64.7 (11/17)	100.0 (5/5)		72.2 (26/36)
EPTs < 2cm	0.0 (0/13) ■	0.0 (0/17) ■	0.0 (0/28) ■	25.0 (4/16) ■	0.0 (0/14) ■	0.0 (0/15) ■	18.2 (4/22) ■	14.3 (1/7) ■	NS	33.3 (12/36) ■
EPTs > 2cm	50.0 (6/12)	30.4 (7/23)	51.5 (17/33)	68.0 (17/25)	53.9 (7/13)	47.4 (9/19)	61.5 (16/26)	87.5 (7/8)		76.0 (38/50)

^aContingency table analysis.

^bNS, not significant.

■ significant ($0.01 < P < 0.05$).

■ highly significant ($P < 0.01$).

demonstrated regional loss of chromosome 6q. Sixteen patients showed two signals per nucleus, ie, disomy, for each chromosome 6 target used and no evidence for allelic losses with the microsatellite or CGH analysis. In contrast patients 12, 37, 69, and 89 displayed disomy for chromosome 6 by FISH and no chromosome 6 losses by CGH analysis, whereas showing LOH in the microsatellite analysis. In patients 12, 37, and 69 this concerned only an isolated LOH of one marker, so that differences in target sequence (microsatellite *versus* FISH analysis) and sensitivity (microsatellite *versus* CGH analysis) might explain the different outcome. Additionally, patients 28 and 56 showed a disomy for chromosome 6 target by FISH but a loss by CGH analysis. These tumors turned out to be aneuploid with an underrepresentation of chromosome 6. Five patients with benign (patients 1, 13, 31, 51, and 63) and one patient with a nonmetastasizing malignant EPT (patient 64) showed trisomy or tetrasomy of chromosome 6 in the FISH analysis (Figure 3, b and c). Altogether microsatellite, FISH and CGH analysis demonstrated allelic loss at 6q in 55 of the 93 patients investigated (59.1%).

Genetic Alterations and Clinical Data

The identified genetic aberrations were correlated with the available clinical data and especially with the biological behavior of the tumors. Separating benign from malignant tumors revealed an overall 6q LOH frequency of 37.0% (10 of 27) in benign and 76.6% (36 of 47) in malignant tumors with marked differences in the maximum number of affected loci (two loci in benign and up to

seven loci in malignant tumors). The difference in LOH frequency was significant in eight of nine markers (*P* values from 0.0001 to 0.0145) (Table 4). When differentiating metastasizing from nonmetastasizing tumors the difference in LOH frequency was significant in only six of nine markers (*P* values from 0.0003 to 0.028). The complete loss of chromosome 6 as detected by FISH analysis was exclusively found in malignant tumors and even more often in the metastasizing ones (Table 3). When comparing functioning *versus* nonfunctioning tumors they exhibited a significant 6q LOH difference at D6S239 on 6q13 (11.1% *versus* 57.1%; *P* = 0.016). There was no correlation between allelic loss at 6q and patient age or sex. On the other hand, a diameter of the primary tumor of ≥ 2 cm was significantly correlated with allelic loss of 6q microsatellite markers. Combining the results of allelotyping, FISH, and CGH analysis revealed significantly more losses at 6q in the EPTs above this threshold (*P* = 0.0001, Table 4).

Discussion

We performed a detailed mapping of allelic losses on the long arm of chromosome 6 in a large series of sporadic human EPTs to identify the potential location of TSGs that might be involved in the pathogenesis of these tumors. Our results demonstrate a high percentage of 6q losses in EPTs, and we were able to narrow down two common regions of allelic deletion at 6q22.1 (D6S262) and at 6q23-q24 (D6S310–UTRN). These regions were significantly more often deleted in malignant than in benign EPTs indicating that these loci might harbor TSGs criti-

cally involved in the malignant progression of EPTs. Interestingly, losses of larger regions on 6q or the entire chromosome 6 were strongly associated with metastatic disease. Thus, analysis of 6q losses might help to determine the malignant potential and possible fate of a given sporadic EPT in a routine diagnostic setting.

In the present study LOH at 6q was identified in 62.2% of the patients with two commonly affected regions on D6S262 at 6q22.1 and on D6S310 to UTRN at 6q23-q24, being deleted in 50.0% and 41.2 to 56.3% of the EPTs, respectively. The demonstration of isolated LOH at these loci in benign EPTs suggests, that these loci might be involved already early in the oncogenesis of EPTs. However, because a number of these benign tumors also harbored additional aberrations at other chromosomal loci, it is reasonable to assume that some clinically benign tumors might already have a malignant potential and that they are detected before invasion or metastases have been established. This would fit with our earlier reported correlation between 6q losses detected by CGH and malignant progression, as well as with the finding in this study, that allelic deletion of 6q was more frequently identified in EPTs >2 cm than in EPTs <2 cm. Eight of nine microsatellite markers proved to be significantly more often deleted in the larger EPTs. These data correspond well with the new World Health Organization classification system for EPTs, that recommends a diameter of 2 cm to discriminate between benign EPTs and tumors with an uncertain or low malignant behavior.³

Several candidate TSGs are located in the common region of deletion identified in our tumors. In the vicinity of 6q22, eg, three interesting TSGs have been localized, namely the genes "absent in melanoma 1" (AIM1), "cyclin C" (CCNC), and "receptor-type protein-tyrosine phosphatase kappa" (PTPRK). AIM1 and CCNC were mapped to 6q21 and PTPRK to 6q22.2-q22.3.²²⁻²⁷ CCNC is up-regulated by 1 α ,25-dihydroxyvitamin D³, inhibits cellular growth, and induces apoptosis,²⁵ whereas AIM1 possibly exerts its effects through interactions with the cytoskeleton.^{23,28} With respect to our common region of allelic deletion at 6q22.1, PTPRK is the most likely candidate TSG. Yang and colleagues²⁶ described that transforming growth factor- β 1 inhibits human keratinocyte proliferation *in vitro*, possibly through induction of PTPRK gene expression. They suggested that PTPRK might be involved in the regulation of cell contact and adhesion via dephosphorylating β -catenin and γ -catenin/plakoglobin or cadherins, thereby contributing to the formation and maintenance of intact adherens junctions. Furthermore, there might be an additional involvement of PTPRK in cell proliferation, tumor invasiveness, and metastatic spread.²⁹ The recently cloned TSG hZAC (also called Lot 1: lost on

transformation), a widely expressed zinc finger protein that inhibits tumor cell growth through induction of apoptotic cell death and G₁ arrest, is located at 6q24-q25 and might therefore be an additional candidate TSG for EPTs.^{30,31} Expression of hZAC has been demonstrated in the pancreas and the hZAC protein seems to function as a transcriptional regulator of the type 1 receptor for pituitary adenylate cyclase-activating polypeptide, an important mediator of autocrine control of insulin secretion in the pancreatic islet.^{31,32}

Losses of chromosome 6q have been detected in many other human neoplasms, including malignant melanoma, carcinomas of the salivary gland, ovary, prostate, stomach, liver, and hematological neoplasms.³³⁻³⁹ Because of the close intimacy of the exocrine and endocrine pancreas, the identification of three commonly deleted regions in exocrine pancreatic adenocarcinomas, namely at 6q21 (69%), 6q23-q24 (60%), and 6q26 (51%) is highly interesting.⁴⁰ However, besides 6q losses the molecular pathways leading to cancer in these two tumor entities seem to be clearly different.^{15,41} Another interesting observation is the occurrence of 6q losses in several different endocrine tumor types, including EPTs,^{15,42} parathyroid adenomas,⁴³ pheochromocytomas,⁴⁴ and adrenocortical carcinomas,⁴⁵ implicating that in these tumors the same TSG may be involved. The presence of several genes on the long arm of chromosome 6, some of them coding for hormones and their receptors as the α polypeptide of chorionic gonadotropin, 5-hydroxytryptamine receptor-1E, and vasoactive intestinal peptide, further underlines this possible link to endocrine organs.

Four benign insulinomas (patients 1, 13, 31, and 63) and an additional nonfunctioning malignant EPT without metastasis (patient 64) were found to be aneuploid, harboring a trisomy or tetrasomy for chromosome 6. This suggests that some EPTs (insulinomas) may use an alternative oncogenic pathway leading to aberrant chromosome numbers already early in tumor development. Furthermore, in 17 unequivocally malignant EPTs no 6q losses could be identified by any of the methods used in this study, suggesting that the loss of TSGs on other chromosomes may be crucial as well in EPT development. Indeed, other TSGs have been reported on chromosomes 1, 3p, 11q, 17p, and 18q,^{8-12,14} and the combined results of these studies and the present one underlines that EPTs may exploit different genetic pathways leading to malignant progression.¹⁵ Especially allelic losses at 6q and/or 3p seem to be critical for the malignant progression of EPTs. Thus, with a combination of 6q- and 3p-allelotyping we are able to detect 88.1% (52 of 59) of malignant EPTs (Barghorn and colleagues¹¹ and present study). This might serve useful in predicting

Figure 1. Patient 16 (69-year-old male; malignant insulinoma, 3.5 cm in diameter). **a:** Allelotyping, LOH for two of five markers (D6S268 and D6S310, **red arrowheads**; ARG1 is not informative; UTRN and IGF2R-II show retention of heterozygosity). **b:** CGH-analysis on chromosome 6: digital image (**left**) and fluorescent ratio profile (**right**). The **red bar** on the **left** of the chromosome ideogram (**center**) indicates region of loss. **c:** FISH analysis, loss of one of the 6q-specific green spots in two of three probes (6q21 and 6q22) in DAPI-stained tumor nuclei. At 6q27 both centromere and 6q-specific probes are detected. The FISH analysis confirms the allelotyping results.

Figure 2. Examples of benign EPTs with isolated allelic losses. **a:** Patient 1, LOH at IGF2R-II. **b:** Patient 91, LOH at D6S310.

Figure 3. **a:** Patient 43, additional LOH with tumor progression. LOH only in the metastasis (M) but not in the primary tumor (T). **b:** Patient 33. FISH-analysis at 6q22 demonstrates monosomy. **c:** Patient 1. FISH analysis at 6q22 demonstrates trisomy (upper nucleus) and tetrasomy (lower nucleus). Tetrasomy was an exceptional finding and not representative for the FISH analysis in this patient.

the possible biological behavior of an EPT in a given clinicopathological setting.

In conclusion, our data indicate that ~60% of a significant collection of sporadic EPTs have losses on chromosome 6q and that putative TSGs at 6q22.1 and 6q23-q24 may be involved not only in the initiation but eventually also in the malignant and metastatic progression of these tumors. Because also clinically benign EPTs can already show allelic losses in these critical regions, the use of these markers in differentiating benign from malignant lesions might be of clinical importance.

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