Allelic alterations in pancreatic endocrine tumors identified by genome-wide single nucleotide polymorphism analysis

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Abstract

Pancreatic endocrine tumors (PETs) are uncommon and the genetic alterations in these indolent tumors are not well characterized. Chromosomal imbalances are frequent in tumors but PETs have not been studied by high-density single nucleotide polymorphism (SNP) array. We used genome-wide high-density SNP array analysis to detect copy number alterations using matched tumor and non-neoplastic tissue samples from 15 patients with PETs. In our study, whole or partial loss of chromosomes 1, 3, 11, 22 was present in 40, 47, 53, 40% of tumors respectively, and gain of chromosomes 5, 7, 12, 14, 17, and 20 was present in 47, 60, 47, 53, 53, and 47% of tumors respectively. One tumor had loss of heterozygosity of chromosome 3 and another of chromosome 22 without copy number alterations, suggesting uniparental disomy due to non-disjunction and deletion or to chromosomal recombination. Chromosomal aberrations of the autosomal chromosomes were correlated with chromosomal loss or gain of other chromosomes ($r>0.5$, $P<0.5$). About 60% of PETs had high allelic imbalances (AI) defined by more than four chromosomal aberrations, and 40% of tumors had low AI. The PETs with high AI were larger: the mean tumor size with high AI was 5.4 ± 3.1 cm compared with 2.3 ± 1.3 cm for low AI ($P=0.03$). Our study shows that genome-wide allelotyping is a powerful new tool for the analysis of AI in PETs.

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Introduction

Pancreatic endocrine tumors (PETs) are uncommon, mostly well-differentiated and indolent neuroendocrine neoplasms, with an age-adjusted annual incidence of <1 per 100 000 (Buchanan *et al.* 1986). The molecular mechanisms of tumor genesis of PETs are poorly understood but have been the focus of many recent reports (Görtz *et al.* 1999, Bartsch *et al.* 2000, Serrano *et al.* 2000, Lubomierski *et al.* 2001, Chan *et al.* 2003, House *et al.* 2003, Wang *et al.* 2005). Previous studies using comparative genomic hybridization and microsatellite allelotyping have shown that PETs have a high frequency of chromosomal allelic alterations that are located on chromosomes 3q, 6q, 11q, 11p, 16p, 20q, 21, and 22q (Chung *et al.* 1998, Rigaud *et al.* 2001).

Determination of chromosomal copy number alterations can help in localizing the chromosomal locations of oncogenes and tumor suppressor genes in malignancies. Allelic imbalances (AI) may be detected by a variety of methods including karyotyping, comparative genomic hybridization, and microsatellite analysis, but these are either of low resolution or laborious to conduct on a genome-wide scale. In contrast, single nucleotide polymorphism (SNP) allelotyping is a sensitive method to detect DNA copy number and chromosomal loss of heterozygosity (LOH; Matsuzaki *et al.* 2004, Zhao *et al.* 2004, Irving *et al.* 2005, Lu *et al.* 2005, Nannya *et al.* 2005, Teh *et al.* 2005). This method has been used to determine AI in a variety of tumors (Lu *et al.* 2005, Teh *et al.* 2005), leukemias (Irving *et al.* 2005), and in a variety of tumor cell

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lines (Matsuzaki *et al.* 2004, Zhao *et al.* 2004, Nannya *et al.* 2005).

We report on genome-wide high-density SNP allelotyping of all the autosomal chromosomes and X chromosome to provide high resolution determination of copy numbers. We identified several whole and partial chromosomal aberrations and uniparental disomy as an alternative to LOH in PETs, and found that the alterations were more extensive in advanced tumors.

Material and methods

Characteristics of specimens and patients

Frozen tumor and non-neoplastic tissue of 15 patients who underwent resection for a PET were obtained from surgical specimens in the frozen section laboratory of the Department of Pathology at the M D Anderson Cancer Center. Surveillance Committee (institutional review board) approved this study. The patient records and histopathological findings were reviewed. The tumors were classified as PETs using established criteria as previously reported (Liu *et al.* 2005). The functional status of each tumor was ascertained by serum measurements of hormones and/or clinical syndrome due to hormonal production.

DNA extraction

DNA from both tumor and non-neoplastic pancreatic parenchyma in microdissected fresh-frozen specimens was extracted using a commercial kit (Qiagen DNA extraction kit, Qiagen Inc.), after a hematoxylin and eosin-stained slide from a frozen block was reviewed. The tumor cell cellularity was at least 70% in all samples. All the tumor and non-neoplastic tissue was obtained from the primary tumor and surrounding non-neoplastic pancreatic tissues.

XbaI mapping array hybridization

In this study *Xba1* GeneChip Mapping 50K Assay Kit (Affymetrix Inc., Santa Clara, CA, USA) was used. This array covers 58 960 SNP loci distributed on all the autosomal chromosomes and X chromosome. The average heterozygosity for these SNPs is 0.3. The analyses were performed according to the manufacturer's instructions. In brief, 250 ng of genomic DNA was digested with *Xba1* restriction enzyme, ligated to adaptors, and amplified by PCR. The resulting amplicons were fragmented, end-labeled with biotinylated dideoxy ATP using terminal deoxynucleotidyl transferase, and hybridized to the *Xba1* GeneChip Mapping 50K array. Hybridization was detected by incubation with streptavidin–phycoerythrin conjugates, followed by scanning the array for phycoerythrin fluorescence and quantization with the Affymetrix GeneChip Scanner 3000 using the GeneChip DNA Analysis Software, version 3.0 (Affymeterix Inc).

Data analysis and DNA copy number

The signal detection rate was the percentage of SNPs that passed the discrimination filter. The mean signal detection rates were 93.8% ± 3.8 in non-neoplastic DNA samples and 93.0 ± 2.0 in tumor samples. The

Table 1 Clinical and histopathological characteristics of patients with pancreatic endocrine tumor

No.	Gender	Age (year)	Size of tumor (cm)	Histologic type[a]	Liver metastasis	Lymph node metastasis	Clinical features
14	M	80	5.0	WDNC	−	+	Gastrinoma
15	F	56	1.5	WDNTUMP	−	−	Non-functional
16	M	55	10.0	WDNTUMP	−	−	Non-functional
20	F	37	1.0	WDNTUMP	−	−	Gastrinoma, MEN-1
25	F	67	2.0	WDNC	−	+	Non-functional
26	F	19	1.0	WDNTUMP	−	−	Non-functional
27	F	53	2.0	WDNTUMP	−	−	Non-functional
28	M	76	1.7	WDNC	−	+	Non-functional
51	F	55	8.0	WDNC	−	−	Non-functional
53	M	53	6.0	WDNTUMP	−	−	Non-functional
54	M	67	3.0	WDNC	+	−	Non-functional
59	M	50	3.0	WDNC	−	+	MEN-1, non-functional
67	F	56	3.0	WDNC	+	+	Non-functional
68	M	73	4.0	WDNC	−	+	Non-functional
69	M	63	8.0	WDNC	+	+	Non-functional

[a]WDNC, well-differentiated neuroendocrine carcinoma; WDNTUMP, well-differentiated neuroendocrine tumor, uncertain malignant potential.

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copy number was estimated by the Chromosome Copy Number Analysis Tool, version 2.0 (Affymterix Inc.). The software used the log intensity as the basic measurement with appropriate chip-wise normalization. The log 2 of the arithmetic average of the perfect match and mismatch intensities across 40 probes was used as the basic measurement for any given SNP. The copy number was estimated by comparing the normalized intensity for the SNP to the expected intensity for two chromosomes in the reference set using the copy number response curve determined from dosage–response data. The software also determined the significance of the copy number variation by comparing with the reference set (± log 10 *P* value). The non-neoplastic/tumor pairs were compared by determining the ratio of non-neoplastic copy number to tumor copy number multiplied by two at each SNP locus. In addition, the average chromosomal copy numbers of non-neoplastic and tumor samples were calculated for the entire chromosomes and chromosomal loci of interest.

Detection of LOH

The LOH calls were based on the SNP calls of the paired non-neoplastic and tumor samples of the same individual. The possible SNP calls made by Affymetrix software were AB (heterozygous SNP), AA and BB (homozygous SNP), and no call (when the software was unable to decide on the calls). Conversion of a heterozygous SNP in normal sample to a homozygous SNP in tumor sample indicated LOH (AB in normal, AA or BB in tumor). When normal samples have homozygous SNP or no call, these SNP loci were not informative.

SNP and gene position

The SNPs and the genes were positioned according to the same genome build: UCSC genome browser http//genome.ucsc.edu/October 2005 assembly. The October 2005 human reference sequence (UCSC version hg17) was based on NCBI build 35.

Statistical analysis

All statistical analysis was performed using SPSS (SPSS Inc., Chicago, IL, USA, USA). Comparisons of categorical variables were made using χ^2-test and Fisher's exact test. Continuous data, including age and tumor size, were evaluated by Student's *t*-test. Correlations among chromosomal aberrations in tumors were evaluated by means of Spearman's rank correlation coefficient.

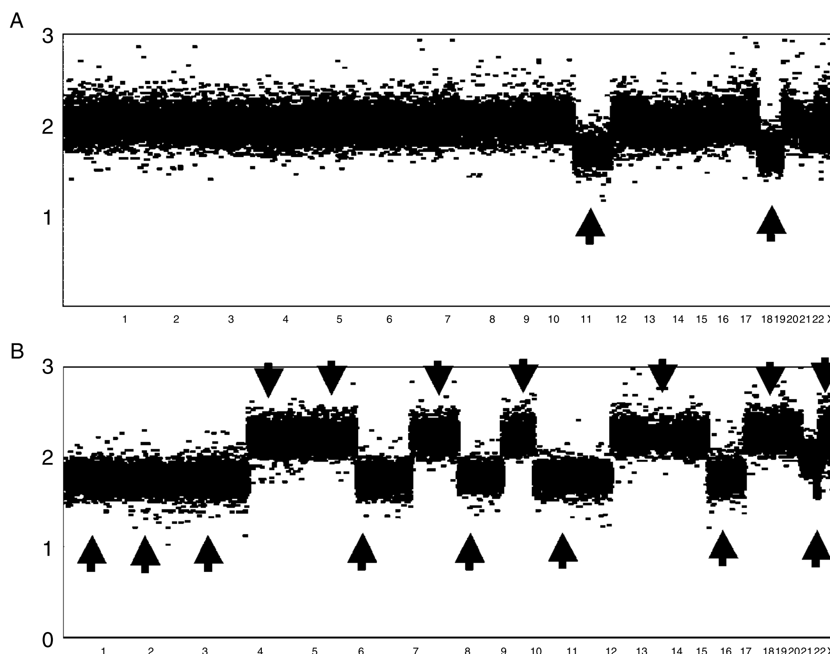

Figure 1 Copy number analysis for whole genome for samples no. 27 (panel A) and no. 68 (panel B). X-axis shows single nucleotide polymorphisms on all the autosomal and X chromosomes from p-terminal to q-terminal. The ratio of tumor copy number to non-neoplastic copy number multiplied by two at each SNP locus is shown on the Y-axis. Sample no. 27 has loss of chromosomes 11 and 18, and sample no. 68 has loss of chromosomes 1, 2, 3, 6, 8, 10, 11, 15, 16, 21, and 22, and gain of chromosomes 4, 5, 7, 9, 12, 13, 14, 15, 17, 18, and 20.

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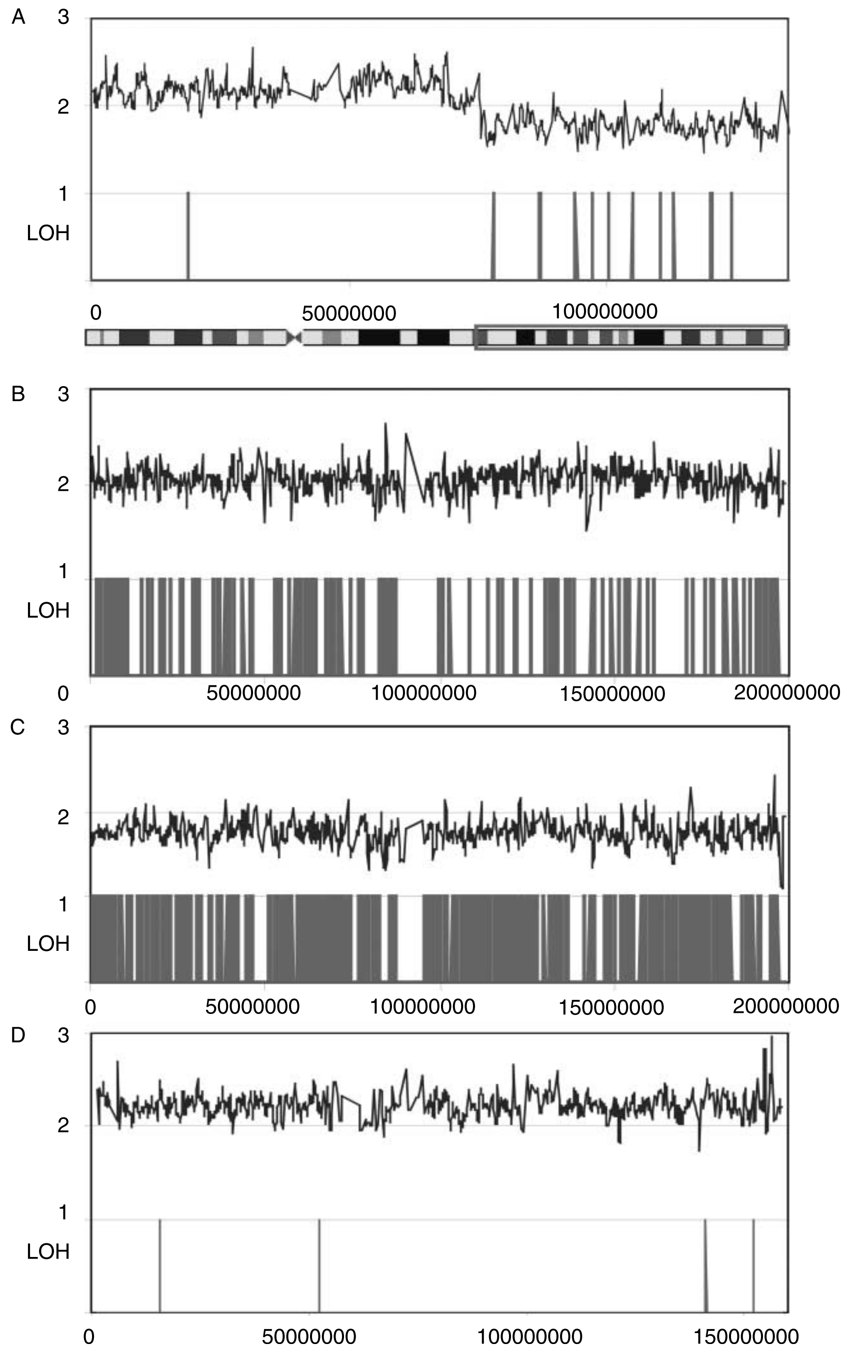

Figure 2 Examples of copy number aberrations of chromosomal loci and allelotyping. X-axis shows single nucleotide polymorphisms on chromosomes from p-terminal to q-terminal. The ratio of tumor copy number to non-neoplastic copy number multiplied by two at each SNP locus is shown on the Y-axis. LOH denotes loss of heterozygosity of heterozygous single nucleotide polymorphisms. Panel A shows partial copy number loss with loss of heterozygosity of chromosome 10 in sample no. 68, panel B shows loss of heterozygosity without copy number loss of chromosome 3 in sample no. 59, panel C shows whole chromosome loss with loss of heterozygosity of chromosome 3 in sample no. 51, and panel D shows whole chromosome gain without loss of heterozygosity of chromosome 7 in sample no. 51.

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Results

Clinicopathologic features

The clinicopathological features of the 15 patients with PETs have been reported previously (Table 1; Chan *et al.* 2003, Liu *et al.* 2005, Wang *et al.* 2005). There were nine well-differentiated neuroendocrine carcinomas and six neuroendocrine tumors of uncertain malignant potential. Both liver and lymph node metastasis were present in two patients (13%), liver metastasis alone in one patient (7%), and lymph node metastasis alone in four patients (27%). There were two gastrinomas and all other tumors were non-functional. Two patients had multiple endocrine neoplasia type 1 (MEN-1).

Alterations of copy number

AI were identified by copy number variation from the reference set and from the copy number of the matched non-neoplastic samples. The average copy number for all the non-neoplastic samples was 2.0 ± 0.05. Chromosomal gains and losses were identified by comparing the ratio of tumor copy number to the non-neoplastic copy number multiplied by two at each SNP locus (examples in Fig. 1). The mean of the copy number ratio with deletion of a chromosome or chromosomal loci was 1.79 ± 0.07, and with amplification of chromosome or chromosomal loci was 2.28 ± 0.13. A comparison was made between the SNP allelotyping data and the copy number estimation simultaneously (examples in Fig. 2). All but two chromosomes with copy number losses had LOH, suggesting that the LOH was due to hemizygous deletion (Fig. 2A). One tumor had LOH of chromosome 3 and another had LOH of chromosome 21 without alterations of copy number (Fig. 2B). These findings suggest that these chromosomes have allelic loss of one chromosome and duplication of the other parental chromosome.

Chromosomal aberrations in PETs are summarized in Fig. 3 and Table 2. All twenty-two autosomal chromosomes and chromosome X had partial or whole loss or amplification in one or more tumors. All but one tumor had at least one chromosomal aberration. Loss of chromosome 11 was the most frequent and present in eight tumors (53%), loss of chromosome 3 was present in seven tumors (47%), and loss of chromosomes 1 or 22 in six tumors each (40%). Gain of Chromosome 7 was the most frequent and present in nine tumors (60%), gain of chromosomes 14 or 17 in eight tumors each (53%), and gain of chromosomes 5, 12 or 20 in seven tumors each (47%). The aberrations of autosomal chromosomes were correlated with each other: loss or gain of a chromosome or chromosomal arm was associated with loss or gain of other chromosomes or chromosomal arms ($r > 0.55$, $P < 0.5$, Fig. 4).

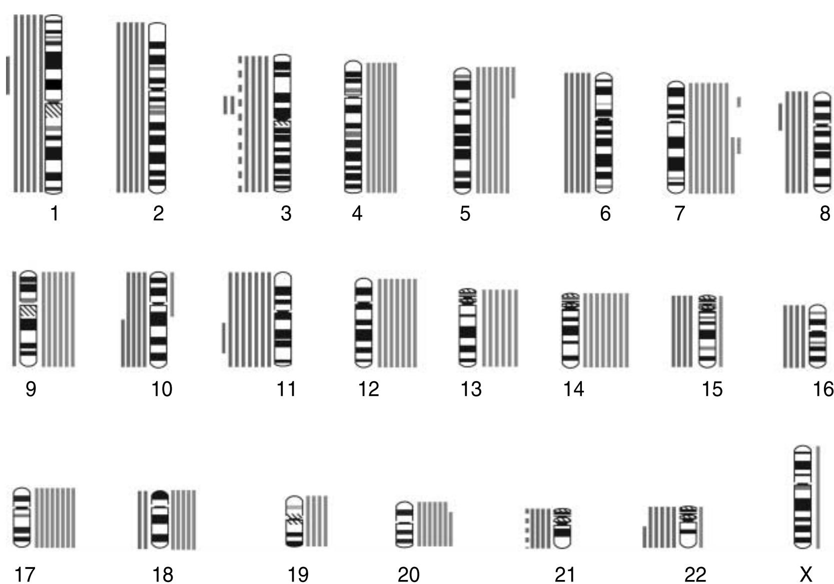

Figure 3 Summary of DNA copy number changes and loss of heterozygosity of 15 pancreatic endocrine tumors. Each line represents chromosomal aberration in one tumor. The vertical lines to the right of the chromosome ideograms indicate gain of chromosome or chromosomal region, lines to the left indicate loss of chromosome or chromosomal region. Dotted lines indicate loss of heterozygosity without copy number alterations of chromosome 3 and 21.

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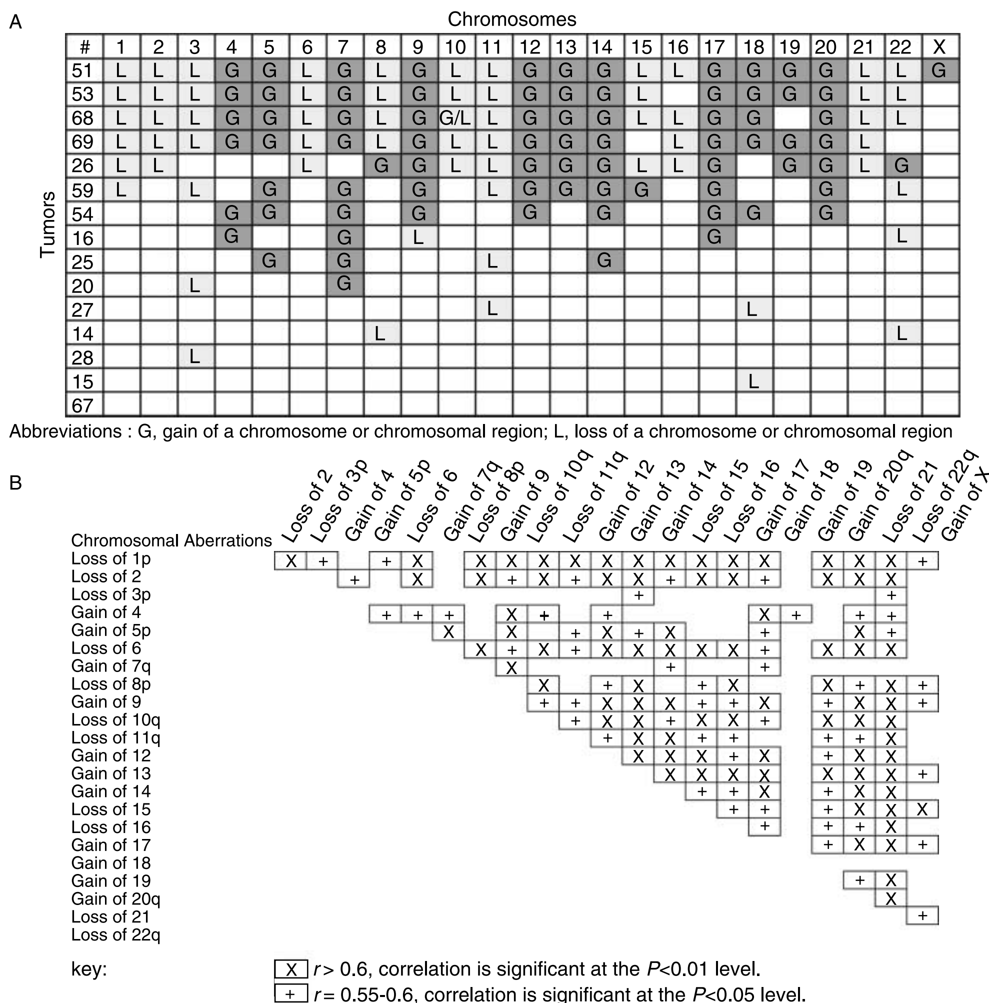

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#	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
51	L	L	L	G	G	L	G	L	G	L	L	G	G	G	L	L	G	G	G	G	L	L	G
53	L	L	L	G	G	L	G	L	G	L	L	G	G	G	L		G	G	G	G	L	L	
68	L	L	L	G	G	L	G	L	G	G/L	L	G	G	G	L	L	G	G		G	L	L	
69	L	L	L	G	G	L	G	L	G	L	L	G	G	G		L	G	G	G	G	L		
26	L	L				L		G	G	L	L	G	G	G	L	L	G		G	G	L	G	
59	L		L		G		G		G		L	G	G	G	G		G			G		L	
54				G	G		G		G			G		G			G	G		G			
16				G			G		L								G					L	
25					G		G				L			G									
20			L				G																
27											L							L					
14								L														L	
28			L																				
15																		L					
67																							

Figure 4 Panel A shows summary of chromosomal aberrations in tumors. Panel B shows chromosomal aberrations that had significant correlation with loss or gain of other chromosomes or chromosomal regions ($r=>0.55$–1.0, $P<0.5$).

We further compared the chromosomal deletions by LOH using SNP allelotyping from this study and microsatellite markers of chromosomes 9p, 11p, 16q, and 18 as reported previously (Chan *et al.* 2003, Wang *et al.* 2005). LOH of chromosome 9 was present in one tumor (no. 16) by both techniques, of chromosome 11q in seven tumors (nos. 26, 27, 51, 53, 59, 68, and 69), of chromosome 16q in three tumors (nos. 26, 51, and 69), and of chromosome 18 in one tumor (no. 27). However, LOH by SNP allelotyping but no loss by microsatellite markers was present in three tumors: one tumor (no. 25) had partial loss of chromosome 11, another (no. 15) had loss of chromosome 18, and the third (no. 68) had loss of chromosome 16. In addition, losses of chromosomes or chromosomal loci were more extensive by SNP allelotyping when compared with loss by microsatellite markers (data not shown). We have previously reported methylation of ras association domain family 1A, isoform A (*RASSF1A*) in these tumors (Liu *et al.* 2005). Methylation of *RASSF1A* gene was present in five of six (83%) PETs with loss of chromosome 3 and also in seven of nine (78%) PETs without loss of chromosome 3.

We divided the PETs into two groups based on number of chromosomal aberrations: low AI with four or fewer chromosomal aberrations, and high AI with more than four chromosomal aberrations. In our study, six (40%) of PETs had low AI and nine (60%) had high AI. The mean size of tumors with high AI was 5.4±3.1 cm compared with 2.3±1.3 cm for tumors with low AI ($P=0.03$). However, there was no correlation between AI and patient's age, sex, histological type, liver metastasis, lymph node metastasis, MEN-1, and hormonal statuses.

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Table 2 Summary of copy number deletions and gains in 15 pancreatic endocrine tumors

Chromosome	Whole chromosome aberration, tumor no.	Partial chromosome aberration, tumor no.	Chromosomal region	Base range	Potential tumor suppressor genes or oncogenes
Loss					
1	26, 51, 53, 68, 69	59	p31.1-p12	79 269 825–120 225 489	*DDA3*
2	26, 51, 53, 68, 69				
3	51, 53, 59, 68, 69[a]	20	p21.31-p21.2	49 870 085–51 884 070	
		28	p21.31-p21.2	49 525 778–51 884 070	Interferon-related developmental regulator 2 (*IFRD2*) *N*-acetyltransferase 6 (*NAT6*) Ras association domain family 1A, isoform A (*RASSF1A*) Cytokine-inducible SH2-containing protein (*CISH*)
6	26, 51, 53, 68, 69				
8	51, 53, 68, 69	14	p22-q11.23	18 983 547–53 650 298	*BNIP3L*
9	16				
10	26, 51, 53, 69	68	q22.2-q26.3	75 266 020–135 228 726	Phosphatase and tensin homolog (*PTEN*) *FAS* Deleted in malignant brain tumors 1 (*DMBT1*)
11	26, 27, 51, 53, 59, 68, 69	25	q14.3-q25	89 823 692–194 619 751	Ataxia telangiectasia mutated protein (*ATM*) Serine/threonine protein phosphatase 2A (*PPP2R1B*) 65 kDa regulatory subunit A, beta isoform
15	26, 51, 53, 68				
16	26, 51, 68, 69				
18	15, 27				
21	26, 51, 53, 68, 69[a]				
22	16, 51, 53, 59, 68	14	p11.23-q13.33	23 484 416–48 053 047	*CHEK2* Meningioma region PK1 3 placental 39.2 *NF2* Progesterone receptor-associated p48 (*ST13*)
Gains					
4	16, 51, 53, 54, 68, 69				
5	51, 53, 54, 59, 68, 69	25	p15.33-p12	0–44 516 774	Telomerase reverse transcriptase (*TERT*)
7	16, 51, 53, 54, 59, 68, 69	20	p13-p11.2	45 020 224–55 392 724	Epidermal growth factor receptor (*EGFR*) Insulin-like growth factor binding protein 3 precursor (*IGFBP-3*)
		20	q11.23-q21.3	75 568 643–96 382 227	Cyclin-dependent protein kinase 6 (*CDK6*)
		25	q11.23-q36.3	75 568 543–158 554 645	
8	26				
9	26, 51, 53, 54, 68, 69				
10		68	p15-q22.2	0–75 191 257	*RET*
12	26, 51, 53, 54, 59, 68, 69				

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Table 2 *continued*

Chromosome	Whole chromosome aberration, tumor no.	Partial chromosome aberration, tumor no.	Chromosomal region	Base range	Potential tumor suppressor genes or oncogenes
13	26, 51, 53, 59, 68, 69				
14	25, 26, 51, 53, 54, 59, 68, 69				
15	59				
17	16, 26, 51, 53, 54, 59, 68, 69				
18	51, 53, 54, 68, 69				
19	26, 51, 53, 69				
20	26, 51, 53, 54, 68, 69	59	p11.23-q13.33	18 623 480–62 342 703	Breast carcinoma amplified sequence 1 (*BCAS1*)
22	26				
X	51				

[a]LOH without copy number loss.

Discussion

In this study, we determined the chromosomal copy number alterations in PETs using a genome-wide high-density SNP allelotyping array and compared the copy numbers in tumor and non-neoplastic tissues. This method overcomes some of the limitations imposed due to the contamination of tumor by non-neoplastic cells in tumor samples, and experimental variations in the samples due to signal-to-noise ratio (Nannya *et al.* 2005). We found mostly whole and a few partial chromosomal aberrations in these tumors and these alterations were correlated with each other suggesting that these are not random events. In our study, SNP allelotyping was more sensitive in determining allelic loss compared with LOH studies using microsatellite analysis. Also the combination of both allelotyping and copy number analysis for each locus allowed us to identify uniparental disomy of chromosomes in two tumors due to either a non-disjunction event followed by chromosomal duplication or to a recombination. These alterations have been reported in acute lymphoblastic leukemias (Irving *et al.* 2005), and are common in non-seminomatous germ cell tumors (Lu *et al.* 2005) and basal cell carcinomas (Teh *et al.* 2005). These alterations cannot be detected by methodology that utilizes chromosomal copy number alone, such as comparative genomic hybridization or *in situ* hybridization. The uniparental origin of these chromosomes may have important implications for a role of imprinted genes present in these chromosomes in tumorigenesis of these malignancies.

In our present study, loss of chromosomes 1, 3, 11, or 22 was frequent in PETs. This finding corroborates previously reported studies of functional and non-functional PETs that showed frequent loss of chromosome 3, 6q, 11, and 22q by comparative genomic hybridization (Speel *et al.* 1999, Hessman *et al.* 2001). In previous studies, the allelic status of PETs was dependent on the tumor characteristics, including the hormonal status of tumors, and differed between MEN-1 associated and sporadic tumors. In a previous study, non-functional PETs had frequent loss of chromosome 6q and 11p compared with functional PETs, but chromosome 20p and 21 losses were frequent in both groups of tumors by allelotyping using microsatellite markers (Rigaud *et al.* 2001). Similarly, MEN-1 associated PETs had LOH of chromosome 11 but also had frequent loss of chromosomes 3, 6, 8, 10, 18, and 21 (Hessman *et al.* 2001). In our study, gain of chromosomes 5, 7, 12, 14, 17, and 20 was frequent. Similarly, previously reported studies showed frequent gain of chromosomes 5q, 7, 9q, 12, 14, 17, and 20

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(Terris *et al.* 1998, Speel *et al.* 1999, 2001, Tönnies *et al.* 2001, Zhao *et al.* 2001). In one study, gain of chromosomes 4 or 7 was more common in metastasis compared with the primary tumors (Zhao *et al.* 2001).

In our study, one frequently deleted region on chromosome 3p21 harbors *RASSF1A* gene involved in renal (Dreijerink *et al.* 2001), lung, breast, and ovarian carcinomas (Agathanggelou *et al.* 2001). We have previously reported that the *RASSF1A* gene was methylated in 66% of PETs and was associated with lymph node metastasis (Liu *et al.* 2005).

In our study, we classified PETs into high AI and low AI, and tumors with high AI had larger tumor size. It has been reported that the total number of chromosomal aberrations is more frequent in PETs with metastasis, in tumors more than 2 cm in size, and in non-functional compared with functional tumors (Speel *et al.* 1999, 2001). Other studies reported that tumors with high AI were associated with adverse prognosis (Rigaud *et al.* 2001), larger tumor size, and more advanced stage (Speel *et al.* 1999).

In the present study, using a limited number of PETs we were unable to find any association of chromosomal alterations with clinical parameters or prognosis. However, previous studies (Speel *et al.* 1999, 2001, Rigaud *et al.* 2001) and our current study have shown that PETs are genetically heterogenous and that may explain in part difficulties encountered in histologic classification of these tumors and predicting clinical behavior or prognosis.

In summary, our data demonstrated the feasibility of utilizing SNP allelotyping for genome-wide evaluation of LOH and chromosomal copy numbers in PETs including uniparental disomy.

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