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Patterns of Chromosomal Imbalances in Parathyroid Carcinomas

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In this study we have characterized chromosomal imbalances in a panel of 29 parathyroid carcinomas using comparative genomic hybridization (CGH). The most frequently detected imbalances were losses of 1p and 13q that were seen in >40% of the cases. The commonly occurring regions of loss were assigned to 1p21-p22 (41%), 13q14-q31 (41%), 9p21-pter (28%), 6q22-q24 (24%), and 4q24 (21%), whereas gains preferentially involved 19p (45%), Xc-q13 (28%), 9q33-qter (24%), 1q31-q32 (21%) and 16p (21%). The distribution of CGH alterations supports the idea of a progression of genetic events in the development of parathyroid carcinoma, where gains of Xq and 1q would represent relatively early events that are followed by loss of 13q, 9p, and 1p, and by gain of 19p. A sex-dependent distribution was also evident for two of the common alterations with preferential gain of 1q in female cases and of Xq in male cases. When the CGH profiles for the 29 carcinomas were compared with our previously published results for sporadic parathyroid adenomas, highly significant differences were revealed. Loss of 1p, 4q, and 13q as well as gains of 1q, 9q, 16p, 19p and Xq were significantly more common in the carcinomas than in the adenomas. In contrast, loss of the 11q13 region, which is the most common CGH abnormality in sporadic adenomas, was not detected in any of the carcinomas. Taken together, the findings identify several candidate locations for tumor suppressor genes and oncogenes that are potentially involved in parathyroid carcinogenesis. ***(Am J Pathol 2000, 157:579–586)***

The vast majority of parathyroid tumors are benign, whereas malignant parathyroid tumors are seen in <1% of patients with primary hyperparathyroidism.[1,2] Clinical findings such as a large tumor in the neck and very high serum levels of calcium and parathyroid hormone indicate that the hyperparathyroidism could be because of a parathyroid carcinoma. However, in cases with early presentation or in subtle cases where histopathology lacks evidence of invasion to adjacent organs or structures the diagnosis of parathyroid carcinoma can be very difficult.

Most parathyroid carcinomas occur sporadically, but familial forms of the disease are also recognized. The hyperparathyroidism-jaw-tumor syndrome and a subset of familial isolated hyperparathyroidism are both linked to chromosomal region 1q21-q32, and are associated with an increased risk of parathyroid carcinoma.[3–8] Loss of heterozygosity involving the wild-type allele for markers in 1q21-q32 has been detected in tumors from 1q-linked families suggesting the inactivation of a tumor suppressor gene in this region.[5,7,8]

Recently, loss of heterozygosity and comparative genomic hybridization (CGH) studies have identified chromosomal regions putatively involved in tumorigenesis of sporadic parathyroid adenoma. Loss of the *MEN1* region at 11q13 is the most common abnormality found[9–12] and in half of these cases a somatic *MEN1* mutation can be demonstrated.[13–15] Losses of chromosomes 1p, 6q, 9p, 11p, 13q, and 15q as well as gains of chromosomes 7, 16p, and 19p have frequently been demonstrated.[11,12,16] Because parathyroid carcinoma is so infrequently encountered (<1% of all hyperparathyroidism) and often the diagnosis is difficult to establish only a few genetic studies have been reported. Therefore the genetic mechanism underlying the development of malignant parathyroid tumors remains primarily elusive and no specific genetic alterations are known. Somatic loss of the retinoblastoma gene, *Rb1*, as well as loss of pRb immunostaining was reported in some carcinomas, but was also demonstrated in parathyroid adenomas.[17–19] Furthermore, alterations of the *TP53* gene have been found in a few carcinomas.[20]

CGH studies have proven to be powerful in identifying regions harboring oncogenes and tumor suppressor

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Table 1. Clinical Data on the 29 Parathyroid Carcinomas

Case no.	Id	Sex (M/F)	Age at operation	Familial/sporadic	Tissue analyzed
Unequivocal					
1	US-1	F		Sporadic	
2	S-1	F	48	Sporadic	Local rec
3	UK-1	M	43	Familial*	Local rec
4	F-1	M	52	Sporadic	Primary
5	F-2	F	32	Sporadic	Primary
6	S-2	F	78	Sporadic	Local rec
7	S-3	F	55	Sporadic	-
8	US-2	F	78	Sporadic	Primary
9	S-4	M	74	Sporadic	Primary
10	F-3	M	78	Sporadic	Primary
11	S-5	M	57	Sporadic	Primary
12	S-6	M	59	Sporadic	Primary
13	US-3	M	48	Sporadic	Local rec
14	S-7	F		Sporadic	-
15	J-1	F	52	Sporadic	Primary
16	J-2	M	37	Sporadic	Metastasis
17	J-3	M	54	Sporadic	Local rec
18	J-4	M	45	Sporadic	Metastasis
19	J-5	F	71	Sporadic	Local rec
Equivocal					
20	S-8	M	46	Sporadic	Primary
21	B-1	F	29	Sporadic	Primary
22	HK-1	F	58	Sporadic	Primary
23	F-4	M	62	Sporadic	Primary
24	S-9	F	51	Sporadic	Primary
25	S-10	M	53	Sporadic	Primary
26	F-5	F	58	Sporadic	Primary
27	US-4	M	52	Sporadic	Primary
28	S-11	F	28	Sporadic	Primary
29	S-12	F	73	Sporadic	Primary

*Affected member of a family with the hyperparathyroidism-jaw tumor syndrome.

genes of importance for tumor development. Agarwal et al[21] have previously reported differences in numerical chromosomal imbalances detected in a set of 10 parathyroid adenomas and 10 carcinomas. With the hope of gaining a better understanding of the molecular tumorigenesis of parathyroid carcinomas we have furthered the CGH studies by analyzing a total of 29 parathyroid carcinomas.

Materials and Methods

Tumor Specimens

Twenty-nine parathyroid carcinomas from 29 patients (28 sporadic and one familial) were fully characterized by CGH (Table 1). The tumors were divided into two groups, unequivocal carcinoma and equivocal cases, because of their histopathological features and clinical course. Cases 1 to 14 and 20 to 29 were all evaluated at the Karolinska Hospital by one of the authors (LG) and classified according to the criteria previously reported.[22] However, because these cases were collected at different locations from multiple centers worldwide and then returned to the respective clinics, no histopathological re-evaluation was performed in connection to the present study and the detailed information for each case is not presently available. Cases 15 to 19 came from the same endocrine surgical unit at Tokyo Women's Medical University and all had a clinical course with distant and/or lymphatic metastasis making the carcinoma diagnosis certain.[23] In addition to microscopically infiltrative growth pattern and/or evidence of recurrence, the 19 cases of unequivocal carcinomas (cases 1 to 19; Table 1) also often showed other pathological features occurring in parathyroid carcinoma such as marked fibrosis often with hyaline bands splitting the parenchyma and focally spread necrosis as well as cytological features such as marked cellular atypia, macronucleoli, and large nuclei. Ten patients had tumors that showed histopathological features of carcinoma, as described above, but lacked microscopically infiltrative growth pattern as well as evidence of recurrence, ie, equivocal cases (cases 20 to 29; Table 1). These cases were therefore classified as equivocal in line with the previously published classification criteria.[22] By histopathological investigation all tumor samples were shown to contain a minimum of 70% tumor cells. The study was approved by the local ethics committee.

Comparative Genomic Hybridization

DNA was extracted from fresh-frozen tumor tissue in five cases and from formalin-fixed paraffin-embedded tumors in the remaining 24 cases. DNA extraction from 20 to 30 paraffin sections (thickness, 3 to 4 μm) was performed using the QIAamp Tissue Kit (Qiagen, GmbH, Germany).

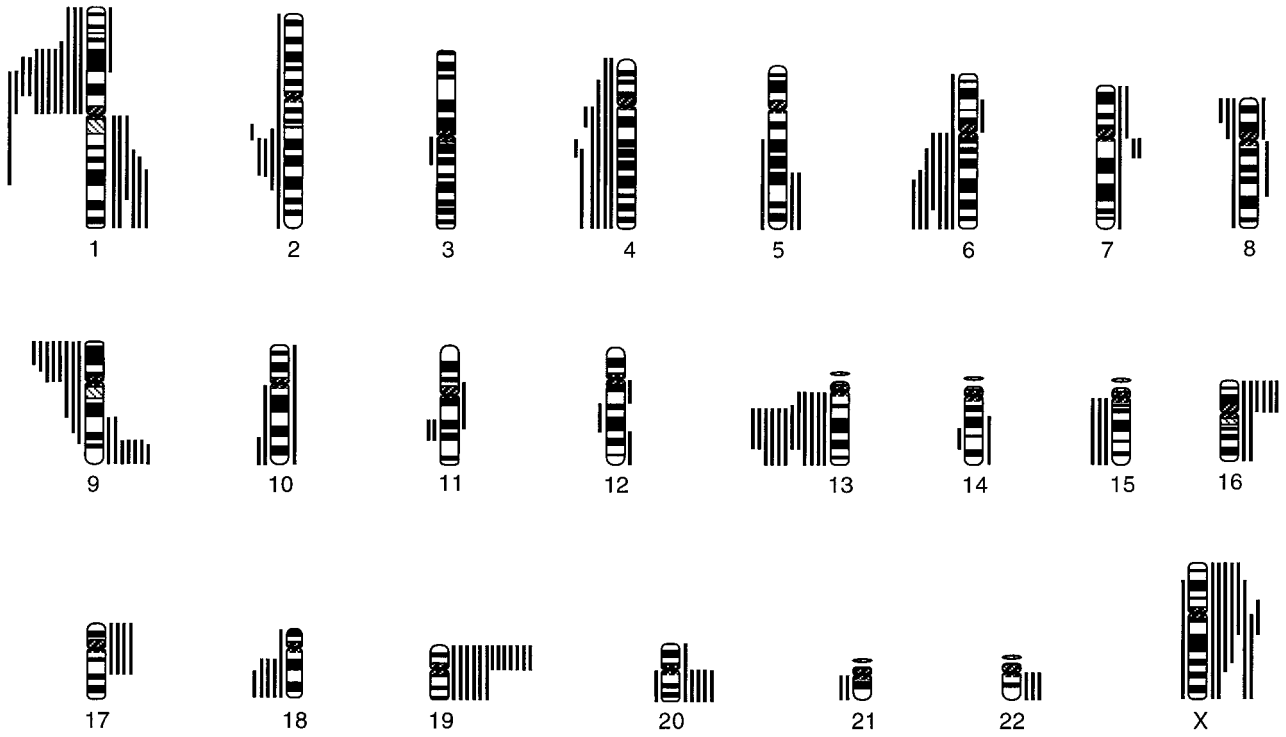

Figure 1. Summary of DNA copy number alterations detected by CGH in the 29 parathyroid carcinomas analyzed. Each line represents one alteration detected in one tumor, with losses illustrated to the **left** and gains to the **right** of the ideograms.

The yield of DNA was maximized with a prolonged proteinase-K digestion according to a previously published protocol.[24]

CGH was performed as previously described.[25] Briefly, tumor DNA samples were labeled with fluorescein isothiocyanate-dUTP (DuPont, Boston MA) by nick translation, and normal reference DNA was labeled with Texas Red (Vysis Inc., Downers Grove, IL). In each case the tumor and reference DNA samples were always sex-matched. Tumor and reference DNA were mixed with unlabeled Cot-1 DNA (Gibco BRL), denatured, and applied onto slides with denatured metaphases of normal lymphocytes (Vysis Inc.). After hybridization at 37°C for 48 hours, the slides were washed in 0.4× standard saline citrate (SSC)/0.3% Nonidet P-40 at 74°C for 2 minutes and in 2× SSC/0.1% NP-40 at room temperature for 1 minute. After air drying, the slides were counterstained with 4,6-diamino-2-phenylindole (Vysis Inc.). Two control hybridizations were also performed including normal female DNA against normal male DNA and DNA from a previously characterized breast cancer cell line (MPE 600; Vysis Inc.) against normal female.

Digital Image Analysis

Six to 10 three-color digital images (4,6-diamino-2-phenylindole, fluorescein isothiocyanate, and Texas Red fluorescence) were collected from each hybridization using a Zeiss Axioplan 2 (Carl Zeiss Jena GmbH, Jena, Germany) epifluorescence microscope and Sensys (Photometrics) charge-coupled-device camera interfaced to a IPLab Spectrum 10 workstation (Signal Analytics Corp., Vienna, VA). Relative DNA sequence copy number changes were detected by analyzing the fluorescence intensities of tumor and normal DNAs along the length of all chromosomes in each metaphase spread. The absolute fluorescence intensities were normalized so that the average green-to-red ratio of all chromosomes in each metaphase was 1.0. The final results were plotted as a series of green-to-red ratio profiles and corresponding standard deviations (SDs) for each human chromosome from p-telomere to q-telomere. At least 12 ratio profiles were averaged for each chromosome to reduce noise. Green-to-red ratios >1.20 were considered as gains of genetic material, and ratios <0.80 as losses. Heterochromatic regions, the short arm of the acrocentric chromosomes and chromosome Y were not included in the evaluation.

Comparison of CGH Alterations in Carcinomas versus *Adenomas*

Individual chromosome copy number changes of parathyroid adenomas[12] and parathyroid carcinomas were compared using the Fisher's exact test in the StatView 4.02 software. Probabilities of <0.05 were accepted as significant. The two groups of tumors were previously classified histopathologically as adenomas and carcinomas by one of the authors (LG), and analyzed by CGH side by side by two of the authors (SK and FF) using identical laboratory procedures and cut-off levels for identification of gains and losses.

Table 2. The Most Frequent Losses and Gains in Relation to the Total Number of Alterations in the 29 Parathyroid Carcinomas

Case no.	No. of changes	Losses and gains involving chromosomal regions						
		Xc-q13 gains	1q31–q32 gains	13q14–q31 losses	19p gains	9p21-pter losses	1p21–p22 losses	6q22–q24 losses
8	0							
28	0							
29	0							
21	0							
13	1	Xpter-q24						
2	1		1q24-qter					
14	1							
18	1							
1	2		1q23-qter	13q				
9	3	X			19p	9p		
11	3	X			19p	9p13-pter		
12	3	Xp21-qter						
27	3	Xpter-q23		13q				
3	4		1q31-qter	13c-q31		9p21-pter		
4	4						1p	
10	4	Xpter-q13		13q14-qter				6c-q24
25	4	Xq		13q14-qter	19p			6q22-qter
5	5				19	9pter-q21	1c-p31	6
15	5		1c-q32				1p	
16	5				19p		1c-p31	
22	5		1q		19p		1c-p31	
23	6			13q14-q31	19	9p		
19	8				19		1c-p32	
20	8	Xp11.2-q13		13q14-qter	19		1c-p22	
17	10			13q	19p		1p	
6	12			13q14-q31	19	9pter-q32	1c-p31	6q14-qter
26	13			13q13-q31	19	9p	1p21-p31.1	6q21-qter
7	15		1q	13q14-qter	19p		1p21-p31.1	6q
24	15			13q		9pter-q22	1p22-q31	6q

Results

CGH Alterations in Parathyroid Carcinomas

DNA samples from all five fresh frozen tumors and from 24 of the 30 paraffin embedded tumors were successfully analyzed by CGH (success rate 100% and 80%, respectively). The chromosomal regions with increased and decreased DNA sequence copy numbers are illustrated in Figure 1 and detailed for each tumor in Table 2. The 29 cases of parathyroid carcinomas were subdivided into cases with unequivocal and equivocal diagnosis of carcinoma, however there were no differences in the numbers of aberrations detected or the subchromosomal regions involved in the two groups of tumors. The number of detected alterations fell within a range of 0 to 15 with a mean value of 4.9 aberrations per sample. Chromosomal imbalances were identified in 25 of the 29 tumors analyzed (86%), with gains and losses detected in comparable frequencies (67 out of 141 and 74 out of 141, respectively).

The commonly occurring regions of loss could be defined to subchromosomal regions 1p21-p22 (41%), 13q14-q31 (41%), 9p21-pter (28%), 6q22-q24 (24%), and 4q24 (21%), whereas gains preferentially involved 19p (45%), Xc-q13 (28%), 9q33-qter (24%), 1q31-q32 (21%), and 16p (21%) (Figure 2). The pattern of CGH alterations in the individual tumors varied depending on the total number of detected alterations (Table 2). Gains of Xq and 1q were both detected as single aberrations in two cases. However, the four most frequent aberrations, ie, loss of 13q, gain of 19p, loss of 9p, and loss of 1p were seen in tumors with at least two or more aberrations. Furthermore loss of 6q and 4q, as well as gain of 9q and 16p were only detected in tumors having a total of four or five alterations.

Studies of the sex distribution of the 10 most common imbalances among the 28 sporadic cases, revealed clear sex differences for two of the abnormalities (Table 3). Gain of Xc-q13 was detected in eight of the 13 tumors from male patients, but in none of the 15 female cases ($P = 0.0004$; Table 3). On the other hand, gain on 1q31-q32 was only seen in tumors from female patients ($P = 0.04$ Table 3).

CGH Alterations in Parathyroid Carcinomas as Compared to Adenomas

The distribution of losses and gains detected in the 29 carcinomas were compared with our previously published results for 26 sporadic parathyroid adenomas[12] (Figure 2). This comparison revealed highly significant differences between the two types of tumors. Loss of 1p, 4q, and 13q as well as gains of 1q, 9q, 16p, 19p, and Xq were significantly more common in the carcinomas than in the adenomas (Figure 2). In contrast loss of 11 was much more common in the adenomas as compared to

Losses and gains involving chromosomal regions				
4q24 losses	9q33-qter gains	16p gains	Other losses	Other gains
				1p31-pter
				8c-q22
			8p21-pter,10q24-qter	
			20q	
			18q12-qter	
			2q14.2-q21, 14q22-q24, 15q	
			11q14-q22	
				7c-q11.2
4q24-qter			3c-q13, 10q	
	9q32-qter	16p		20q
	9q32-qter		Xp21-qter	
	9q33-qter		2q22-q31	17pter-q21
4q22-q24	9q32-qter		15q	5q23-qter, 17pter-q21, 22q
4		16	2q21-q32	20q
4	9q22-qter		18	5q23-qter, 7p, 10, 20 high
		16p	4c-q13, 15q, 18q12-qter	17pter-q21, 20q, 22q
	9q32-qter	16p	8p, 18q12-qter	6c-p21, 7c-q11.2, 17pter-q21, 22q
4q	9q22-qter	16p	2q22-q31, 5q14-qter, 8, 11q14-q22, 21q	12c-q13, 12q22-qter
4p15.1-qter		16	2, 12q14-q21, 18q12-qter, 21q	7, 8p, 11p11.2-q14, 14q21-qter, 20q

the carcinomas (Figure 2). The different genetic profiles of adenomas and carcinomas were even more evident when the minimal regions involved were considered. For example, losses involving 1p are characteristic of carcinomas but are also frequent in adenomas. However the minimal regions on 1p involved are clearly different, with involvement of 1p21-p22 in the carcinomas (Figure 2) and of 1p34-pter in the adenomas.[12] Furthermore, the *MEN1* gene region in 11q13 is a major target for losses of chromosome 11 in adenomas, whereas the *MEN1* gene locus was not involved in any of the two 11q losses detected in carcinomas (Figure 1).

Discussion

The tumorigenesis of malignant hyperparathyroidism is poorly understood. In this study a large panel of parathyroid carcinomas were investigated by CGH in an attempt to obtain a general picture about the prevalence and location of chromosomal imbalances in these tumors. One or more genomic alterations were demonstrated in the vast majority of cases (25 out of 29 cases or 86%). In the four cases without demonstrable chromosomal imbalances, submicroscopic or balanced genetic alterations might still be present although they could not be detected by the method applied. The pattern of CGH alterations detected in the parathyroid carcinomas was clearly different from the pattern previously reported for sporadic adenomas (Figure 2). This finding suggests that the two entities may have diverse pathogenesis. CGH analyses of adenomas *versus* carcinomas has been previously reported for other tumor types (eg, colon, ovary, and adrenocortical), and in all these cases the adenomas presented with few CGH imbalances whereas the carcinomas, in addition to the alterations present in the adenomas, also demonstrated multiple numerical alterations.[26–28]

The distribution of genetic alterations on the most frequently involved chromosome arms support the idea of a progression of genetic events in the development of parathyroid carcinoma (Table 1). Because gains of Xq and 1q were both detected as single alterations it is likely that alterations of genes in these regions are relatively early events in tumorigenesis. Similarly 13q loss, 9p loss, and 19p gain can be regarded as intermediate events, whereas 6q loss, 4q loss, 9q gain, and 16p gain would represent events occurring late in the tumor development.

The most frequently detected abnormality in this study was gain of 19p (45%). Gain of the same chromosome arm has also been described in benign parathyroid tumors, although at significantly lower frequencies (Figure 2). The 19p region harbors a locus for familial hypocalciuric hypercalcemia[29] making this a possible candidate gene for development of a range of parathyroid tumors including sporadic adenomas and carcinomas, irradia-

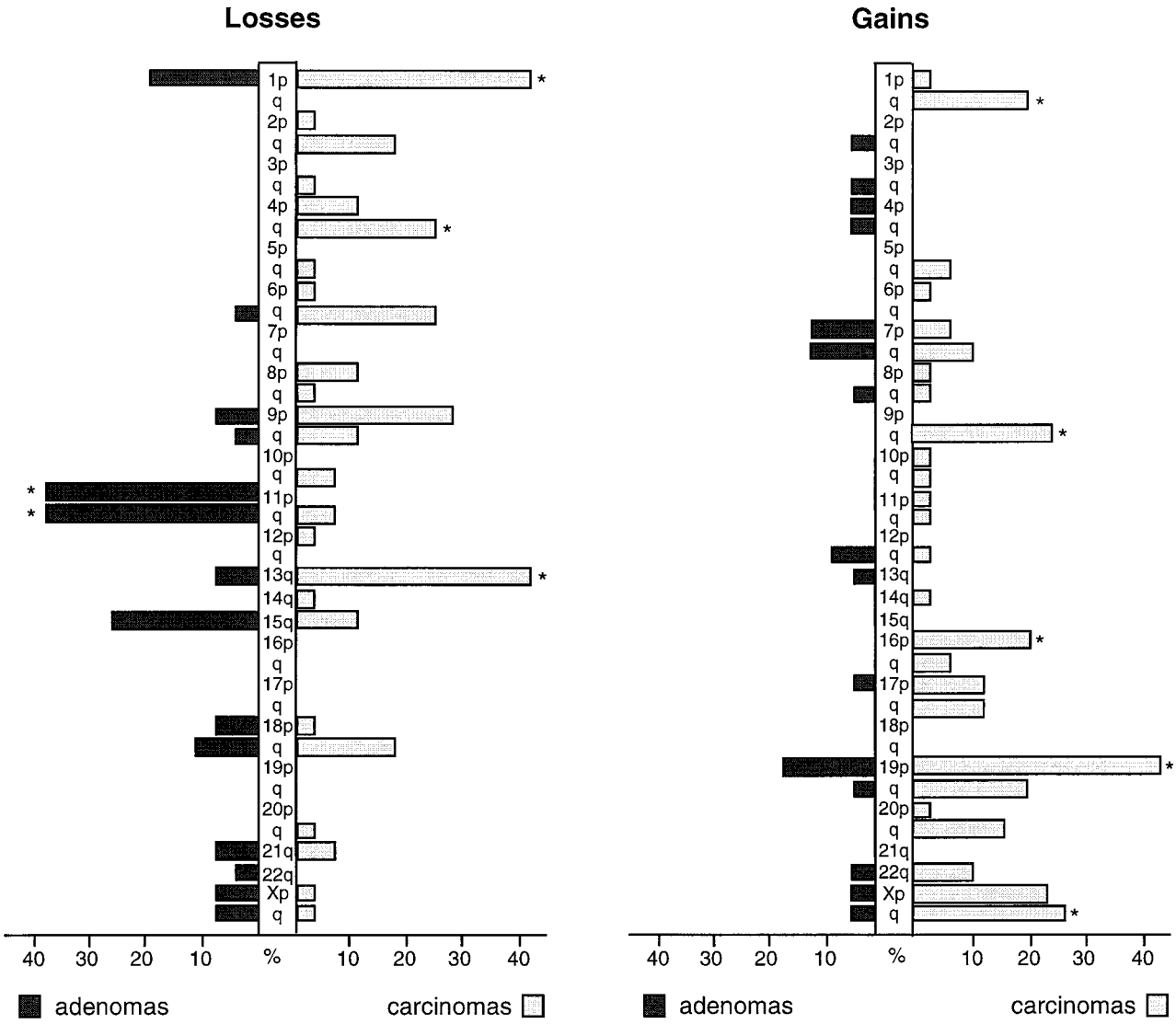

Figure 2. The distribution of the frequencies of losses and gains by chromosomal arms in 26 parathyroid adenomas[12] and 29 parathyroid carcinomas. *, significant difference ($P < 0.05$) in the frequencies between the adenomas and the carcinomas.

tion-associated adenomas, and adenomas from familial cases.

One of the most frequently detected losses involved the 13q14-q31 region. This region harbors the *RB1* gene at 13q14.3, which is known to be altered in several different human malignancies. Whether *RB1* is involved in parathyroid malignant transformation is still unknown. On the one hand, losses including the *RB1* locus were significantly more common in the carcinomas than in the adenomas (Figure 2). However, on the other hand the minimal region of loss includes large parts of chromosome 13, leaving several other genes as possible candidates eg, the *BRCA2* tumor suppressor gene.

Loss of 1p was also seen in almost half of the carcinomas (41%). The common minimal region of loss was assigned to 1p21-p22, which is clearly different from the 1p34-pter region preferentially involved in parathyroid adenomas.[11,12,30] The tumor suppressor gene or genes in this region contributing to the development of these tumors have not been identified yet. The distal portion of 1p is frequently deleted in neuroblastoma and other tumors and overlaps with the location of the p73 tumor suppressor gene, which consequently can be excluded as involved in parathyroid carcinomas.[12,31] However, the 1p21-p22 minimal region of loss defined in this study overlaps with the 1cen-p31 region of loss that we have previously seen in pheochromocytomas and abdominal paragangliomas.[32] These findings would support the existence of a tumor suppressor gene within 1p21-p22 that is involved in tumorigenesis of endocrine tumors.

Gains of 1q31-q32 and of Xcen-q13 were significantly more common in carcinomas than in adenomas. Sex-dependent occurrences were also apparent with exclusive involvement of 1q in female cases and of Xq in male cases. Interestingly, the 1q31-q32 region of gain overlaps with the *HRPT2* locus for the hyperparathyroidism-jaw-tumor syndrome and some forms of familial isolated hyperparathyroidism, which are characterized by a predisposition to parathyroid adenoma/carcinoma in combination with ossifying jaw fibromas and renal hamartomas.

Table 3. Sex Distribution of the Most Frequent CGH Alterations in Sporadic Parathyroid Carcinomas

Alteration	Male cases (n = 13)	Female cases (n = 15)	P value*
Gain 19p	7/13 (54%)	6/15 (40%)	n.s.
Loss 1p21-p22	4/13 (31%)	8/15 (53%)	n.s.
Loss 13q14-q31	6/13 (46%)	5/15 (33%)	n.s.
Loss 9p21-pter	3/13 (23%)	4/15 (27%)	n.s.
Gain Xc-q13	8/13 (62%)	0/15 (0%)	0.0004
Loss 6q22-q24	2/13 (15%)	5/15 (33%)	n.s.
Gain 9q33-qter	3/13 (23%)	4/15 (27%)	n.s.
Loss 4q24	2/13 (15%)	4/15 (27%)	n.s.
Gain 1q31-q32	0/13 (0%)	5/15 (33%)	0.04
Gain 16p	2/13 (15%)	4/15 (27%)	n.s.
Summary	3.7/13 (28%)	4.5/15 (30%)	n.s.

*n.s., not significant.

Furthermore, a reduced penetrance for the parathyroid component is characteristic of the disease in female members of families linked to the *HRPT2* locus.[5,7] This circumstance has been suggested to indicate the involvement of an additional locus on the X chromosome in the tumor development. Tumors from *HRPT2*-linked families frequently demonstrate loss of heterozygosity of the wild-type alleles for polymorphic markers in the region that could indicate that the disease gene is a tumor suppressor gene. However, the finding of gain in the 1q31-q32 region by CGH in both familial and sporadic cases, suggests a more complex mechanism of tumor development. Considering the above circumstances it is tempting to speculate about a model where the tumor development is promoted in a dose-dependent manner both by the loss of the wild-type gene as well as by the amplification of the mutated allele.

Whether sporadic parathyroid carcinomas develop from benign adenomas, or if they occur as a separate disease has not been confirmed. Although adenomas are common, carcinomas are extremely unusual, which would in itself speak against a progression in the majority of cases. The diverse genetic profiles of the two entities are not supportive of a progression pathway. In agreement with previous CGH studies of parathyroid tumors,[11,21] loss of the 11q13 region is the most common abnormality in adenomas but was not demonstrated in a single carcinoma (Figure 1). This strongly indicates that adenomas developing along the *MEN1* pathway do not have high potential for malignant transformation. This observation is also in agreement with the notion that families with hyperparathyroidism related to a constitutional *MEN1* mutation hardly ever develop carcinomas. However, the genetic basis of sporadic adenomas without involvement of the *MEN1* gene locus is still unknown. Therefore the available genetic information cannot be used to establish or exclude a genetic relationship between those adenomas and the group of sporadic parathyroid carcinomas. On the other end of the spectra, affected members of 1q-linked families have a high risk of parathyroid carcinoma. These cases are usually diagnosed as benign tumors in the initial phase, whereas parathyroid malignancy is only recognized during follow-up, which could possibly be related to a malignant progression. Whether some sporadic parathyroid tumors also develop along the *HRPT2* gene pathway and are therefore characterized by a malignant potential remains a central question which will finally be answered after identification of the gene involved.

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