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Genomic Localization of Novel Candidate Tumor Suppressor Gene Loci in Human Parathyroid Adenomas[1]

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ABSTRACT

Only one oncogene, *cyclin D1/PRAD1*, has an established role in parathyroid tumorigenesis, and parathyroid tumor suppressor genes on chromosome arms 1p and 11q, which still have not been identified, have also been implicated by loss of heterozygosity analysis. To investigate whether other putative tumor suppressor genes are involved in the pathogenesis of parathyroid adenomas, we performed a more comprehensive analysis of allelic losses in these tumors. Using 39 polymorphic markers, we examined each chromosome arm, excluding the short arms of the acrocentric chromosomes. In 25 parathyroid adenomas, frequent loss of heterozygosity, in >25% of the informative cases, was observed on chromosome arms 6q (30%), 11p (27%), and 15q (35%), in addition to previously reported 1p (30%) and 11q (38%) allelic losses. To more specifically localize the smallest shared regions of molecular genetic deletion, we examined the following chromosomes in greater detail: chromosome 6 (9 additional markers), chromosome 11 (8 additional markers), and chromosome 15 (15 additional markers). The regions most commonly deleted in these tumors were 6q22–23, 6q26–27, 11q13, 15q11–21, and 15q26–qter. All tumors with 11p loss had patterns consistent with monosomy for chromosome 11. These findings provide novel evidence for the existence of tumor suppressor genes on chromosome arms 6q and 15q that contribute commonly to the pathogenesis of parathyroid adenomas.

INTRODUCTION

Parathyroid adenomas are common, benign monoclonal tumors that cause hypercalcemia by the excessive secretion of parathyroid hormone (1). Thus far, three molecular genetic abnormalities have been implicated in the pathogenesis of parathyroid adenomas. The *cyclin D1/PRAD1* oncogene was discovered by its clonal rearrangement and overexpression in parathyroid adenomas (2–4) and was then implicated in other tumors, including breast cancer and B-cell lymphoma (5–8). In addition, common regions of allelic loss have localized still unidentified candidate parathyroid tumor suppressor genes to chromosome arms 1p (9) and 11q (10–12), the latter usually including the multiple endocrine neoplasia type 1 (*MEN1*)[3] gene region mapped to 11q13. Given the molecular heterogeneity demonstrated for many human tumors, we decided to examine the possibility that other tumor suppressor genes might contribute to the development of parathyroid adenomas.

Allelic loss studies have been useful for this purpose because somatic loss of one allele is a common mechanism for the inactivation of tumor suppressor genes, and such losses often include neighboring genetic loci, facilitating detection of the target region (13). Therefore, we performed a comprehensive allelotype analysis of human parathyroid adenomas.

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[3] The abbreviations used are: MEN, multiple endocrine neoplasia; LOH, loss of heterozygosity; FAL, fractional allelic loss.

MATERIALS AND METHODS

Patients and Tumor Samples. For allelotype analysis, parathyroid adenoma tissue and venous blood samples were obtained from 25 unselected patients undergoing parathyroidectomy for the management of hyperparathyroidism, all of whom were surgically and pathologically proven to have parathyroid adenomas by accepted criteria. These patients' clinical features are described elsewhere (9). For detailed mapping of deleted regions on chromosome 15, an additional 15 tumor-blood pairs were examined. These 15 unselected patients ranged in ages from 28 to 76 years (mean, 58 years). None of the patients included in this study had a history of neck irradiation nor a clinical or family history suggestive of multiple endocrine neoplasia. All patients were hypercalcemic and had elevated serum parathyroid hormone levels. After surgical removal, tumor tissue was carefully dissected, frozen in liquid nitrogen, and stored at −70°C until the extraction of DNA. All resected parathyroid tumors were classified as adenomas by clinicopathological assessment; none had any feature suggesting malignancy. High molecular weight genomic DNA was extracted from blood and tumor specimens by standard methods (14).

Allelic Loss Analyses. Primers for PCR amplification of microsatellite markers, chosen to represent every chromosome arm except the short arm of acrocentric chromosomes, were obtained from Research Genetics (Huntsville, AL) or synthesized on a DNA synthesizer (Applied Biosystems, Foster City, CA; Table 1). Some were also selected because of their proximity to either known or suspected tumor suppressor genes. Typically, one of each pair of primers was end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (New England BioLabs, Beverly, MA). Genomic DNA (100 ng) was then amplified in a 20-μl volume using a reaction mixture containing 1.5 mM MgCl2, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 200 μM concentration of each deoxynucleotide triphosphate, 0.01 μg/ml of BSA, 1.25 pmol of ^{32}P end-labeled sense primer, 5 pmol of unlabeled antisense primer, and 0.6 unit of AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) or 0.6 unit of Pyrostase DNA polymerase (Molecular Genetic Resources, Tampa, FL). PCR was carried out for 30–35 cycles in a GeneAmp PCR System 9600 (Perkin Elmer Cetus). Each cycle consisted of denaturation at 94°C for 30 s, annealing at 53–63°C for 30 s, and extension at 72°C for 45 s; for each polymorphism, the PCR reaction was optimized by adjusting the annealing temperature (Table 1). PCR products were then mixed with a formamide gel-loading solution, heat denatured at 94°C, separated on a denaturing 6–8% polyacrylamide, 32% formamide gel (15), and visualized by autoradiography for 0.5–48 h.

Two polymorphisms were detected by Southern blotting of *Msp*I-digested paired leukocyte control and tumor DNA (5 μg) from each patient: *D6S37* (pYNZ132) and *D15S1* (pMS1–14). Both plasmids were obtained from the American Type Culture Collection. Allelic loss was scored as described previously (9).

RESULTS

Table 1 shows the primers used for the initial allelotype analysis of the 25 parathyroid adenomas, chromosomal localizations of the polymorphic loci, and the frequency of LOH at each locus. Representative examples of autoradiograms demonstrating LOH are shown in Fig. 1. Of the 39 chromosomal arms analyzed, 24 (61.5%) revealed LOH in one or more tumors. Among the loci that showed LOH, the frequency varied widely, ranging from 5% on 4q, 7p, 17q, 18p, 19p, and 22q to 38% on 11q. Allelic losses at frequencies >25% were observed on chromosomal arms 1p (30%), 6q (30%), 11p (27%), 11q (38%), and 15q (35%), as shown in Fig. 2. A more detailed study of 1p in this set of tumors revealed previously that 40% of them had LOH (9). The

Table 1 *Frequency of LOH at individual chromosomal sites in 25 parathyroid adenomas*

Chromosome	Locus	Chromosomal localization	Annealing temperature (°C)	LOH/informative cases	% of LOH
Allelotype analysis					
1	*D1S243*	1p36	60	6/20	30
	D1S215	1q21–23	60	2/11	18
2	*D2S155*	2p15–16	58	1/17	6
	D2S102	2q33–37	53	2/20	10
3	*D3S11*	3p22–24.2	60	0/16	0
	Glut 2	3q26.1–26.3	58	0/23	0
4	*D4S2397*	4p13	60	1/18	6
	D4S415	4q32–35	58	1/19	5
5	*D5S392*	5p15–pter	55	0/19	0
	D5S409	5q21	60	1/17	6
6	*D6S344*	6p24–25	58	0/22	0
	D6S310	6q22–23	58	7/23	30
7	*D7S507*	7p21–pter	55	1/20	5
	D7S479	7q22–31	53	0/17	0
8	*D8S277*	8p22–pter	55	0/18	0
	D8S272	8q24–qter	58	0/15	0
9	*D9S126*	9p21	55	3/16	19
	D9S146	9q13–21	58	1/9	11
10	*D10S249*	10p15–pter	60	1/14	7
	D10S187	10q22–24	55	2/22	9
11	*D11S554*	11p11.2–12	58	6/22	27
	PYGM	11q13	63	6/16	38
12	*D12S77*	12p12–13	53	2/18	11
	D12S392	12q24–qter	60	1/16	6
13	*D13S168*	13q14.2–14.3	58	2/18	11
14	*D14S45*	14q32	53	0/23	0
15	*D15S87*	15q26	55	7/20	35
16	*D16S418*	16p13–pter	58	0/22	0
	D16S413	16q24–qter	58	0/22	0
17	*D17S796*	17p13	60	0/17	0
	D17S791	17q12–21	58	1/22	5
18	*D18S59*	18p11–pter	58	1/20	5
	D18S46	18q12.2–21.1	60	1/17	6
19	*D19S216*	19p13–pter	55	1/22	5
	D19S210	19q13–qter	58	1/18	6
20	*D20S95*	20p13–pter	55	0/16	0
	D20S25	20q13.3	58	0/20	0
21	*D21S156*	21q22.3	58	0/16	0
22	*D22S351*	22q11.2	58	1/19	5
Additional deletion mapping					
6	*D6S282*	6p21	55	0/22	0
	D6S286	6q14	55	1/17	6
	D6S300	6q16.3–21	58	3/17	18
	D6S287	6q	55	4/22	18
	D6S311	6q24–25	55	4/6	67
	D6S441	6q	55	1/11	9
	D6S305	6q25.2	58	2/15	13
	D6S264	6q26–27	58	5/12	42
	D6S37	6q26–27	VNTR[a]	4/5	80
11					
	D11S861	11p15.2	58	5/21	24
	D11S907	11p13	53	3/16	19
	D11S1383	11q12	58	4/5	80
	D11S787	11q13.4	58	4/6	67
	D11S527	11q13.5	58	5/7	71
	D11S873	11q14	55	6/23	26
	D11S614	11q22–23	58	5/22	23
	D11S874	11q24–25	58	3/13	23

[a] VNTR, variable number tandem repeat.

Table 1 *Continued*

Chromosome	Locus	Chromosomal localization	Annealing temperature (°C)	LOH/informative cases	% of LOH
15					
	D15S97	15q11–12	60	8/32	25
	GABRB3	15q13–14	58	7/31	23
	D15S165	15q	58	7/32	22
	D15S144	15q	58	7 /9	78
	ACTC	15q	58	6/36	17
	D15S118	15q	60	5/16	31
	D15S1	15q14–21	RFLP	4 /5	80
	CYP19	15q21.1	60	5/33	15
	D15S108	15q	58	3 /8	38
	D15S125	15q	58	5/24	21
	D15S114	15q23–25	58	4 /4	100
	D15S116	15q26	58	7/21	33
	D15S127	15q26	58	7/10	70
	D15S100	15q26	58	5/31	16
	D15S107	15q26	58	6 /8	75

frequency of allelic loss varied in individual tumors (Table 2). Allelic losses were not detected in 9 tumors, whereas the remaining 16 tumors showed loss(es) in 1–19 chromosomal arms. Among these 16 tumors that have allelic loss(es), 14 tumors showed losses in at least two different chromosomal arms.

FAL was calculated as the ratio of chromosomal arms that showed loss among all informative arms for each patient, as described by Vogelstein *et al.* (16) (Table 2). The FAL values varied from 0 to 0.594, with a median of 0.087 in 25 tumors. Neither FAL values nor deletion in any particular chromosomal arm was significantly associated with any clinical parameter studied, including tumor size, patient age, sex, level of serum parathyroid hormone, level of serum calcium, or clinical symptoms.

In addition to losses of 1p and 11q regions reported previously, our allelotype analysis identified three autosomal arms, namely 6q, 11p, and 15q, on which >25% of parathyroid adenomas had LOH. To more specifically define the smallest shared regions of loss, in which the putative tumor suppressor genes are expected to reside, we constructed detailed deletion maps of chromosomes 6, 11, and 15. For chromosome 6, we examined all 25 adenomas for allelic loss at 9 additional polymorphic loci spanning the chromosome. All 25 tumors were informative at one or more loci on chromosome 6, and overall, 8 (32%) of 25 adenomas had allelic loss at one or more of these loci. Fig. 3 shows a detailed map of the allelic loss data on chromosome 6 for these eight adenomas. All of these tumors had losses on 6q but not 6p, including four tumors (cases 12, 13, 16, and 28), with loss at all informative 6q loci. Of the four cases that showed partial deletion on 6q, two tumors (cases 23 and 48) had allelic loss telomeric to *D6S286* at 6q14. Moreover, two other tumors (cases 42 and 47) showed allelic loss at the *D6S310* locus (6q22–23) and loss telomeric to the *D6S264* locus (6q26–27), respectively. To add experimental weight to the conclusion that the PCR assays were detecting true allelic losses rather than another form of allelic imbalance (*e.g.*, amplification of the "retained allele"), we performed conventional Southern blot analysis using a variable number tandem repeat marker, *D6S37* (6q26–27). In each positive case, true allelic loss was observed (Fig. 1), supporting the interpretations of the PCR-based data. These results suggest that at least one and perhaps two regions of common deletion exist on 6q, at 6q22–23 and 6q26–27.

For chromosome 11, final results reflect the examination of all 25 adenomas using a total of 10 widely spaced markers. Eight of 25 adenomas (32%) showed allelic loss of at least one locus on chromosome 11 (Fig. 4). The only marker that revealed allelic loss in all informative tumors among these eight was *PYGM* at 11q13; one tumor (case 49) had allelic loss confined to this shortest region of overlap. *PYGM* is linked tightly to the *MEN1* putative tumor suppressor gene, and *MEN1* is likely to be the tumor suppressor gene inactivated in these sporadic parathyroid adenomas as well (10, 17). It is of interest that all tumors with allelic losses on 11p also showed LOH at all other informative loci, consistent with monosomy 11.

To construct a deletion map of chromosome 15, we analyzed a total of 40 adenomas for allelic loss using 16 polymorphic markers, including 15 microsatellite markers and one Southern-based RFLP marker, *D15S1*. Overall, 12 (30%) of 40 adenomas showed loss of at

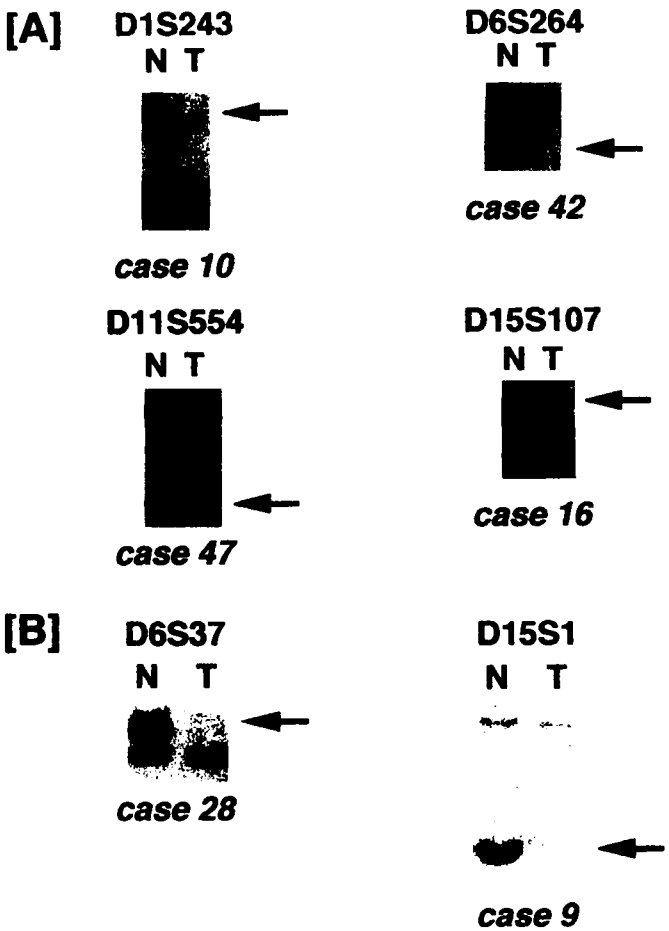

Fig. 1. Representative autoradiograms of microsatellite markers (*A*) and from Southern blot analyses (*B*), demonstrating LOH. *T*, DNA samples isolated from tumor tissue; *N*, DNA samples isolated from the corresponding normal peripheral leukocyte. Chromosomal loci are indicated above the autoradiograms. *Arrows*, tumor-specific loss of one allele.

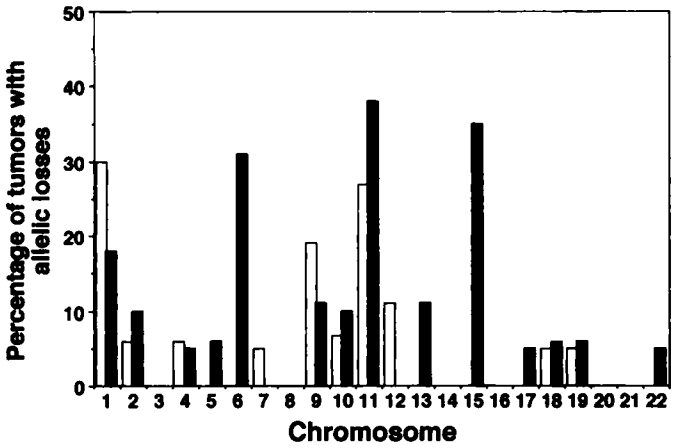

Fig. 2. Frequency of allelic loss on each chromosomal arm in 25 parathyroid adenomas. □ and ■, the short arm (p) and long arm (q) of each chromosome, respectively. All primers used in allelotype analysis are listed in Table 1.

Table 2. *Allelic losses in chromosomal arms and FAL in individual tumors*

Tumor	Chromosomal arms with LOH	No. of informative arms	FAL
1		30	0
6		29	0
8		31	0
9	1p, 12p, 15q	24	0.125
10	1p, 1q, 2p, 2q, 9p, 15q	29	0.207
11		30	0
12	1p, 6q, 9p, 11p, 11q, 15q	32	0.188
13	6q, 11p, 11q, 15q	29	0.138
15	11p, 11q	26	0.077
16	6q, 15q	32	0.063
18	11p, 11q	32	0.063
20	1p, 10q	28	0.071
23	6q, 13q	28	0.071
25		30	0
26		26	0
27		26	0
28	1p, 6q, 11p, 11q, 15q	33	0.152
41	1p, 12p, 15q	28	0.107
42	6q, 7p, 11q	30	0.100
43		25	0
45	13q	31	0.032
46		26	0
47	1q, 2q, 4p, 4q, 5q, 6q, 9p, 9q, 10p, 10q, 11p, 11q, 15q, 17q, 18p, 18q, 19p, 19q, 22q	32	0.594
48	6q, 15q	29	0.069
49	11q	32	0.031

least one locus on 15q. Southern blot data were again consistent with true allelic loss rather than with another form of allelic imbalance. Fig. 5 shows a detailed map of the allelic loss data for these 12 cases. Three tumors (cases 9, 47, and 48) had patterns consistent with monosomy for chromosome 15q, whereas the other nine cases had partial deletion on 15q. Allelic loss patterns were quite complex, with five tumors (cases 13, 41, 61, 63, and 65) having one deleted region and four tumors (cases 10, 12, 16, and 28) having two separately deleted regions. No single polymorphic locus showed allelic loss in all informative tumors among these 12. The observed patterns of complex losses on chromosome 15 are consistent with the existence of at least 2 and perhaps 3 distinct regions of common deletion, one being on distal 15q (15q26–qter) and one or two on proximal 15q (15q11–21). It would appear that study of more tumors and markers will be necessary to further delineate the critical regions of loss on chromosome 15 in parathyroid adenomas.

DISCUSSION

The aim of this study was to identify chromosomal regions likely to contain important tumor suppressor genes involved in the development of human parathyroid adenomas. The analysis revealed frequent LOH on five chromosomal arms, including sites of deletions identified previously in other neoplasms and novel sites that have thus far not been described in any other tumor system. Frequent LOH on chromosomes 1p and 11q in human parathyroid adenomas has been reported previously (9, 10–12). In addition to these sites, we detected LOH in >25% of informative cases on chromosome arms 6q, 11p, and 15q. This suggests the possible involvement of more tumor suppressor genes in the pathogenesis of parathyroid adenomas than was appreciated previously.

Frequent allelic loss on chromosome 6q has been reported in other types of tumors, including renal cell carcinoma (18), breast cancer (19), melanoma (20), ovarian cancer (21), lymphoma (22), and hepatocellular tumors (23). Our results show that 6q losses in parathyroid adenomas cluster in two discrete regions of common deletion. On the basis of the available physical mapping data for the markers, these areas of minimal common deletion correspond to regions 6q22–23

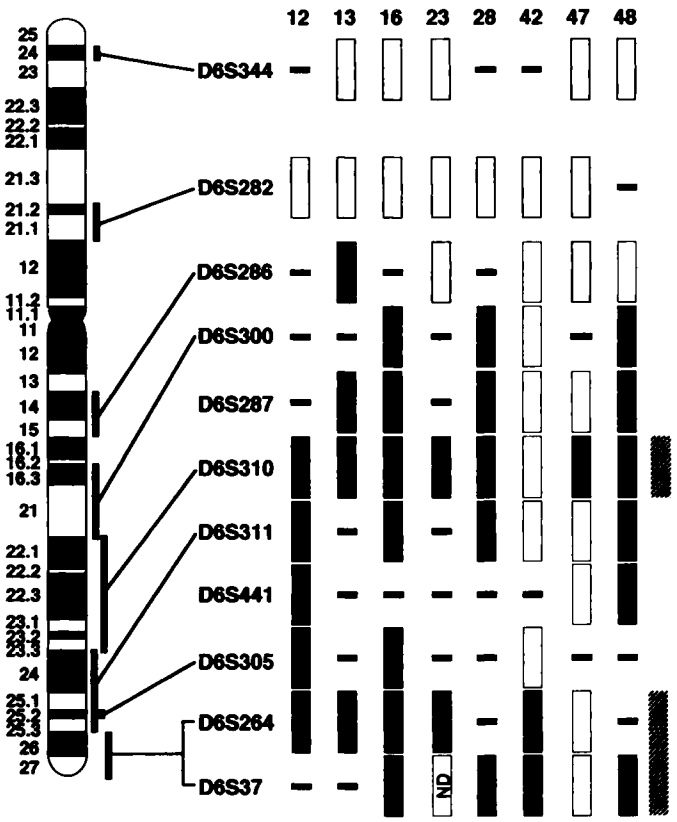

Fig. 3. Deletion map of chromosome 6. The eight tumors demonstrating chromosome 6 loss are illustrated. *Left*, markers are listed in order according to the published linkage and physical mapping data (43–45). *Top*, case numbers. At a given locus: ■, tumor-specific allelic loss; □, retention of both alleles; *thick bar*, constitutional homozygosity; *ND*, not done. *Hatched rectangles* on the extreme *right*, two commonly deleted regions, 6q22–23 (*D6S310* locus) and 6q26–27 (telomeric to *D6S264*).

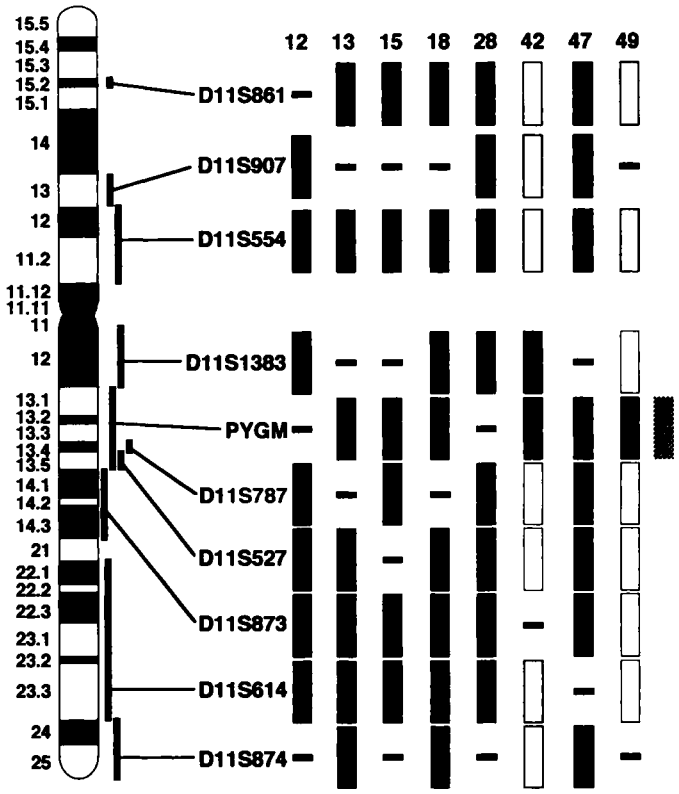

Fig. 4. Deletion map of chromosome 11. The eight tumors demonstrating chromosome 11 loss are illustrated. *Left*, markers are listed in order according to the published linkage and physical data (46). *Top*, case numbers. At a given locus: ■, tumor-specific allelic loss; □, retention of both alleles; *horizontal thick bar*, constitutional homozygosity. *Hatched rectangle* on the extreme *right*, one commonly deleted region, 11q13.

and 6q26–27. 6q22–23 overlaps with a commonly deleted region described in malignant melanoma (20) and B-cell non-Hodgkin's lymphoma (22). It remains to be defined whether the inactivation of the same gene is important in all these diseases; but interestingly, *cyclin D1* (*PRAD1*), originally identified as a parathyroid oncogene, has also been implicated in B-cell lymphomas, breast cancer, and several other malignancies (5, 6, 24, 25). Region 6q26–27 should also be taken into consideration to potentially harbor a critical tumor suppressor gene, in view of the specific deletions in this region described in renal cell carcinoma (18), malignant melanoma (20), ovarian cancer (21), and lymphoma (22). Although there are no obvious candidate tumor suppressor genes in these two areas, microcell-mediated chromosome transfer studies have indicated that wild-type chromosome 6 carries a gene that can reverse the tumorigenicity of a melanoma cell line (26), and that chromosomal regions 6q21–23 and 6q-26–27 induce alterations of *in vitro* growth properties and suppress tumorigenicity of breast cancer cell lines (27). Our findings are certainly compatible with these concepts that at least two tumor suppressor genes may exist on chromosome 6q.

As reported previously by others (10, 12, 28), we have implicated allelic loss at 11q13 as a clonal lesion in approximately 30% of sporadic parathyroid adenomas, presumably reflecting inactivation of the *MEN1* gene. Allelic loss at RFLPs in the *MEN1* region has also been shown in a small minority of parathyroid glands from patients with uremic hyperparathyroidism (29). The *MEN1* susceptibility locus has been mapped to chromosome 11 very close to the *PYGM* marker by genetic linkage analysis (30). The findings of 11q13 allelic loss in *MEN1*-related pancreatic islet cell tumors and parathyroid neoplasms provided evidence that the gene, as yet unidentified, functions as a tumor suppressor (10, 30). Furthermore, LOH of the 11q13 region has been detected in other types of tumors, including renal cell carcinoma (18), squamous carcinoma of the head and neck (31), and male germ cell tumors (32). However, the relationship of *MEN1* and these tumor-associated genes located in the vicinity of 11q13 remains to be determined. In the present study, we showed that several tumors with 11p loss also had patterns consistent with monosomy 11 more frequently than was reported previously (10–12, 28). It is possible that even these extensive losses are unmasking only the single *PYGM*-linked tumor suppressor gene, or alternatively an additional tumor suppressor gene on 11p may occasionally act in concert with the 11q13 loss, providing an extra selective advantage.

Frequent allelic losses on chromosome 15 have been reported in a few tumor types, including osteosarcoma (33) and epithelial ovarian cancer (34). In the present study, we have constructed deletion maps of parathyroid adenomas and identified two distinct commonly deleted regions. One of these regions lies between loci defined by *D15S97* and *D15S1* at 15q11–21 and the other lies between *D15S107* and *D15S87* at 15q26–qter. As noted above, patterns of allelic loss on chromosome 15 were complex, but the clonality and high frequency of such lesions strongly favors their pathogenetic importance. In the last several years, important human disease genes have been mapped to chromosome 15q. Among them, Bloom syndrome is a rare autosomal recessive disorder in which a variety of neoplasms, most notably carcinomas and leukemias, pose an increased risk. Recently, it has been reported that the Bloom syndrome gene is tightly linked to *FES*, a gene the chromosome position of which is known to be 15q26.1 (35). However, parathyroid adenomas have not been recognized as part

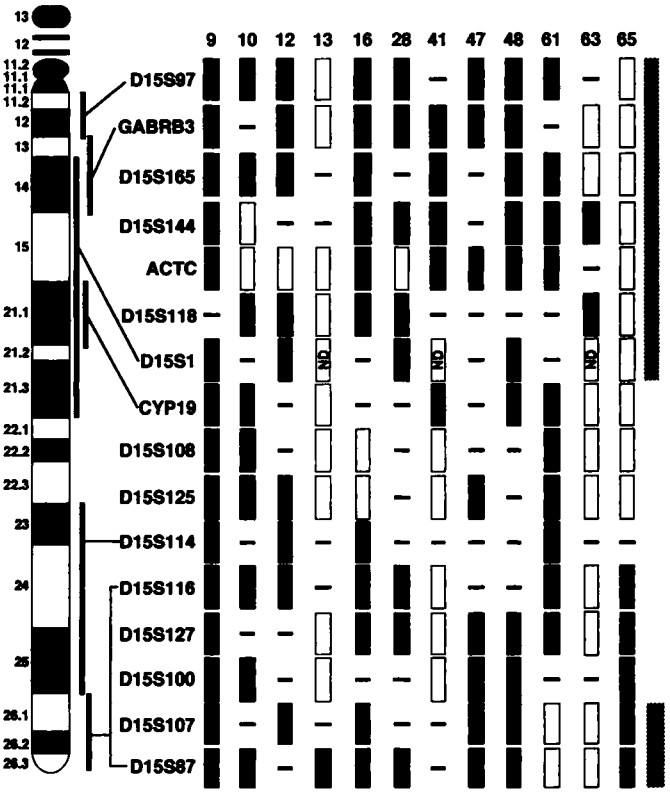

Fig. 5. Deletion map of chromosome 15. The 12 tumors demonstrating chromosome 15 loss are illustrated. *Left*, markers are listed in order according to the published linkage and physical data (47–49). *Top*, case numbers. At a given locus: ■, tumor-specific allelic loss; □, retention of both alleles; *horizontal thick bar*, constitutional homozygosity; *ND*, not done. *Hatched rectangles* on the extreme *right*, two commonly deleted regions, 15q11–21 and 15q26-qter.

of the Bloom tumor predisposition and have low indices for genetic instability, so it seems most likely that a different 15q gene(s) will prove relevant for parathyroid tumorigenesis.

The occurrence of distinct allelic losses in a given tumor type has been evaluated by its FAL value (36). The average FAL value (8.6%) in parathyroid adenomas was much lower than that in colorectal carcinoma (20%) or in other types of malignancies (36–40). A FAL value has been reported for only one other type of benign tumor, to our knowledge, namely a FAL of 24% for actinic keratoses (41); however, many chromosomal arms were not examined. In colorectal carcinoma, high FAL values were strongly associated with poor prognosis (36); no clear correlation of the much lower FAL values in parathyroid adenomas with their clinicopathological features has been observed nor would this necessarily be expected. However, the variability in FAL values among parathyroid tumors suggests that the development of even these benign neoplasms is a multistep process and is heterogeneous at the molecular level. Generally low background FAL values in parathyroid adenomas do heighten the likelihood that the novel candidate tumor suppressor gene regions we have identified are indeed of pathogenetic significance.

In summary, the comprehensive allelotyping of parathyroid adenomas has provided novel guideposts for future efforts at positional cloning and positional candidate cloning (42) of human tumor suppressor genes that will be of certain significance to endocrine tumors, and most likely, to other human tumors as well.

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