Different Patterns of Allelic Loss (Loss of Heterozygosity) in Recurrent Human Pituitary Tumors Provide Evidence for Multiclonal Origins[1]

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ABSTRACT

Sporadic human pituitary tumors are benign adenomas of monoclonal origin. This implies that they arise from *de novo* somatic mutation(s) within a single pituitary cell. The availability of original and recurrent/regrown tumors from the same patient allowed testing of the prediction that recurrent/regrown tumors have identical genetic abnormalities as the original tumor sample. We used PCR amplification, from archival slide-extracted DNA, to allelotype microsatellite polymorphisms as an indication of clonality and confirmed this by X chromosome inactivation analysis in samples from women. Tumors from 33 of 49 (67%) patients with two or more specimens showed loss of heterozygosity (LOH) of at least one marker in at least one of their samples. Two patterns of LOH were observed. In pattern A in 14 of 33 (42%) of patients, the LOH pattern of the first tumor was preserved in the second recurrent sample, with some recurrent tumors also showing additional LOH. In these patients, the original and second tumors are presumed to arise from the same original clone with or without progressive accumulation of LOH. In pattern B [19 of 33 (58%) patients], LOH seen in the first tumor was not preserved in the second or subsequent tumors, as evidenced by retention of heterozygosity compared with the first tumor. The simplest explanation is that the second tumor, although still monoclonal, arises from another independently abnormal clone. This was confirmed by X chromosome inactivation analysis in all 11 women where this was informative. These results show that initial and recurrent tumors, of a benign tumor type, are frequently derived from separate independent clones. This suggests that either: (*a*) more than one abnormal clone is present from the outset though only one dominates; or (*b*) several clones arise independently at different times. In both scenarios, the initiating event(s) that predisposes to transformation might result in multiclonal hyperplasia, possibly as a consequence of exogenous stimulation.

INTRODUCTION

Although pituitary tumors are generally benign adenomas, they frequently recur or regrow after initial surgery. Large nonfunctional tumors are more often invasive and difficult to remove completely at initial surgery and are therefore frequently treated by radiotherapy to prevent regrowth. Nevertheless, despite this, some regrow and require further surgery.

The initiating events in sporadic tumorigenesis of the pituitary are still largely unknown, although these adenomas are monoclonal (1–3) and therefore are thought to arise from *de novo* somatic mutations, providing a growth advantage to a single cell that, by clonal expansion, results in the adenoma. Apart from the somatotroph-specific *Gsp* oncogene, activation of classical oncogenes is not a common or early event in pituitary tumorigenesis (4–6), occurring only rarely in pituitary carcinomas (6). Other potential initiating mechanisms are loss of TSG[3] function. Of the commonly studied TSGs, the retinoblastoma (*Rb*) gene is not deleted, despite LOH in this region of chromosome 13q in invasive tumors and pituitary carcinomas (7, 8). Similarly, the *MEN-1* gene seems to be expressed in most sporadic tumors, despite frequent LOH of closely linked markers on 11q13 (9–11). Also, LOH on 9p close to the *MTS1/CDKN2A/p16* gene appears to be a frequent event (12) in about 30% of noninvasive and invasive pituitary tumors, suggesting that this is an early event in pituitary tumorigenesis, and this gene is also retained.

In the light of the above findings, we have proposed previously that pituitary tumor invasiveness (defined radiologically), and possibly biological aggressiveness, may be a conse-

Received 6/8/00; revised 8/7/00; accepted 8/8/00.
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[1] We thank the North Staffordshire National Health Service Trust and the West Midlands Regional Research and Development Directorate for financial support and provision of facilities.
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[3] The abbreviations used are: TSG, tumor suppressor gene; LOH, loss of heterozygosity; MEN-1, multiple endocrine neoplasia type 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUMARA, human androgen receptor allele.

quence of an increasing accumulation of loss of TSG function (4), and that the hypothesis (13) of accumulating genetic changes in advancing tumorigenesis may apply to pituitary tumors. We tested this hypothesis in specimens from patients with tumor recurrence or regrowth by comparing the pattern of LOH between successive samples. Moreover, this approach also provides an opportunity to determine whether a recurrent/regrown tumor is derived from the same or a different clone from the original tumor.

PATIENTS AND METHODS

We retrospectively obtained paraffin-embedded samples from patients who had had two or more pituitary operations separated by at least 1 year (Table 1). The indications for second and subsequent operations were variable, including regrowth of residual tumor requiring debulking before radiotherapy, regrowth sufficient to cause new or progressive visual impairment despite radiotherapy, regrowth causing significant headache, worsening or drug-resistant hormone hypersecretion in prolactinomas, acromegaly, or Cushing's disease. In the majority of patients, the regrowth/recurrent tumor was large (>1-cm diameter). The patients ranged in age from 16 to 78 years at first operation (32 males; 17 females). The interval between first and second operation varied from 1 to 25 years (median, 3 years). All cases were sporadic cases with normocalcemia and no family history of endocrinopathy. No patient was from a known MEN-1 family. Clinical subtypes were: 32 nonfunctioning; 7 somatotrophinomas; 5 prolactinomas; 4 corticotrophinomas; and 1 thyrotroph adenoma. All were confirmed by immunohistochemistry. Thirty-six patients received radiotherapy at some stage, and in 21 patients at least 1, occasionally more, the sample was available for analysis after radiotherapy. The extent of tumor invasiveness was based on radiological criteria, defined by computed tomography or magnetic resonance imaging scans judged by the individual clinical center, as described previously (8). Most initial and recurrent tumors were invasive, meaning the tumor was present in one or both cavernous sinuses or invading into the sphenoid sinus. Blood samples for constitutive DNA extraction were obtained from all subjects after informed consent was obtained. The study was approved by the North Staffordshire District and other institutional ethical committees.

Tumor DNA Preparation

Eleven consecutive 5-μm sections were taken from the paraffin-embedded tumor. A single section was subjected to H&E staining, and tumor was identified and encircled by marker pen by a pituitary histopathologist. This area was removed from 10 sequential unstained sections by microdissection using a sterile scalpel, thereby leaving behind any "normal" tissue. This procedure provides a microscopically homogeneous sample with minimal contamination from nonneoplastic cells (14). DNA was extracted by prolonged (3–5 days) proteinase K (0.2 mg/ml) digestion in 50 mM Tris-HCl (pH 8.5), 1 mM EDTA, and 0.5% Tween 20. Samples were heated to 99°C for 10 min and subjected to brief centrifugation. Supernatants were removed to fresh tubes and stored at 4°C. In addition, constitutive DNA was extracted from matched blood samples using commercially available reagents (Nucleon 1; Scotlab, Strathclyde, Scotland).

LOH Analysis

Loss at known or putative TSG loci was assessed by microsatellite analysis of microdissected tumor and matched patient blood DNA. The rationale for selection of loci for analysis was based on previous studies of frequent loss and/or association with invasive pituitary adenomas or pituitary carcinomas (7, 8, 12, 14, 15). The microsatellite markers on chromosomes 9p, 10q, 11q, and 13q, their map position, primer sequences, and the PCR conditions have been described in recent studies (8, 12, 14). Primer sequences, obtained from the Genome Database, were designed to yield products of <200 bp. PCRs were carried out in 25 μl volumes with 1.5 mM $MgCl_2$, 200 μM each of dATP, dGTP, dTTP, and dCTP, 2 pmol of each primer, template DNA (as serial dilutions), and 1 unit of Taq DNA polymerase. PCR was carried out for 25–28 cycles. Constitutive and tumor DNA products were run adjacently and separated on 8% nondenaturing polyacrylamide gels, fixed in 10% methylated spirit/0.5% acetic acid for 6 min, and then incubated in 0.1% aqueous silver nitrate for 15 min. After two brief washes in distilled water, products were visualized by development in 1.5% sodium hydroxide/0.1% formaldehyde. All samples from the same patient were PCR amplified in the same experiment and run in adjacent lanes on the same gel.

Allelic loss was identified by a reduction in band intensity of >80% by visual inspection or the absence of one of the expected allele PCR products in the amplified DNA. All LOH assignments were agreed by two observers and confirmed by an independent observer unaware of the case sample order. In case of disagreement, the analysis was repeated, and if there was still doubt about assignment, the result was declared noninformative. In this way, the effect on the conclusion regarding assignment would be "neutral." We have shown previously in "mixing" experiments that 30% contamination with normal DNA is required to obscure allele loss (12). Template DNA was serially diluted prior to PCR amplification, allowing a direct comparison of dilutions that produced similar product intensities between constitutive DNA and the retained allele(s) in the matched tumor sample. For samples showing LOH, this was confirmed by at least one, often two, repeat analyses on a separate DNA extract from additional slides from the same paraffin block. Where the block contained spatially discrete tumor fragments large enough, these were extracted separately to compare LOH within the same tumor specimen. We confirmed, in five such cases with at least one LOH, that this was identical in two or more fragments, confirming that for that the sample the cells within it were derived from the same clone. There were no samples with sufficient adjacent normal pituitary tissue to confirm that the LOH was confined to the tumor, although we have previously failed to show any LOH in normal pituitary tissue processed in an identical manner (8, 12, 14).

Because we were concerned that extraction of DNA from paraffin-embedded tissue might lead to preferential loss of the larger allele at a given locus, we coamplified the housekeeping gene *GAPDH* from several of the samples. The *GAPDH* amplicon was designed to yield a product of 252 bp, which was larger than those amplified by the microsatellite analysis. In all instances, the larger *GAPDH* fragment amplified irrespective of which allele was lost. An example of this is shown in Fig. 1.

Table 1 Patient details

No.[a]	Sex	Age[b]	Diagnosis[c]	Grade[d]	Op date	Post-op scan[e]	Radiotherapy[f]	Surgical opinion[g]
1.1	M	31	C	3	1984	Residual	No	Recurrence
1.2				3	1986	Residual	Yes	Regrowth
1.3				4	1991	Residual	Yes	Regrowth
1.4				4	1994	NA	NA	
2.1	F	16	C	1	1991	No tumor	No	
2.2					1993	NA	No	
3.1	M	20	NF	3	1981	NA	No	Recurrence
3.2				3	1984	Residual	Yes	Regrowth
3.3				3	1986	Residual	No	Regrowth
3.4				3	1991			Regrowth
3.5				3	1992	Residual	No	Regrowth
3.6				3	1995		No	
4.1	M	78	NF	2	1991	NA	No	Residual
4.2				3	1992	NA	Yes	
5.1	F	35	NF	2	1993	Intrasellar only	No	Regrowth
5.2				2	1996	No residual	No	
6.1	M	29	NF	2	1989	Residual	No	Regrowth
6.2				2	1993	Residual	Yes	
7.1	F	64	NF	2	1990	Residual	Yes	Regrowth
7.2				3	1993	Residual	No	
8.1	F	36	NF	3	1993	Residual	No	Recurrence
8.2				2	1994	NA	No	
9.1	M	34	NF	3	1984	NA	Yes	Recurrence
9.2				3	1994	NA	No	
10.1	M	59	NF	2	1981	NA	No	Recurrence
10.2				2	1984	NA	No	Regrowth
10.3				3	1991a	NA	Yes	NA
10.4				3	1991b	NA	No	Regrowth
10.5				3	1992	NA	No	
11.1	M	54	NF	NA	1973	No	No	Regrowth
11.2				2	1975	NA	Yes	Recurrence
11.2				2	1979	NA	No	Recurrence
11.3				NA	1980	NA	No	Recurrence
11.4				3	1985	No residual	Yes	No tumour
12.1	M	56	C	3	1985	Residual	No	Regrowth
12.2				3	1986	NA	Yes	
13.1	M	29	T	2	1983	NA	Yes	NA
13.2				3	1989	Residual	No	
14.1	M	50	P	2	1982	NA	Yes	NA
14.2				3	1992	NA	No	NA
14.3				3	1993	NA	No	
15.1	M	48	A	4	1990	NA	Yes	NA
15.2				4	1991	NA	No	
16.1	M	48	NF	2	1985	NA	No	NA
16.2				3	1989	NA	No	NA
16.3				3	1991	Residual	Yes	
17.1	M	60	NF	3	1983	Residual	Yes	Regrowth
17.2				2	1987	Residual	No	
18.1	F	66	NF	2	1983	NA	Yes	NA
18.2				2	1985	NA	No	
19.1	M	20	NF	3	1978	NA	Yes	NA
19.2				3	1989	NA	No	NA
19.3				3	1991	NA	No	NA
20.1	F	37	NF	2	1986	NA	Yes	Recurrence
20.2				2	1988	NA	No	
21.1	M	58	NF	3	1990	Residual	Yes	Regrowth
21.2				3	1995	NS	No	
22.1	M	29	NF	3	1973	Residual	Yes	Recurrence
22.2				2	1985	Uncertain	No	Regrowth
22.3				2	1986	No tumor	No	
23.1	F	30	NF	2	1990	Residual	No	Regrowth
23.2				2	1994	Residual	Yes	
24.1	M	62	NF	3	1990	No residual	No	Recurrence
24.2				2	1995	Residual	Yes	
25.1	M	45	NF	2	1989	Residual	No	Regrowth
25.2				2	1994	Residual	Yes	
26.1	F	45	NF	3	1975	NA	Yes	Recurrence
26.2				3	1991	NA	No	NA

Table 1 Continued

No.[a]	Sex	Age[b]	Diagnosis[c]	Grade[d]	Op date	Post-op scan[e]	Radiotherapy[f]	Surgical opinion[g]
26.3				3	1995	Residual	No	NA
27.1	F	35	NF	2	1993	No tumor	No	Regrowth
27.2				3	1995	Residual	Yes	
28.1	M	57	NF	2	1990	Residual	No	Regrowth
28.2				3	1996	No residual	Yes	
29.1	F	38	NF	2	1991	Residual	No	Regrowth
29.2				2	1996	No residual	No	
30.1	M	38	A	NA	1980	NA	Yes	NA
30.2				3	1996	Residual	No	Regrowth
31.1	M	36	NF	3	1995	Residual	No	Residual
31.2				3	1997	NA	No	Complete removal
32.1	M	40	NF	3	1996	Residual	No	Complete removal
32.2				3	1996	Residual	Yes	Residual
33.1	M	45	NF	NA	1972	NA	Yes	NA
33.2				3	1997	Residual	No	Complete removal
34.1	F	25	A	3	1987	NA	No	Residual
34.2				3	1988	NA	Yes	Complete removal
35.1	M	17	P	3	1989	Residual	Yes	Residual
35.2				3	1994	Residual	No	NA
36.1	F	18	P	3	1992	Residual	No	Uncertain
36.2				3	1993	Residual	No	Uncertain
36.3				3	1994	Residual	No	NA
36.4				3	1997	NA	No	Complete removal
37.1	M	54	NF	3	1996	No residual	No	Complete removal
37.2				3	1997	Residual	No	Complete removal
38.1	M	19	A	3	1994	Residual	Yes	Residual
38.2				3	1997	Complete	No	NA
39.1	M	42	NF	3	1985	NA	No	Residual
39.2				3	1987	NA	Yes	Uncertain
40.1	F	29	P	2	1983	No residual	No	Uncertain
40.2				3	1997	NA	No	Complete
41.1	F	52	P	3	1996	Residual	No	Residual
41.2				3	1997	No residual	No	Complete removal
42.1	M	54	NF	3	1994	Residual	Yes	Residual
42.2			C	3	1997	Residual	No	Complete removal
43.1	F	38	NF	3	1986	NA	No	Residual
43.2				3	1988	Residual	No	Uncertain
44.1	M	25	A	NA	1982	Residual	Yes	Uncertain
44.2				1	1997	Residual	No	Uncertain
45.1	F	47	NF	2	1993	No residual	No	Complete removal
45.2				2	1997	NA	No	Complete removal
46.1	M	54	NF	3	1991	NA	Yes	Residual
46.2				3	1997	NA	No	Complete removal
47.1	M	35	A	3	1993	NA	No	Uncertain
47.2				3	1997	NA	No	Complete removal
48.1	M	33	C	3	1990	NA	No	NA
48.2				NA	1992	NA	Yes	NA
48.3				3	1996	No residual	No	No residual
49.1	F	22	A	3	1986	No residual	No	Uncertain
49.2				NA	1988	No residual	Yes	Uncertain

[a] Patient number.
[b] Age at first surgery.
[c] Clinical and immunohistochemical subtype: C, corticotrophinoma/Cushing's disease; A, somatotrophinoma/acromegaly; P, prolactinoma/hyperprolactinaemia; T, thyrotrophinoma/thyrotoxicosis; NF, nonfunctioning tumor/clinically silent.
[d] Radiological grade: 1, microadenoma (<10 mm); 2, macroadenoma (>10 mm) confined to sella turcica with or without suprasellar extension; 3, as 2 but with sphenoid or cavernous sinus invasion; 4, as 3 but with intra- or extracranial spread (*i.e.*, carcinoma).
[e] Within 6 months of surgery for presence or absence of tumour. NA, not available.
[f] Conventional external DXR (40–50 cGy) delivered in 20–30 fractions. Yes indicates given after surgery on that occasion.
[g] Surgeon's view of whether the tumor had regrown from residual tumor or recurred from apparently completely removed tumor.

Clonal Analysis of Pituitary Tumors by PCR for X Chromosome Inactivation

Human Androgen Receptor Short Tandem Repeat. Clonality of the initial and subsequent pituitary tumor samples from the same subject was analyzed from the microdissected tumor cell population and matched patient blood samples. Because the methylated, inactive *HUMARA* is preserved after *Hpa*II digestion, a PCR-based technique was used to determine clonality using the recently identified, highly polymorphic (~90%), short tandem repeat within *HUMARA* (16). Tumor and

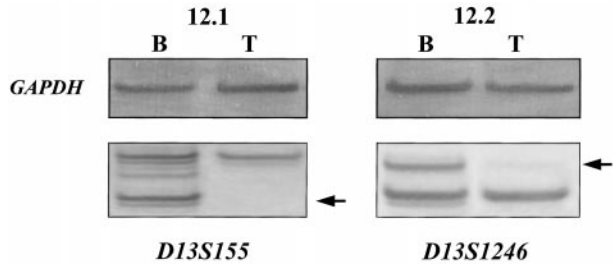

Fig. 1 Coamplification of *GAPDH* with microsatellite analysis at loci *D13S1246*, where the upper allele is lost, and *D13S155*, where the lower allele is lost in samples from patient 12 (see also Fig. 3). In each instance, the *GAPDH* amplicon is seen as the *uppermost band* on the gel.

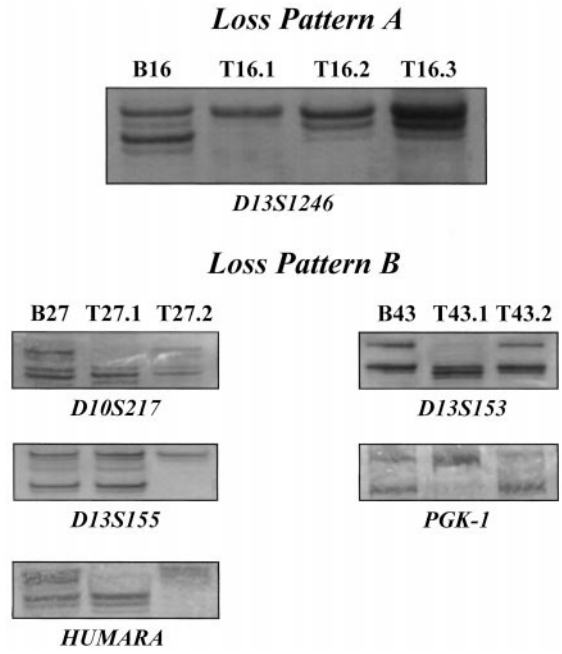

Fig. 2 Representative examples of LOH, as assessed by microsatellite analysis in subjects showing either loss pattern A (patient 16) or loss pattern B (patients 27 and 43). In each case, first and subsequent tumors (*T*) are compared with a matched patient blood DNA sample (*B*). Subject identity is shown *above* the gels, and the microsatellite markers are *below*. Losses are assigned as >80% decrease in the intensity of one of the alleles in tumor DNA relative to the two alleles present in the matched blood DNA sample. For patient 27, X inactivation was demonstrated with the human androgen receptor polymorphism (*HUMARA*), whereas for patient 43, this was shown with *PGK*-1.

matched blood DNA were digested in a total volume of 10 μl for 16 h with 10 units of the methylation-sensitive restriction enzyme *Hpa*II. To ensure complete digestion of the DNA, an additional incubation (16 h) with an additional 10 units of *Hpa*II restriction enzyme was performed. Approximately 200 ng of digested and undigested DNA were subjected to a primary PCR amplification of a region that encompasses a *Hpa*II methylation restriction site in addition to the *HUMARA* trinucleotide ([CAG]n) repeat element. PCR amplification was achieved using specific oligonucleotides (sense, 5′- GCTGTGAAGGTT-GCTGTTCCTCAT-3′; antisense, 5′-TCCAGAATCTGTTC-CAGAGCGTGC-3′) and limited to 12 cycles. One-tenth volume of this PCR reaction was then used in a secondary hemi-nested PCR amplification and limited to 25 cycles (sense, 5′- GCGTGCGCGAAGTGATCC-3′). PCR products were electrophoresed on 8% nondenaturing polyacrylamide gels and visualized as described above.

***PGK*-1 Gene.** In addition, clonality of initial and subsequent tumor samples was analyzed using the *PGK*-1 PCR-based method of clonal analysis. This method is based on a RFLP of the X chromosome phosphoglycerokinase (*PGK*-1) gene and on the differential methylation of the *PGK*-1 gene because of random inactivation of one of two X chromosomes in females heterozygous for the *Bst*X1 polymorphism (17).

Approximately 200 ng of undigested or *Hpa*II-digested DNA samples from female patients (see above) were used for PCR amplification of the *PGK*-1 locus with primers 1A (sense, 5′-CTGTTCCTGCCCGCGCGGTGTTCCGCATTC-3′) and 1B (antisense, 5′-ACGCCTGTTACGTAAGCTCTGCAGGCCTC-C-3′). Samples were amplified for 12 cycles, and one-tenth volume of this reaction was used in a secondary PCR reaction with internal primers 2A (sense, 5′-AGCTGGACGTTAAAGG-GAAGCGGGTTCGTTA-3′) and 2B (antisense, 5′-TACTCCT-GAAGTTAAATCAACATCCTCTTG-3′) and amplified for 25 cycles. After amplification, samples were digested with 10 units of *Bst*XI restriction enzyme for 16 h. Restriction fragments were electrophoresed on 6% nondenaturing polyacrylamide gels and visualized by silver staining. Patients were deemed informative for the *PGK*-1 polymorphism if PCR amplifications and subsequent digestion demonstrated the presence of two fragments of 433 and 530 bp, resulting from alleles with and without the *Bst*XI polymorphism, respectively. However, when samples were first digested with *Hpa*II prior to amplification and subsequent post-PCR digestion with *Bst*XI, a single band indicative of monoclonality was observed. Assignment of X chromosome allelic loss was on the same basis as autosomal LOH.

RESULTS

Pattern of Allelic Loss in Initial and Subsequent Samples

The details of the LOH analysis with the nine microsatellite markers used in this study are shown in summary form in Figs. 3 and 4, and representative examples of LOH are shown in Fig. 2. Figs. 3 and 4 shows the pattern of allelic loss in the first and subsequent tumors for each of the 33 patients exhibiting LOH. On the basis of LOH pattern in the first and subsequent tumors, two common patterns were seen: pattern A, where LOH observed in the original tumor was identical (preserved) in the patients subsequent tumor(s) (Fig. 2, T16), and in some cases this was accompanied by additional losses (Fig. 3, *e.g.*, T7, T9, T12, and T23); and pattern B, where LOH was observed in the first sample, and both alleles were retained (loss to retention) in a subsequent sample(s) (Fig. 2, *e.g.*, T 27 and T43). In some cases, this was accompanied by additional losses (Fig. 4). Figs. 3 and 4 show that 14 patients showed loss pattern A, and 19 patients showed loss pattern B (loss to retention), respectively.

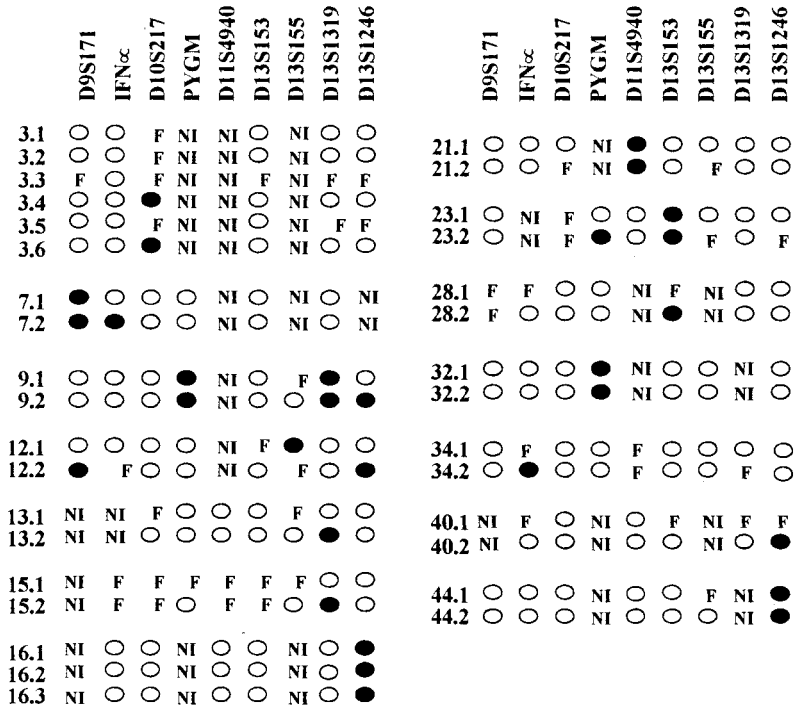

Fig. 3 Allelogram of subjects showing loss pattern A. Patient numbers for first and subsequent tumors are shown to the *left* of the loss patterns and the microsatellite markers *above*. ●, losses; ○, retentions. *NI*, noninformative samples; *F*, failed to amplify. Tumor subtype by immunohistochemistry: nonfunctional cases, 3, 7, 9, 16, 21, 23, 28, and 32; somatotrophinomas 15, 34, and 44; prolactinoma, 40; corticotrophinoma, 12; thyrotrophinoma, 13. Males, 3, 9, 12, 13, 15, 16, 21, 28, 32, and 44; females, 7, 23, 34, and 40.

Clonality on the Basis of LOH Pattern

Loss Pattern A: Evidence for a Monoclonal Origin. From the patterns of LOH observed, we suggest that LOH pattern A is consistent with a single monoclonal origin, because within each individual patient, the loss pattern seen in the first tumor is preserved in subsequent samples (Figs. 2 and 3). For example, the three samples from subject 16 are likely to be from the same clone because of loss of marker *D13S1246* on all occasions and retention of all other informative markers. Equally, pattern A is seen in subject 23, where loss at the marker *D13S153* is seen in the first (23.1) and subsequent (23.2) tumor, with an additional loss at the marker *PYGM*, consistent with a progressive accumulation of losses with time (Fig. 3). In some cases, failed determinations of LOH confounded this interpretation. For example, in patients 28, 34, and 40, LOH analysis (Fig. 3) at markers that failed in the first tumor revealed loss in the subsequent tumor. We therefore assigned these patients to loss pattern A because we consider that these would be similar to patients 13 and 15 if the marker was retained, or to patients 16, 21, and 32 if the marker was lost. The clinical characteristics of the patients were 10 males (ages, 20–57) and 4 females (ages, 25–34). Tumor subtype distribution was 8 nonfunctioning tumors (nos. 3, 7, 9, 16, 21, 23, 28, and 32); 3 somatotrophinomas (nos. 15, 34, and 44); 1 prolactinoma (no. 40); 1 corticotrophinoma (no. 12); and 1 thyrotrophinoma (no. 13). The median interval between first and second/third surgery was 5 years (range, 1–15 years).

Loss Pattern B: Evidence for a Multiclonal Origin. In samples showing retention of heterozygosity in the second or subsequent sample, this could most likely be explained by the second sample being derived from a distinctly separate clone (Figs. 2 and 4). For example in patient 6 (Fig. 4), the first tumor showed loss at 4 of the microsatellite markers, whereas in the second sample (6.2), retention of heterozygosity at three of these markers was observed, making it highly unlikely that this sample is clonally related to the first. The possibility that retention of heterozygosity is attributable to contamination by normal tissue is excluded because LOH is still found in tumor 6.2 at the marker *D13S1246*. Equally, in patient 1 (Fig. 4), the first tumor showed loss at three markers (*IFNA*, *D10S217*, and *PYGM*), whereas all subsequent tumors showed retention of heterozygosity at one of these markers (*IFNA*). However, in this case, the phenomenon of "loss to retention" is repeated between the third (1.3) and fourth (1.4) operation, with retention of heterozygosity at the marker *PYGM*. Thus, the allelic loss pattern in this case is suggestive of the presence of three independent clones. For two tumors, we were able to obtain discrete separate biopsies from the same operation, and the demonstration of identical loss and retention pattern across the nine markers argues against different loss patterns existing in the same tumor and further supports the robustness of the technique (data not shown).

The clinical characteristics of patients with this loss pattern were 12 males (ages, 17–78) and 7 females (ages, 16–45). Tumor subtype distribution was 12 nonfunctioning tumors (nos. 4, 5, 6, 17, 19, 22, 24, 26, 27, 31, 33, and 43); 2 somatotrophinomas (nos. 30 and 49); 3 prolactinomas (nos. 14, 35, and 36); and 2 corticotrophinomas (nos. 1 and 2). The median time between first and second/subsequent surgery was 3 years (range, 1–25 years).

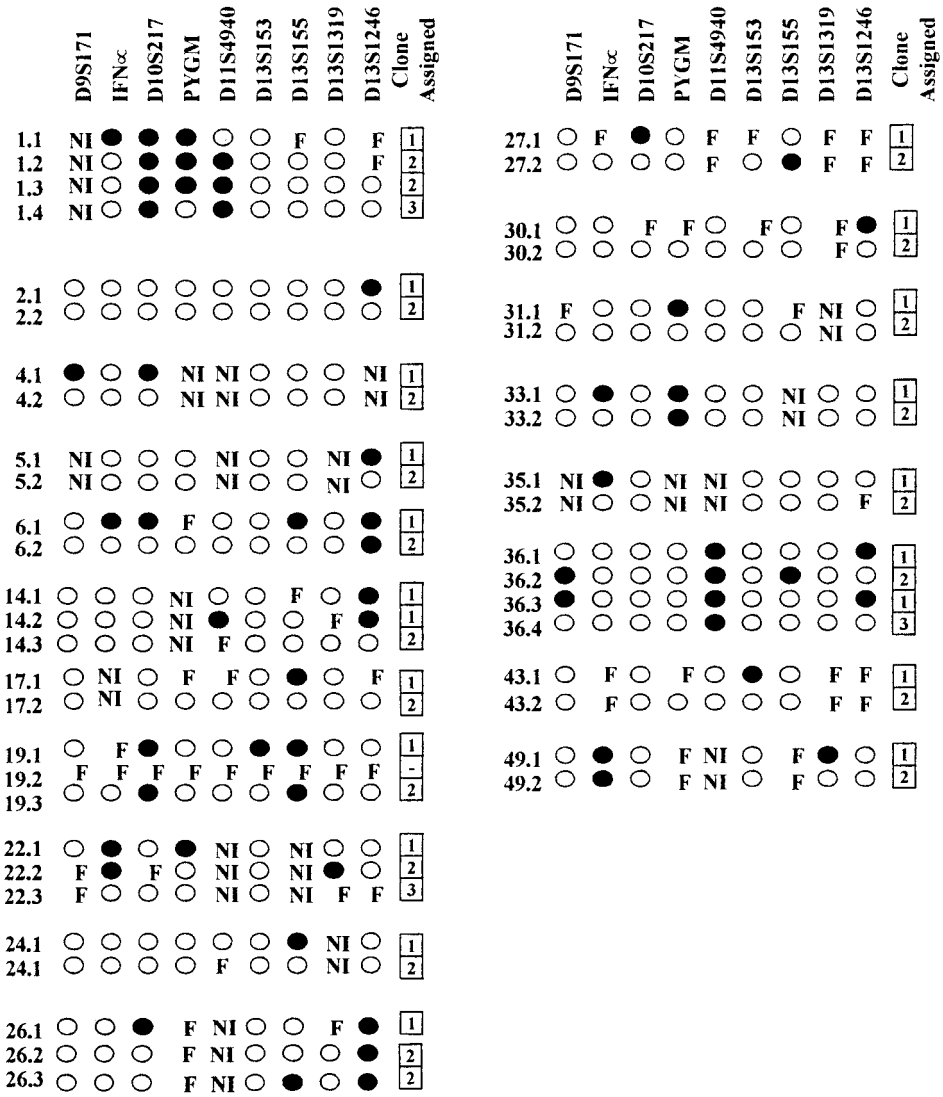

Fig. 4 Allelogram of subjects showing loss pattern B. Patient numbers for first and subsequent tumors are shown to the *left* of the loss patterns and the microsatellite markers *above*. For each subject, the clone (arbitrarily assigned numbers 1–3) giving rise to the observed loss pattern in the first and subsequent tumor(s) are shown on the *right* of the loss pattern. ●, losses; ○, retention. *NI*, noninformative samples; *F*, failed to amplify. Tumor subtype by immunohistochemistry: nonfunctional cases, 4, 5, 6, 17, 19, 22, 24, 26, 27, 31, 33, and 43; somatotrophinomas, 40 and 49; prolactinoma, 14, 35, and 36; corticotrophinoma, 1 and 2. Males, 1, 4, 6, 14, 17, 19, 22, 24, 30, 31, 33, and 35; females, 2, 5, 26, 27, 36, 43, and 49.

Clonal Analysis of Recurrent Pituitary Tumors Assessed by X Chromosome Inactivation

There was sufficient material available from 14 of 22 women, of whom 11 were informative at either *HUMARA* (9 cases) and/or the *PGK*-1 locus (3 cases). The results from these analyses are summarized in Fig. 5. The *HUMARA* or *PGK*-1 polymorphism confirmed a different clonal origin in successive tumors in 5 of 11 patients (Fig. 5), examples of which are shown in Fig. 2 (T27 and T43). *HUMARA* revealed two independent clones in patient 18, evident as the lower allele in the first tumor and the higher allele in the subsequent sample. However, in this case, no losses were found across the autosomal microsatellite markers used here.

Analysis of X chromosome inactivation in tumor 26 with *HUMARA* and *PGK*-1 revealed the presence of independent clones, the first in the initial tumor (26.1), and the second in the subsequent samples (26.2 and 26.3). The LOH data are consistent with this interpretation, in that relative to the initial tumor (26.1), the subsequent tumors show the loss to retention pattern at the marker *D10S217* (pattern B). Thus, in combination with the X chromosome inactivation analysis the additional loss seen in 26.3 (*D13S155*) is a cumulative/progressive loss sustained in the same clone, giving rise to tumor 26.2. The presence of independent clones in the successive tumors from patient 27 as revealed by *HUMARA* analysis again confirms the LOH interpretation of a different clone in the first and subsequent tumor.

Two further tumors (nos. 34 and 43) were informative with *PGK*-1. Tumor 34 showed the presence of an independent clone in the second sample; however, in this case, LOH analysis was unable to unambiguously define clonality because INFA failed to amplify in 34.1. In tumor 43, *PGK* showed the presence of an independent clone in the second sample (43.2; Fig. 2). The presence of independent clones was once again entirely consistent with the LOH studies that showed a loss to retention pattern (pattern B).

Four additional subjects were informative for the

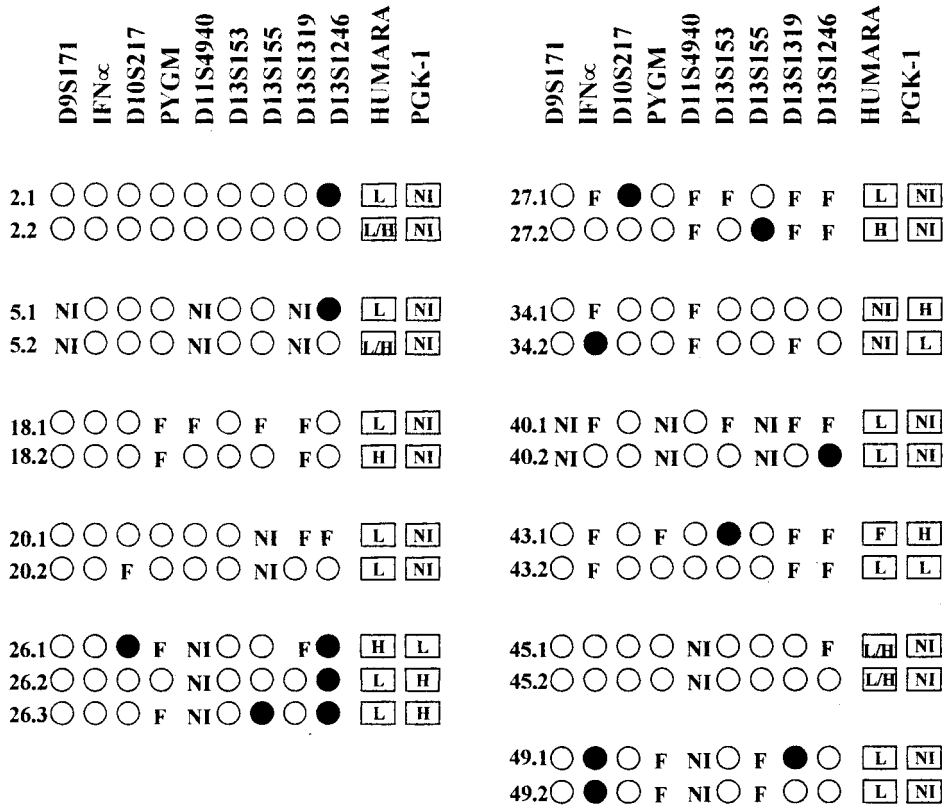

Fig. 5 Combined summary of clonality in female subjects informative for X chromosome inactivation, as assessed by *HUMARA* and *PGK*-1, together with a similar analysis by microsatellite determination of LOH in the same subjects. Patient numbers for first and subsequent tumors are shown to the *left* of the loss patterns and the microsatellite markers *above*. Loss patterns are shown as described in Figs. 2 and 3. Clonality as assessed by *HUMARA* and *PGK*-1 are shown on the *right* of the loss patterns. *L*, presence of a single lower allele; *H*, presence of a single higher allele; *L/H*, presence of two alleles in the tumor sample. *NI*, noninformative specimens. Tumor subtype by immunohistochemistry: nonfunctional cases, 5, 18, 20, 26, 27, 43, and 45; somatotrophinomas, 34 and 49; prolactinoma, 40; corticotrophinoma, 2.

HUMARA polymorphism; three showed the same clone in each case in successive tumors (nos. 20, 40, and 49). In two cases (nos. 20 and 40), the LOH and X chromosome inactivation interpretation of single monoclonal origin were entirely consistent. In subject 49, the LOH data (pattern B) is consistent with two independent clones, but X chromosome inactivation failed to confirm this. The likely explanation is that 49.1 and 49.2 do represent different clones, but in this case the same X chromosome is inactivated. In patients 2 and 5, the first tumors were monoclonal, as evidenced by a single lower band (Fig. 5, *band L*), whereas the subsequent tumors (2.2 and 5.2) gave a pattern consistent with the presence of more than one clone (Fig. 5, *bands L* and *H*). The most likely interpretation of these data is the presence of two clones in the second samples, one of which has not sustained LOH at the microsatellite marker *D13S1246*. Thus, the presence of this coexisting clone would mask and negate the loss seen in the first tumors. In subject 45, the initial and subsequent tumor is either biclonal or perhaps multiclonal. The simplest explanation, in this case, is the coexistence of at least two clones in each tumor sample not sustaining loss at any of the markers used in this study. Conversely, the individual clones have sustained loss at some of these markers, but these losses are not coincident. In this case, retention in one clone and loss in the other would confound microsatellite analysis in the determination of LOH. In summary, X inactivation analysis revealed 5 of 11 patients with tumors of different clonal origins, 3 of 11 from the same clone, and 3 of 11 were composed of two clones.

Relationship of LOH to External Radiotherapy

Of the 49 subjects, 33 (67%) showed LOH at one or more of the markers studied in at least one sample; 16 of 49 (33%) showed no LOH. Considering all of the loci examined, 62 of 92 (66%) allelic losses were observed in samples obtained before radiotherapy, 30 of 92 (33%) in samples after radiotherapy, which was given as 40–50 cGy in 25–30 fractions. Considering the potential of radiotherapy to alter clonality, 8 of 19 (42%) subjects showing a "loss to retention" pattern received radiotherapy after first surgery; therefore, this could possibly have contributed, whereas 11 of 19 (58%) either had radiotherapy after their second surgery ($n = 6$) or never received radiotherapy ($n = 5$), and therefore could not have contributed.

DISCUSSION

Human pituitary tumors are benign neoplasms that have been shown to be monoclonal in origin by both X chromosome inactivation (1–3) and LOH analysis (14, 15, 18). Monoclonality strongly suggests that initiating somatic genetic events arise *de novo* in anterior pituitary cells, rather than as a result of abnormal stimulation from extraneous factors (*e.g.*, hypothalamic releasing hormones), which would be expected to affect all cells of one subtype equally and lead to polyclonal expansion/hyperplasia.

In this study of recurrent pituitary tumors, we anticipated that recurrent tumors would be derived from the same clone as the original tumor with perhaps additional LOH, as predicted

from the Fearnon and Vogelstein (13) model of tumorigenesis, where increasing LOH is associated with increased propensity to progression/recurrence. However, not all tumors conformed to this pattern because in some tumors, we observed a pattern of loss to retention between first and subsequent tumors. One possible explanation for the observed LOH patterns was that a proportion of recurrent tumors were derived from entirely independent clones. This was confirmed by the X chromosome inactivation analysis in women. There are at least two interpretations of these findings:

(*a*) Human pituitary tumors are multiclonal from the outset. Initially one clone dominates and the second clone cannot be identified. In this interpretation, the initiating event(s) might give rise to a population of predisposed cells that require a second "hit or insult." The second "hit or insult" gives rise to several clones at a single point in time. Further genetic abnormalities give one clone a growth advantage over another, leading to a dominant clone. When the tumor is removed at first surgery, cells from the dominant clone are mostly eliminated. However, if there is a remnant after surgery, this may comprise an admixture of cells from the original dominant clone and another clone, either of which are capable of expansion. Accordingly, cells in a tumor regrowth or recurrence may be predominantly from the original clone or another independent clone that was not initially dominant. In the latter case, the recurrent tumor is still monoclonal but from a different clone. However, if more than one clone (the original and second emerging clone) now expand equally, LOH and X chromosome inactivation will define the tumor as biclonal or polyclonal. If pituitary tumors do contain several independent clones *ab initio*, it may be possible to demonstrate this by showing different LOH and X inactivation patterns in multiple biopsies from the same tumor. However, in this retrospective study, this was only possible in two subjects, and both samples showed an identical LOH pattern.

(*b*) Recurrent tumors may develop entirely independently at a different point in time, and in this case different clones are temporally separated. Thus, while in the first scenario the second "insult" might target multiple cells at the same point in time, in the second, this event would be temporally discrete, targeting a single cell. In both cases, the second event is responsible for the growth advantage, allowing clonal expansion into a monoclonal tumor. From our data, it is not possible to ascertain which of these two scenarios applies, but that either is possible is shown for the first time from this analysis. In fact, we found that one of the aforementioned explanations was more likely than the progressive accumulation of additional allelic loss within the same original clone, because loss pattern B was more common than loss pattern A.

Thus, we suggest that recurrent human pituitary tumors arise from a separate independent clone in a significant proportion of cases (up to 60%), although within the same operative specimen they are genetically homogeneous, as evidenced by the identical LOH/X chromosome inactivation pattern in each individual aliquot. Furthermore, this conclusion applies to tumors derived from all of the cell lineages within the pituitary, indicating that whichever initiating scenario applies, this is likely to be similar for all tumor subtypes. It remains unclear why, in the same subject, an initially minor clone could become dominant. One possibility could be that the initially dominant clone produces inhibitory growth factors (19) which, in a paracrine fashion, prevent the outgrowth of the minor clone. When these paracrine inhibitory factors are removed by surgery, the minor clone may then be capable of expansion.

It is, at present, unclear what causes the earliest changes that constitute initiating events in pituitary tumorigenesis. Oncogenic mutations are not common, being restricted to a subset of somatotrophinomas only *(Gsp*; reviewed in Refs. 4 and 6). The pituitary tumor transforming gene (*PTTG*; Ref. 20), identified from a clonal rat pituitary cell line, may also play a significant role in human pituitary tumorigenesis and shows increased expression in invasive tumors (21). Although LOH has been identified in the region of recognized tumor suppressor genes, *MEN-1* and *Rb*, neither of these genes are deleted or mutated in the vast majority of sporadic tumors (7–10). Thus, LOH on chromosomes 11q13 and 13q point to as yet unidentified TSGs in these regions, although their overall frequency of loss (30–35%) makes it unlikely that these represent the earliest genetic changes responsible for tumor initiation.

To our knowledge, this is the first report that describes the presence of an emerging independent clone(s) in benign pituitary tumors from an individual subject as assessed by LOH and confirmed by X chromosome inactivation analysis. In three subjects, X chromosome inactivation showed a biclonal pattern that may reflect the presence of at least two codominant clones, each of which has a different X chromosome inactivated. However, these were the only cases in which we could not definitively exclude the possibility that this was attributable to contaminating normal tissue, but our use of microdissected, histologically defined tumor tissue makes this unlikely. In a previous report examining the clonal composition of corticotrophinomas, which were histologically discrete adenomas with no contaminating normal tissue, three of nine showed a polyclonal pattern, indicating that a proportion of pituitary adenomas may be biclonal/polyclonal (22).

It might be expected that the DNA-damaging effects of radiotherapy could have resulted in some of the allelic losses observed and possibly the emergence of new clones. Although this might be feasible in some cases, our results show that this is by no means universal because nearly 60% of patients in whom we propose an independent clone received either no radiotherapy, or the treatment after the sample revealing the separate clone was identified.

There was no difference between the clinical characteristics of patients whose recurrent/regrown tumors appeared to be from an independent clone and those whose were from the same clone, especially with respect to age, sex, tumor subtype distribution, and time between operations. Thus, the observed differences in clonal origins were independent of these factors.

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Different Patterns of Allelic Loss (Loss of Heterozygosity) in Recurrent Human Pituitary Tumors Provide Evidence for Multiclonal Origins

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Clin Cancer Res 2000;6:3973-3982.