0021-972X/97/$03.00/0
Journal of Clinical Endocrinology and Metabolism

Vol. 82, No. 3
Printed in U.S.A.

Allelic Deletion in Pituitary Adenomas Reflects Aggressive Biological Activity and Has Potential Value as a Prognostic Marker*

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ABSTRACT

Tumors of the pituitary gland are usually benign adenomas and account for 10% of all intracranial neoplasms. Five pituitary tumors have previously been reported to harbor multiple allelic deletions. Of these, three displayed particularly aggressive biological behavior, whereas there were no clinical details provided for the others. This study was designed to test the hypothesis that genetic deletions are a marker of invasive behavior and to identify the loci most commonly involved. Accordingly, we studied two cohorts of pituitary tumors, classified radiologically as invasive or noninvasive, for loss of heterozygosity (LOH).

There is a significantly higher frequency of LOH in invasive tumors (10.8% of all loci examined) compared to noninvasive tumors (2.4%; $P < 0.001$). Of the 11 loci investigated, 75% of the allelic deletions identified in invasive tumors were found at 4 loci: 11q13, 13q12–14, 10q, and 1p. Twenty of 47 invasive tumors had evidence of at least 1 allelic deletion, whereas 14 of 20 had more than 1. Of the 6 tumors with only 1 deletion, 5 involved the 11q13 locus, suggesting that this is an early change in the transition from noninvasive to invasive adenoma. Comparison of invasive and noninvasive tumors demonstrates a significantly higher frequency of deletions affecting 11q13 ($P < 0.001$), 13q12–14 ($P < 0.05$), and 10q26 ($P < 0.05$) in invasive tumors. In addition, allelic deletion correlates with increasingly invasive behavior (modified Hardy classification), as 73% of grade 4 tumors compared to 33% of grade 3 and 9.5% of grade 1 and 2 tumors demonstrated LOH at any locus. Furthermore, in some tumors we identified a breakpoint between markers intragenic and extragenic to the retinoblastoma gene (Rb1) on chromosome 13q, suggesting that tumor suppressor genes other than or in addition to Rb1 may be involved in pituitary tumorigenesis. This was further supported by the presence of Rb protein in two of four tumors where the genetic loss extended to include the intragenic marker D13S153.

Early identification of tumors with likely invasive potential by means of genetic analysis (LOH) may provide useful information on potential tumor behavior and aid tumor management in a manner that is not possible using routine histological methods. A large prospective study is required in patients without radiological evidence of invasion to assess the value of LOH in predicting outcome and for planning treatment. (*J Clin Endocrinol Metab* **82:** 818–824, 1997)

A SIGNIFICANT proportion of sporadic adenomas of all histological subtypes have been identified with deletions involving the long arm of chromosome 11, encompassing the site of the multiple endocrine neoplasm type 1 gene (1, 2). Deletions in the region of the retinoblastoma gene (Rb1) have also been found in a small number of pituitary carcinomas (3), although, interestingly, no evidence of Rb1 deletion has been found in three series of sporadic pituitary adenomas unselected in terms of clinical behavior (4–6). p53 immunopositivity has also been identified in invasive nonfunctional tumors and corticotropinomas (7), and this may indicate the presence of mutations in this commonly mutated gene (8). In other types of neoplasia, however, it is known that the accumulation of an increasing load of genetic abnormalities is associated with progression from simple adenomas to tumors with aggressive and frankly malignant behavior (9). This has not yet been recognized in pituitary tumorigenesis, perhaps because of the rarity of frankly malignant tumors. However, we and others have shown that a small number of unusually aggressive adenomas have been shown to harbor deletions involving more than one autosome (2, 10), suggesting that the transition from noninvasive to invasive adenoma may involve multiple tumor suppressor genes. To determine whether this is the case, we examined a large number of pituitary adenomas, divided according to the radiological criteria of Hardy (11) into two cohorts, invasive (47 tumors) and noninvasive (42 tumors), for loss of heterozygosity (LOH) at a number of chromosomal locations known to harbor tumor suppressor genes.

We now show a significant concentration of LOH in tumors with radiological evidence of invasion compared to noninvasive tumors, in which LOH is minimal. Screening of a total of 11 genetic loci from archival pituitary tumors dem-

Received August 22, 1996. Revision received October 31, 1996. Accepted November 11, 1996.

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* This work was supported by the North Staffordshire Hospital Trust.
† Supported by a Sheldon Research Fellowship awarded by the West Midlands Regional Health Authority.
§ Supported by the BUPA Medical Foundation Ltd.

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onstrated that allelic deletion in the invasive tumors is clustered at four loci: 11q13 (multiple endocrine neoplasm type 1), 13q12–14, 10q26, and 1p.

Subjects and Methods

Patients and tumor specimens

Paired samples of normal and tumor tissue were obtained from patients who had undergone surgery for either hormone-secreting or nonfunctional pituitary tumors. Clinical and hormonal characterization was performed by standard endocrinological criteria, and tumor subtype was confirmed by routine immunohistochemistry. Tumor samples were collected retrospectively and randomly from several centers in the United Kingdom and abroad after standard histological assessment. Normal tissue was blood, where available, other normal tissue from the same slide as the tumor, or, in a small number of cases, paraffin-embedded material from other organs taken either for other surgical purposes or postmortem.

Tumors were defined as invasive on the basis of sphenoid sinus invasion on computed tomography and/or cavernous sinus invasion on magnetic resonance imaging reports and graded using criteria modified from Hardy's classification (11). Grade 4 tumors demonstrated central nervous system/extracranial spread on computed tomography/magnetic resonance imaging scan with or without distant metastases, which were often confirmed at autopsy. Grade 3 tumors were locally invasive, with evidence of bony destruction and tumor within the sphenoid and/or cavernous sinus. Grades 2 and 1 consisted of macroadenomas (>1 cm diameter) with or without suprasellar extension and microadenomas (<1 cm diameter), respectively. Grades 3 and 4 tumors were considered invasive. All centers contributing material used the above-mentioned grading scale. A large proportion of tumors obtained from patients outside our own center were grade 4, *i.e.* metastatic. To remove any possibility of bias in the interpretation of allelic deletion, assignment to invasive/noninvasive cohorts was performed after genetic analysis.

Based on these criteria, 47 patients had invasive tumors, with 11 classified as grade 4 and 36 as grade 3. This group consisted of 35 men and 12 women with a median age of 45.9 yr (range, 13–79). Twenty-seven subjects had nonfunctional tumors, 7 subjects had prolactinomas, 5 patients were acromegalic, and 4 patients had Cushing's disease. Of the remainder, there was 1 TSH-secreting adenoma, 1 FSH-secreting adenoma, 1 stem cell adenoma, and 1 gonadotropin-secreting adenoma.

Forty-two patients had noninvasive tumors, of which 35 were classified in grade 2 and 7 in grade 1. The median age was 47.7 yr (range, 15–79), with 19 men and 23 women; 27 members of this group had nonfunctional tumors, 6 were acromegalic, 6 had Cushing's disease, and 3 had prolactinomas.

Of the total of 89 tumors in this series, 13 have been previously reported (2); 6 of these are included in the invasive and 7 in the noninvasive cohort.

Fifteen postmortem pituitary samples confirmed to be normal by routine microscopy (see above) before DNA extraction were included as controls. Of these, nine were from women and six were from men with a median age of 78 yr (range, 27–88). Constitutive DNA was extracted from samples of spleen removed simultaneously and stored at −70 C.

Approval for this study was obtained from the North Staffordshire District ethics committee, and informed consent was obtained from patients or relatives.

Tissue and DNA preparation

Ten 5-μm sections were taken from the original fixed and paraffin-embedded block. Hematoxylin and eosin staining of one slide allowed identification of tumor material, which was carefully removed from the remaining slides. This procedure allowed the clear distinction of tumor from any surrounding nontumorous tissue, thereby minimizing the possibility of contamination and providing a microscopically homogeneous sample. DNA was extracted by prolonged proteinase K (0.2 mg/mL) digestion (up to 120 h) in 50 mmol/L Tris-Cl (pH 8.5), 1 mmol/L ethylenediamine tetraacetate, and 0.5% Tween-20. After brief centrifugation, the supernatant was removed to a fresh tube and heated to 95 C for 10 min.

Constitutive DNA for comparison was extracted from leukocytes or other tissue using commercially available reagents (Nucleon I, Scotlab, Strathclyde, Scotland) or normal paraffin-embedded tissue, as described above.

Location of microsatellite markers and PCR amplification

PCR amplification was carried out using oligonucleotide primers specific for highly polymorphic microsatellite repeat sequences situated at the site of known tumor suppressor gene loci. Nucleotide sequences and chromosomal locations are shown in Table 1. Nested primers were used in some cases to improve sensitivity and specificity (12).

PCR was carried out for 25–30 cycles using the following conditions: annealing at 50–54 C for 2 min, extension at 72 C for 2 min, denaturation at 94 C for 1 min, followed by a final extension of 72 C for 5 min.

Detection of LOH

Constitutive and tumor DNA products were run adjacently and separated on 8–10% nondenaturing polyacrylamide gels, fixed in 10% methanol-10% acetic acid, 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 an aqueous solution of 1.5% sodium hydroxide and 0.1% formaldehyde.

Allele loss is identified by a reduction in intensity of greater than 80% or the absence of one of the expected PCR products. This method has previously been shown to identify LOH in fixed archival material and gives identical results as Southern blot restriction fragment length polymorphism analysis from fresh or frozen tissue in direct comparison from the same tumor (2). Results were independently reviewed by three observers without knowledge of tumor grade, and LOH was recorded only if the reduction in intensity was clear and agreed upon by all three observers. All samples in which LOH was identified were subject to repeat amplification with identical results on at least two occasions.

Immunohistochemical staining

Sections were hydrated and microwaved in citrate buffer, pH 6.0, for 25 min at full power in a 650-watt microwave. They were immunostained essentially as previously described (7), using a labeled streptavidin-biotin-peroxidase technique with a mouse monoclonal antibody, NCL-RB1 (Novacastra Labs, Newcastle upon Tyne, UK), as primary antibody and 3′,3′-diaminobenzidine as chromogen. Negative controls included omission of primary antibody and staining of tissue from a proven retinoblastoma. A breast carcinoma was used as a positive control. Tumors were scored positive if nuclear staining was present and negative if no nuclear staining was present in the tumor, but positivity was present in normal cells.

Statistical analysis

Differences in the frequency of allelic deletions between groups were assessed using Fisher's exact test. Statistical significance was assigned to $P < 0.05$, whereas borderline significance was given to values of less than 0.1 and actual P values were quoted. The odds ratios (OR) and 95% confidence intervals were calculated using Instat (Graph Pad software, San Francisco, CA). This provides an indication of the probability of a tumor demonstrating LOH at a given locus in invasive compared to noninvasive tumors or, alternatively, the probability of a tumor having a 13q12–14 deletion in the presence of LOH affecting 11q13.

Results

LOH in invasive tumors (grades 3 and 4)

Twenty of the 47 (42.6%) tumors studied in this group had evidence of LOH involving at least 1 locus; of these, 6 had evidence of only 1 deletion, of which 5 involved 11q13. Of the total of 14 tumors with 11q13 deletions, 5 also demonstrated LOH involving 13q12–14 (D13S155). Using Fisher's exact test, there was a significant association between LOH at these two loci ($P < 0.05$; OR = 5.6; confidence interval = 1.2–27.4), the remaining 70% of tumors (14 of 20) had sustained at least

TABLE 1. Primer sequences

Locus	Location	Primer sequences	Heterozygosity (%)	LOH in other tumours
D1S190	1p31-35	CAACTCCTTGCATGTATGTGC CTGGGGAAAGGATTCATGGA	94	MEN-II Breast cancer
D3S966	3p21.3	TACCTCCTCACTGTTTCATATTAG ACATAGTATGTCTCGGCTAACAG	77	Thyroid Renal cell (VHL)
D3S1283	3p24	GGCAGTACCACCTGTAGAAATG AGTAACAGAGGCATCGTGTATTC	71	See above
D10S225	10q 11.2	GAAGAGAAGGGACAGAACCA TCAAGAAGCAGAGCAATGAA	>70	Glioblastoma Prostate
D10S217	10q26	TGCAGGATTCACGATTTCAA CTGTGGCACTCAGATCCCAT	82	See above
PYGM	11q13	CCTGACTGCTGTACTTCTGAATTC GCCAGAGTCCACCTACTGTTAGTG	89	MEN-I
PYGM	11q13	GGAATGCTGATTTCCAGGTT CTGTCAGGTAGCAACTGACATC		See above Nested primers
D11S534	11q13	TGCAACCATGGAGAGTCTGGA GGTATATGGAAACTCTCCGTA	89	MEN-I
D13S155	Rb1	GGTATATTCTCAGAGCCTGGA ACAGCCAGCACATTTATTGA	83	Retinoblastoma Breast
D13S155	Rb1	TCACACAGCCTTCCATAGACC TCTGTTTCTTTGACCTCAGATTG		See above Nested primers
TP53	17p13	GAGGGATACTATTCAGCCCGAGGT ACTGCCACTCCTTGCCCCATTC	90	Li-Fraumeni Lung
NM23	17q21	TTGACCGGGGTAGAGAACTC TCTCAGTACTTCCCGTGACC	>70	BRCA1 (familial breast)
D22S156	22q	AGCCTGGGAGTCAGAGTGA AGCTCCAAATCCAAAGACGT	78	NF2 Glioblastoma
IL2RB	22q12-13	TCCCTTGGCTCCTGTGTG GAAAGCCTGAAACTCCTCAA	91	NF2 Glioblastoma

MEN II, Multiple endocrine neoplasia type II; NF2, neurofibromatosis type 2.

2 deletions. The overall frequency of deletions for this cohort at all 11 loci studied in informative cases was 10.8% (50 of 463). However, the majority of losses (37 of 50; 74%) were confined to 4 loci: 11q13, 13q12–14, 10q26, and 1p31–35. Thus, LOH in these tumors is not a general phenomenon, but exhibits relative specificity for these sites. 11q13 is most commonly affected with a frequency of 31.1%. (14 of 45), 13q12–14 was demonstrably involved in 25% of the tumors (11 of 44), 10q26 in 14.9% (7 of 47), and 1p31–35 in 11.1% (5 of 45). The remaining loci were affected in less than 10% of the cases; in particular, there was evidence of LOH in only 4.8% of informative cases (2 of 42) at the p53 locus. Removal of the 13 samples previously studied (6 invasive and 7 noninvasive) did not change the conclusions and had a marginal, but insignificant, effect on calculated P values (see below). Examples of LOH using these techniques are shown in Fig. 1.

LOH in noninvasive tumors (grades 1 and 2)

From this group of 42 tumors, only 4 of 42 (9.5%) demonstrated LOH at any of the sites examined in the invasive cohort. There were 10 deletions in total in these 4 tumors, with 6 occurring in 1, 2 in another, and 1 only in the third and fourth tumors.

Overall, the frequency of any LOH in the noninvasive tumors was 2.4% (10 of 415) at the 11 loci studied, significantly less than that in the invasive cohort (10.8%; $P < 0.0001$; OR = 4.4; CI = 2.2–8.8). Removal of the previously reported tumors (2) resulted in a calculated $P < 0.0003$.

When we compared the noninvasive tumors (grades 1 and 2) with the invasive (grades 3 and 4) tumors, we found that LOH correlates with increasingly invasive behavior. For this comparison we further divided the invasive cohort into those without (grade 3) and those with intra- or extracranial spread (grade 4). Figure 2 shows that in comparison to noninvasive tumors, in which 9.5% (4 of 42) showed LOH at any locus, this figure increased to 33% of the grade 3 tumors (12 of 36; $P < 0.001$) and to 73% of the grade 4 tumors (8 of 11; $P < 0.05$).

For the noninvasive tumors, the frequency of LOH at D13S155 (Rb1 extragenic) was 7.3% (3 of 41), that at D13S153 (Rb1 intragenic) was 2.9% (1 of 35), and that at 1p31–35 was 4.9% (2 of 41). For the other microsatellite markers, only single tumors from the noninvasive cohort showed loss at D3S996 (3.5%; 1 of 29), 10q26 (2.5%; 1 of 40), 1q13 (2.5%; 1 of 40), 17q21 (2.6%; 1 of 39), and D22S156 (2.6%; 1 of 38). Comparison with the invasive tumors at the 11 loci examined is shown in Fig. 3. There was a significantly higher frequency of deletions affecting 11q13 ($P < 0.001$; OR = 17.6; confidence interval = 2.2–141.5), 13q12–14 ($P < 0.05$; OR = 4.2; confidence interval = 1.1–16.4), and 10q26 ($P < 0.05$; OR = 0.8–58.1) in invasive tumors. There was no significant difference in the frequency of deletions affecting any of the other loci studied.

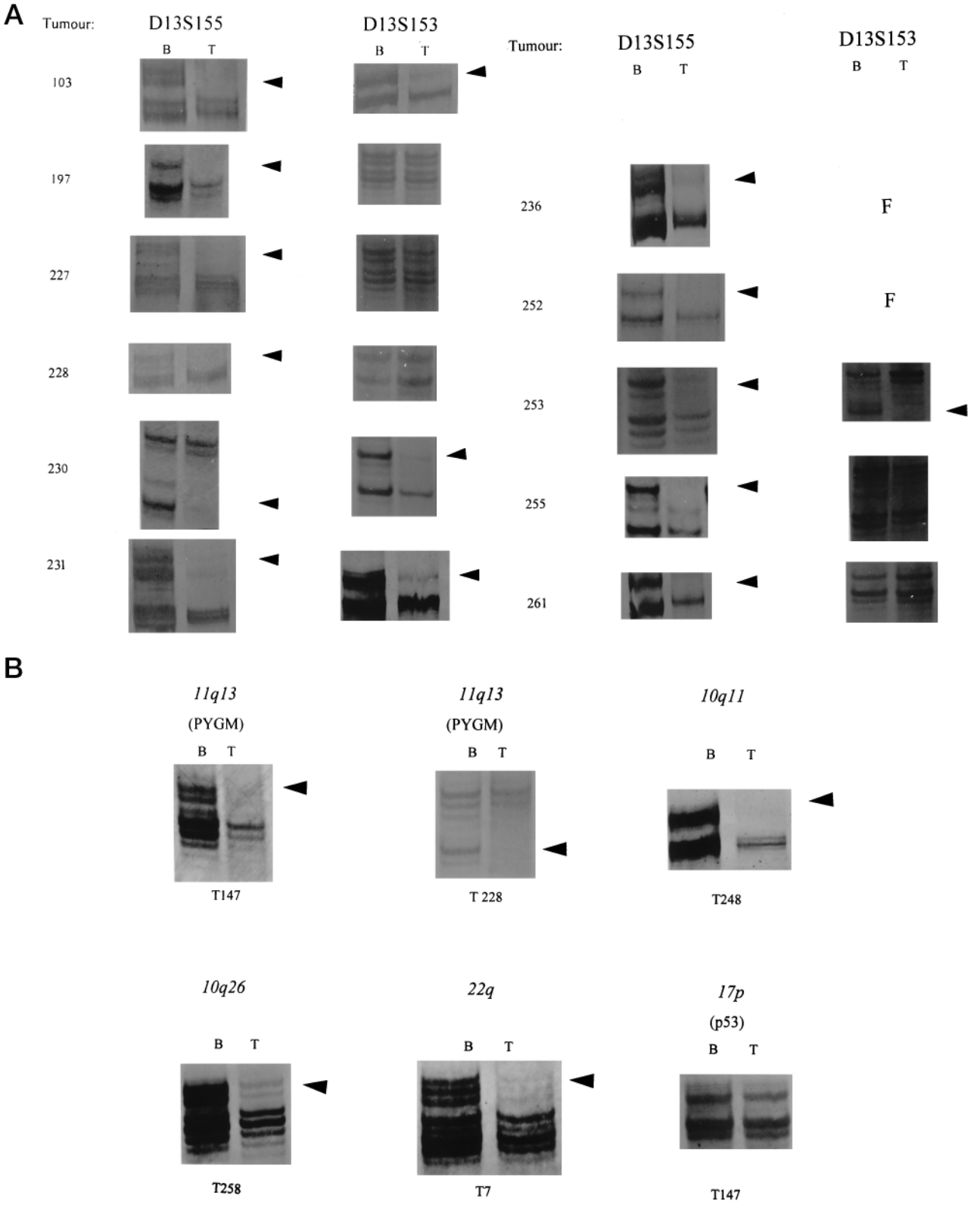

FIG. 1. a, Examples of polyacrylamide gels of PCR-amplified microsatellite polymorphisms, demonstrating the results of PCR amplification of 11 tumor showing LOH at D13S155 (*arrowed*) and either loss (four tumors) or retention (five tumors) at D13S153. B, Blood; T, tumor. b, Representative examples of LOH in tumor tissue compared to matched blood samples at microsatellite markers on chromosomes 10q11, 10q26, 11q13, and 22q. No LOH at the p53 locus on chromosome 17p is evident in the majority of tumors. Legend as above.

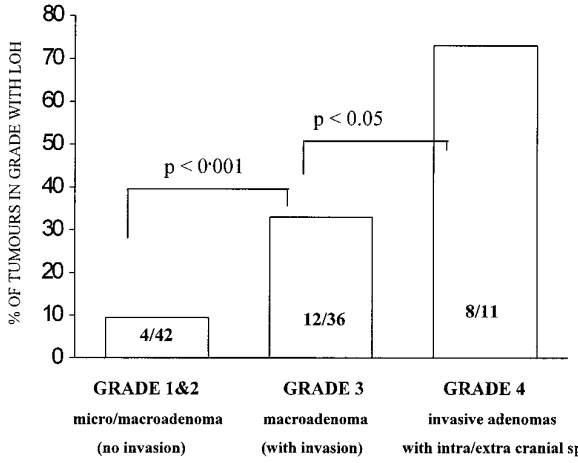

FIG. 2. The percent LOH in the grade 1 and 2 tumors compared to that in grade 3 and 4 tumors. This shows that a significantly greater proportion of invasive and malignant tumors exhibit allelic deletion(s) compared with the noninvasive cohort (grades 1 and 2). Numbers at the *bottom of each column* indicate the number of tumors with at least one allelic deletion (LOH) of the total number studied in the cohort.

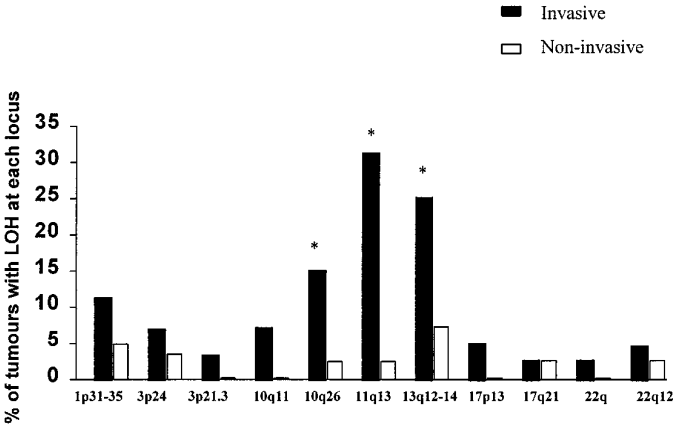

FIG. 3. A comparison of the percentage of tumors showing LOH in invasive (n = 47) and noninvasive (n = 42) cohorts at 11 genetic loci, showing a significant increase ($P = 0.05$) in the former at loci 11q13, 13q12–14, and 10q26. Six of 47 invasive and 7 of 42 noninvasive tumors have been reported previously, of which only a single invasive tumor was shown to harbor a loss on 11q13 (8).

LOH involving the Rb1 locus has only previously been demonstrated in invasive adenomas and carcinomas (3), whereas in 3 series of clinically unselected tumors no evidence of LOH has been found (4–6). One important difference in our series was the use of an extragenic marker, D13S155, which is centromeric to Rb1. Of the 11 invasive tumors demonstrating LOH at D13S155, 4 also had LOH at the Rb intragenic marker D13S153. Five tumors, however, did not reveal any evidence of LOH at D13S153 (2 were not informative), suggesting the presence of a break point centromeric to Rb1 in these 5 tumors. In addition, 3 of the noninvasive tumors showed loss of D13S155, and in 2 of these cases, the intragenic marker to Rb1 (D13S153) was retained (Figs. 1 and 4).

Of the 14 tumors showing loss of the marker D13S155,

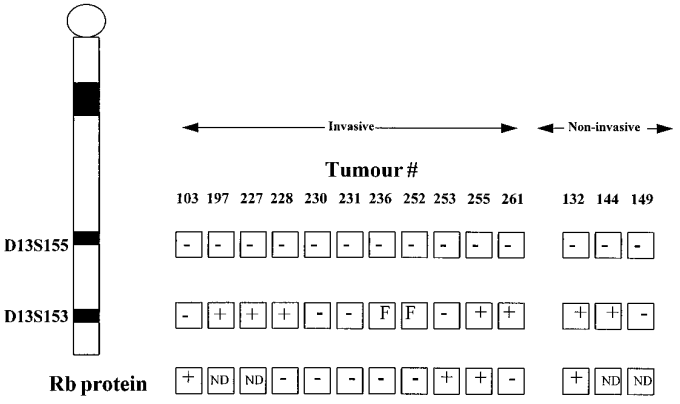

FIG. 4. This demonstrates and summarizes the results of LOH studies at the locus D13S153 (retinoblastoma gene) in those tumors with LOH at D13S155. Five of the 11 invasive tumors and 2 of 3 noninvasive tumors did not show LOH at D13S153, indicating the presence of a breakpoint centromeric to Rb1. Four of the invasive and 1 of the noninvasive tumors demonstrate LOH at both loci. +, Retention of heterozygosity; −, LOH; F, failed to amplify. For 10 of the tumors, immunohistochemical staining for Rb protein is included. +, Immunopositive; −, immunonegative; ND, not done. Each sample derives from a separate individual tumor.

there was only sufficient suitable tumor material for immunocytochemical identification of Rb protein in 10 cases. Four of the 10 tumors showed immunopositivity; the proportion of positive cells varied between cases. In 2 of 4 immunopositive tumors, the area of LOH extended to include the Rb intragenic marker D13S153 (Fig. 4). Two tumors (no. 228 and 261) that showed retention of D13S153 were immunohistochemically negative for Rb protein. No evidence of LOH was found in 15 postmortem pituitary samples using markers for 11q13, 13q12–14, 10q26, 1p31–35, and 17q21, indicating that background LOH is negligible (0 of 75 loci).

Discussion

Modern theories of tumorigenesis suggest that the accumulation of independent genetic events is important for tumor initiation and progression (9). Multiple allelic deletions have been identified in a number of different tumor types (9, 13) although only in a small number of pituitary tumors (2, 10). Of the five pituitary tumors with multiple allelic losses reported in the world literature to date, three appear to have been particularly aggressive, whereas no clinical information is provided regarding the others. These preliminary observations are in accordance with other tumor systems in which the number of genetic events increases with progression from early adenoma through to carcinoma (13, 15). This study is the first to look at different patterns of allele loss in defined subsets of invasive and noninvasive pituitary tumors and has shown that in pituitary tumors, deletions affecting chromosomes 11q13, 13q12–14, and 10q26 occur to a significantly greater extent in invasive than in noninvasive tumors. Moreover, there is a progressive accumulation of allelic loss with tumor progression, confirming for the first time that concepts derived from more common cancers apply to the pituitary.

The frequency of LOH in the invasive tumors is higher than that in previous reported studies (2, 4–6), which did not

specifically differentiate invasive from noninvasive tumors. This together with minimization of false negative results, due to normal tissue contamination, by the use of tumor DNA extracted exclusively from homogeneous tumor material may explain the difference between this study and its predecessors.

It is interesting that in those invasive tumors with LOH affecting only one locus, five of the six were lesions affecting 11q13. The probability that this occurred by chance is small, because these deletions make up only 28% of the total number of allele losses in our series. This suggests that LOH at this site is an important step in the transition from noninvasive to invasive adenoma.

Functional inactivation of the retinoblastoma tumor suppressor gene (Rb1) is an important pathogenetic factor in the development of several human malignancies, for example osteosarcoma and non-small cell lung carcinoma (16). Direct evidence for a pathogenic role for Rb1 is provided in Rb knock-out transgenic mice, which develop neurointermediate lobe pituitary tumors (17). However, three studies have failed to find any evidence of LOH in human pituitary tumors, using intragenic markers for Rb1 (4–6). Immunocytochemistry has also confirmed the presence of Rb1 protein in clinically unselected adenomas and Western blot analysis (18) showed normal amounts of Rb protein compared to postmortem pituitary. LOH using intragenic markers for Rb1 has been found in invasive adenomas and carcinomas (3), but this study also identified Rb1 protein by immunocytochemistry, suggesting that other tumor suppressor genes on 13q may be involved in the pathogenesis of aggressive pituitary adenomas (3). Our study initially used a microsatellite marker (D13S155) that lies between Rb1 and the recently characterized hereditary breast cancer gene (BRCA2) that maps to chromosome 13q12–13 (19). The finding of 25% LOH in invasive pituitary adenomas at D13S155, with 5 of 11 of these tumors and 2 of 3 noninvasive tumors retaining the Rb1 intragenic marker (D13S153), supports the view that a tumor suppressor gene(s) on 13q12–13 other than and centromeric to Rb1 is also involved in pituitary tumorigenesis. This view is further supported by the findings of Rb protein by immunocytochemistry in those tumors with D13S155 deletions. In addition, as reported by others (3), we found no correlation between loss of an Rb intragenic marker (D13S153) and immunohistochemical detection of Rb protein. Our failure to detect RB protein in some tumors may reflect deletion not detected by the microsatellite marker D13S153 or more subtle mutations, to an expressed protein, that is no longer recognized by the antibody.

Of the five pituitary adenomas reported to date with multiple allele losses, four have sustained deletions affecting the long arm of chromosome 10, at 10q26 (2, 10). We have also shown LOH in invasive pituitary tumors affecting the same locus. It is interesting that these deletions do not commonly affect the entire long arm of chromosome 10, as LOH affecting D10S225 (10q11.2) is not usually involved. LOH affecting this region has also been identified in other tumors, such as glial neoplasms (15) and thyroid carcinomas (20). It is noteworthy that in glial tumors, deletions affecting 10q occurred exclusively in high grade gliomas of aggressive phenotype (15). Our own data also suggest that deletions involving 10q occur more frequently in invasive than in noninvasive lesions.

Our data have shown that LOH involving 11q13, 13q, and 10q26 occurs significantly more frequently in invasive pituitary tumors. Although this suggests that tumor suppressor gene inactivation is involved in tumor progression rather than initiation, it remains possible that other tumor suppressor genes are involved at an earlier stage.

Prediction of the biological behavior of pituitary tumors remains difficult on clinical grounds, and radiological and histological criteria are not particularly helpful in this regard. Early identification of which tumors may become invasive may not only provide prognostic information, but may also guide management in a manner not previously possible. We suggest that routine genetic analysis using the markers identified here as being frequently lost in invasive tumors might provide useful information for therapeutic decision making. For example, if a large macroadenoma has been incompletely removed at surgery, the decision as to whether to irradiate that particular tumor is not necessarily automatic, as many such residual tumors are slow growing. If allelic loss at the loci identified in this study was found in the tumor, this would suggest more rapid regrowth and indicate the need for immediate radiotherapy to reduce the chance of recurrence. A long term prospective study is underway to examine this possibility.

Acknowledgments

We thank the following to whom we are indebted for tissue samples: Drs. C. W. Burke and M. Esiri (Oxford, UK), Dr. P. E. Belchetz (Leeds, UK), Prof. A. B. Atkinson (Belfast, UK), Prof. C. R. W. Edwards (Edinburgh, UK), Prof. M. C. Sheppard and Dr. D. A. Heath (Birmingham, UK), Prof. J. A. H. Wass (London, UK), Dr. J. M. MacKenzie (Liverpool, UK), Prof. A. J. Martinez (Pittsburgh, PA), Dr. R. T. Jung (Dundee, UK), and Dr. J. S. Bevan (Aberdeen, UK).

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