Chromosome 13q Deletion Mapping in Pituitary Tumors: Infrequent Loss of the Retinoblastoma Susceptibility Gene (RB1) Locus Despite Loss of RB1 Protein Product in Somatotrophinomas


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ABSTRACT

Two recent studies have described allelic loss of an RB1 intragenic marker on chromosome 13q in aggressive and metastatic pituitary tumors that did not correlate with loss of pRB. The second report also showed that losses were more frequently associated with a more centromeric marker. Because both of these studies suggest the presence of another or other tumor suppressor genes (TSGs) on 13q, we carried out an allelotype analysis encompassing known and recently described TSG loci on 13q, together with immunohistochemical analysis of pRB.

We analyzed 82 nonfunctional tumors and 53 somatotrophinomas subdivided into invasive and noninvasive cohorts. A significantly higher frequency of loss, at one or more of 13 markers, was evident in the invasive nonfunctional tumors (54%, 26 of 48) than in their noninvasive counterparts (29%, 10 of 34). An approximately equal frequency of loss was apparent in invasive (28%, 5 of 18) and noninvasive (31%, 11 of 35) somatotrophinomas at one or more markers. In those tumors harboring deletion, loss at two or more markers was more frequent in invasive nonfunctional tumors 65% (17 of 26) compared with 36% (4 of 11) of their noninvasive counterparts. In somatotrophinomas, 40% (2 of 5) of invasive tumors as compared with 64% (7 of 11) of noninvasive tumors had evidence of two or more deletions. In tumors showing loss at two or more loci, the majority showed large deletions; however, loss of the RB1 intragenic marker D13S1353 was infrequent. In most cases, loss at individual markers was more frequent in invasive tumors than their noninvasive counterparts. A marker 3 cM telomeric to RB1 (D13S1319) showed the highest frequency of deletion in both invasive cohorts (29% of somatotrophinomas and 24% of nonfunctional tumors).

Immunohistochemical analysis of pRB showed frequent loss in somatotrophinomas (27%, 9 of 33) in comparison with 4% (2 of 53) of nonfunctional tumors. Although loss of RB1 did not correlate with loss of an intragenic marker or tumor grade, it was significantly associated with the somatotrophinoma subtype (P = 0.002). These data suggest that chromosome 13q is a frequent target for allelic deletion in pituitary tumors and point to another or other TSG loci in these regions.

INTRODUCTION

Human pituitary tumors are predominantly monoclonal in origin, and the majority of these tumors will remain benign; however, a proportion will develop invasive behavior. Although human endocrine tumors are thought to arise from the progressive accumulation of genetic alterations in both oncogenes and tumor suppressor genes (3–5), a proposal analogous to that demonstrated for colorectal cancer (6), these changes are not clearly defined.

Mice heterozygous for an RB1 mutation do not develop retinoblastomas but have a near complete predisposition to pituitary tumor development (7, 8). Although these are of neurointermediate lobe derivation, they nevertheless prompted studies of the RB1 locus in human pituitary tumors. Early studies using intragenic markers to RB1 suggested that this was not a frequent target of loss in pituitary tumorogenesis (9–11). However, two recent reports (12, 13) have described losses on chromosome 13q in invasive pituitary adenomas, carcinomas, and their metastases in comparison to their noninvasive counterparts. Although both reports describe loss of RB1 intragenic markers, Bates et al. (13) found that losses were more frequently associated with a microsatellite marker 2 cM centromeric to RB1 and that a proportion of tumors failed to express pRB. However, both studies demonstrated lack of correlation between LOH1 at an RB1 intragenic marker and loss of pRB, suggesting the presence of another TSG on chromosome 13q. Similar conclusions have been reached in other common cancers including head and neck (14), breast (15), ovarian (16, 17), and prostate tumors (18). More recent reports have described other TSGs on 13q including BRC2A1 (19) and other putative TSG loci telomeric to RB1 in hematological malignancies, termed DBM (20–22) and centromeric to RB1 in breast cancer termed BRUSH1 (23).

To further define the role of chromosome 13q and RB1 in pituitary tumorigenesis, we have carried out an allelotyping analysis of 135 sporadic human pituitary tumors (53 somatotrophinomas and 82 nonfunctional) using a panel of 13 microsatellite markers encompassing known and putative TSG loci together with IHC analysis of pRB. On radiological criteria, these tumors were subdivided into invasive and noninvasive tumor cohorts (13). The study shows that chromosome 13q is a frequent target for deletion, irrespective of tumor subtype (nonfunctional and somatotrophinoma) or clinical behavior; however, loss of an intragenic marker to RB1 was infrequent. Loss of pRB expression as assessed by immunohistochemical analysis was not associated with LOH at an RB1 intragenic marker or tumor grade; however, we show an association with the somatotrophinoma subtype.

MATERIALS AND METHODS

Patient Material. Eighty-two sporadic, clinically nonfunctional pituitary tumors and 53 somatotrophinomas with matched blood samples were obtained from patients who had undergone hypophysectomy. Within the nonfunctional cohort, 57 tumors have been reported previously for chromosome 9p deletion analysis (24). Tumor tissues were collected retrospectively following standard histological analysis (24). Tumor tissues were collected retrospectively following standard immunohistochemical assessment. Tumors were defined as noninvasive or invasive and graded according to criteria based on a modified Hardy classification (25) as described previously (13). Grade 1 tumors were microadenomas (<1-cm diameter) and grade 2 tumors consisted of enclosed macroadenomas (>1-cm diameter) with or without suprasellar extension. Both grade 1 and grade 2 tumors were defined as noninvasive. Grade 3 tumors were locally invasive with evidence of bony destruction and tumor within the sphenoid and/or cavernous sinus. Grade 4 tumors demonstrate central nervous system/ extracranial spread with or without metastases. Grade 3 and grade 4 tumors were considered to be invasive. Using these criteria, 35 somatotrophinomas were classified as noninvasive (comprising 11 grade 1 tumors and 24 grade 2 tumors).
tumors) and 18 as invasive (16 grade 3 tumors and 2 grade 4 tumors). Similarly, 34 nonfunctional tumors were classified as noninvasive (2 grade 1 tumors and 32 grade 2 tumors) and 48 tumors as invasive (44 grade 3 tumors and 4 grade 4 tumors).

In addition, 15 histologically normal postmortem pituitary and matched spleen samples were obtained, within 12 h of death, and stored at -70°C. Normal pituitary tissues were formalin fixed, paraffin embedded, and confirmed normal by routine microscopy prior to DNA extraction.

**Tissue and DNA Preparation.** Prior to DNA extraction from archival specimens, a single 5-μm section was subjected to H&E staining, allowing tumor identification and microdissection from subsequent slides, therefore providing a microscopically homogeneous sample with minimal nonepithelial cell contamination. DNA was extracted as described previously (13, 24).

**LOH Analysis.** Oligonucleotide sequences to 13 highly polymorphic microsatellite markers spanning chromosome 13q in the following linkage order: D13S1250, D13S1246, D13S260, D13S219, D13S253, D13S263, D13S168, D13S155, D13S125, D13S153, D13S272, D13S1319, and D13S176 (Fig. 1) were obtained from the Genome Database. The marker D13S153 lies in intron 2 of the RB1 gene (26), and the marker D13S260 maps to the TSG BRCA2 (20). The recently described TSG DBM lies 1.6 cm telomeric to RB1 (22); we used the microsatellite marker D13S272 (22, 27, 28) to assess LOH status at this locus. LOH status at the recently described BRUSHI locus was assessed with the microsatellite marker D13S219 as described (23). The total tumor cohort (82 nonfunctional and 53 somatotrophinomas), together with matched blood DNA, were PCR amplified for these microsatellite markers as described previously (13, 24). Products were run on 8% nondenaturing polyacrylamide gels, fixed and visualized by silver staining as described previously (13, 24). Products were run on 8% nondenaturing polyacrylamide gels, fixed and visualized by silver staining as described previously (13, 24).

LOH was identified in patient tumor DNA by a reduction in allele intensity of >80% or by the absence of one of the expected PCR products in the amplified tumor DNA. Lenkocyte and tumor template DNA was serially diluted before PCR amplification, and dilutions were compared that produced similar band intensities between constitutive DNA and the remaining allele(s) in the matched tumor sample. LOH was confirmed after three observers, with no prior knowledge of tumor grade, agreed about the reduction in band intensity. In all cases, the frequency of loss at individual microsatellite markers represents only informative tumors and excludes patients in which tumor and/or matched bloods failed to amplify (see “Results”).

**pRB Immunohistochemistry.** Archival sections were deparaffinized, rehydrated, and microwaved in a citrate buffer (pH 6.0) for 25 min on full power in a 600-W microwave oven. Sections were then stained using a labeled streptavidin-biotin system, with a primary mouse monoclonal antibody NCL-RB1 (Novacastra Labs, Newcastle-Upon-Tyne, UK) as described previously (13). The primary antibody, that recognizes an epitope of pRB located between amino acids 300 and 380, was used at a dilution of 1:25. The secondary antibody was biotinylated anti-rabbit/mouse immunoglobulins, followed by streptavidin-biotin peroxidase (Large volume LSAB kit; Dako, Ltd, Buckinghamshire, United Kingdom), with diaminobenzidine as chromogen. Negative controls were the substitution of pre-immune rabbit serum for the primary antibody and the staining of tissue sections from a proven retinoblastoma. A breast carcinoma was used as a positive control. Tumors were scored Negative controls were the substitution of pre-immune rabbit serum for the antibody was biotinylated anti-rabbit/mouse immunoglobulins, followed by streptavidin-biotin system, with a primary mouse monoclonal antibody NCL-RB1 (Novacastra Labs, Newcastle-Upon-Tyne, UK) as described previously (13, 24).

**RESULTS**

**Allelic Loss at One or More Microsatellite Markers on Chromosome 13q.** Fifty-three somatotrophinomas (35 noninvasive and 18 invasive) and 82 nonfunctional tumors (34 noninvasive and 48 invasive) were studied for evidence of LOH using 13 polymorphic markers spanning known and putative TSG loci on chromosome 13q (Fig. 1). The somatotrophinoma cohort (Fig. 1a) showed an approximately equal frequency of loss in invasive (28%, 5 of 18) and noninvasive (31%, 11 of 35) tumors at one or more microsatellite markers. However, in the nonfunctional tumor cohort (Fig. 1b), there was a significantly higher frequency of loss, at one or more microsatellite markers, in invasive tumors (54% (26 of 48) compared with their noninvasive counterparts (P = <0.05). Representative examples of LOH at informative microsatellite markers are shown in comparison to matched leukocyte DNA in each case (Fig. 2).

**Pattern of Loss in Pituitary Tumors.** In the nonfunctional cohort of those tumors that had sustained deletion, 4 of 11 (36%) of noninvasive tumors showed losses at two or more loci, and this increased to 17 of 26 (65%) of their invasive counterparts (Fig. 1b). In the somatotrophinoma cohort, 7 of 11 (64%) of noninvasive tumors as compared with 2 of 5 (40%) of their invasive counterparts had sustained multiple deletions involving two or more loci (Fig. 1a). In tumors showing LOH, at two or more loci, the majority showed large deletions frequently involving juxtaposed markers. In two tumors, both invasive somatotrophinomas (103 and 253), losses were evident at all informative loci (with one exception), implying loss of the complete arm of chromosome 13q. However, retention of heterozygosity at a chromosome 13p marker showed that the whole of the chromosome had not been lost (data not shown). Invasive and noninvasive tumors had a broadly similar pattern of overall losses. A strikingly common region of loss, centered on the microsatellite marker D13S1319, which lies ~3 cM telomeric to RB1, was the most frequently deleted locus in invasive somatotrophinomas (4 of 14; 29%) and invasive nonfunctional tumors (9 of 38; 24%; Fig. 1).

**Allelic Loss at the RB1 Intragenic Marker D13S153.** The majority of tumors studied showed retention of the RB1 intragenic marker D13S153. The highest frequency of LOH at D13S153 was apparent in invasive somatotrophinomas (2 of 15; 13%), followed by invasive nonfunctional tumors (3 of 46; 6.5%). Loss of D13S153 was detected in only 2 of 32 (6%) noninvasive somatotrophinomas, and no losses were detected in 33 noninvasive, nonfunctional tumors (Fig. 1). Of the six pituitary carcinomas available for study, one of five informative tumors (nonfunctional) showed loss of the RB1 intragenic marker.

**Frequency of Loss in Invasive and Noninvasive Pituitary Tumors.** Fig. 1 summarizes the frequency of LOH across the 13 microsatellite markers in somatotrophinomas and nonfunctioning tumors. The figures also show multiple regions of allelic deletion distinct from RB1 that are significantly associated with invasive tumors in comparison to their noninvasive counterparts. In the somatotrophinomas, two markers, D13S1319 and D13S176, show a significantly higher frequency of loss in invasive tumors in comparison with their noninvasive counterparts. Similarly, in the nonfunctional tumors, three markers comprising D13S260, D13S155, and D13S1319 show a significantly higher frequency of LOH in invasive tumors compared with their noninvasive counterparts. For the remaining markers, the majority were more frequently lost in invasive tumors in comparison with their noninvasive counterparts; however, this did not reach statistical significance (Fig. 1). Microsatellite mapping of 15 historically normal postmortem pituitaries with the 13 polymorphic microsatellite markers used in this study failed to show allelic deletion on chromosome 13q.

**pRB Immunohistochemistry.** Thirty-three of 53 somatotrophinomas and 53 of 82 nonfunctional adenomas were evaluable for immunohistochemical analysis of pRB expression. Of the evaluable tumors, 27% (9 of 33) of somatotrophinomas and 4% (2 of 53) of nonfunctional tumors failed to stain for pRB irrespective of LOH status at any of the microsatellite markers used in this study. Loss of pRB was found at approximately equal frequency in invasive (5 of 11) and noninvasive tumors (6 of 11). Fig. 1 summarizes the pRB expression status in tumors showing chromosome 13q deletions. In the total tumor cohort (nonfunctional and somatotrophinomas), seven tumors showed loss at the intragenic RB1 intragenic marker (Fig. 1); of these, four were available for staining. In two cases, loss at RB1 was accompanied by an absence of pRB (Fig. 1). Because so few tumors showed loss of the RB1 intragenic marker and loss of pRB, we were unable to
Fig. 1. Deletion mapping of somatotrophinoma and nonfunctional tumors. LOH was analyzed using 13 polymorphic microsatellite markers to chromosome 13q. Deletion status of somatotrophinomas (a) and non-functional tumors (b) are shown. The relative positions and linkage order of each microsatellite marker are shown on the left of both figures, together with the approximate map positions of the TSGs RB1, BRCA2, DBM, and BRUSH1. Tumors are subdivided into invasive and noninvasive. To aid interpretation, tumors showing a single loss are shown to the left of those showing more than one loss. The frequency (%) LOH at each of the markers is shown to the right of each of the loss patterns for invasive and noninvasive somatotrophinomas and nonfunctional pituitary tumors. The asterisk shows markers demonstrating significantly increased frequency of LOH in invasive tumors compared with noninvasive counterparts, where * denotes \( P < 0.05 \) and ** denotes \( P < 0.01 \) (Fisher’s Exact test). The figure also shows the IHC staining status for those tumors sustaining LOH on 13q. Staining status for tumors without LOH on this chromosome are not shown (see “Results”).
However, a significant correlation was found between loss of pRB and the somatotrophinoma subtype ($P < 0.002$). Ten histologically normal postmortem pituitaries were also examined for pRB and showed nuclear staining in all cases.

**DISCUSSION**

In this study, we confirmed and extended recent investigations that define a role for chromosome 13q aberrations in pituitary tumors (12, 13). Using a panel of highly polymorphic markers to chromosome 13q, our analysis showed that the nonfunctional and somatotrophinoma tumor cohorts show LOH at one or more markers, irrespective of clinical behavior. However, in nonfunctional tumors, the frequency of loss (at one or more markers) was significantly higher in invasive (54%) than in their noninvasive counterparts (29%), whereas an approximately equal overall frequency of loss was apparent in somatotrophinomas (28% versus 31%, respectively). In those tumors that had sustained deletion, loss at two or more loci was more frequent in invasive nonfunctional tumors (65%) than their noninvasive counterparts (36%).

Previous studies by our own group and others (13, 29–31) have shown that multiple allelic deletions (in this case, on two or more chromosomes) are associated with increasingly invasive behavior. For the somatotrophinoma cohort, the reverse was true in that 64% of noninvasive tumors compared with 40% of invasive tumors had evidence of two or more deletions on chromosome 13q. The difference may reflect a subtype-specific phenomenon; however, it does not exclude the possibility of an increase in the frequency of losses involving other chromosomes segregating with invasive somatotrophinomas. In this context, we recently reported an approximately equal frequency of loss (~31%) on chromosome 9p in invasive and noninvasive nonfunctional pituitary tumors (24), leading us to suggest that chromosome 9p is an early target in pituitary tumorigenesis. Subsequent studies have shown losses on this chromosome to be an infrequent event in somatotrophinomas (32). Taken together, these findings further support the concept of subtype-specific genetic aberrations. Noninvasive tumors, irrespective of subtype, showed highly infrequent loss of an $RB1$ intragenic marker, confirming our previous findings (13). However, earlier studies appeared to discount a role for $RB1$ deletion in noninvasive tumors (9–12). The low frequency of loss found in this and our previous study (13) most likely reflects the larger number of tumors investigated. Loss of an $RB1$ intragenic marker was found at a higher frequency in invasive tumors, regardless of tumor subtype in comparison with their noninvasive counterparts. These findings are in agreement with our earlier studies (13); however, they are somewhat at variance with a recent study by Pei et al. (12), who reported loss of $RB1$ in all of 13 highly invasive or malignant pituitary tumors. Although both studies used intragenic markers, the size of the gene (>200 kb) cannot exclude microdeletions as a mechanism accounting for these different findings or perhaps reflect the different tumor subtypes studied. Interestingly, Pei et al. (12) showed in two of their tumors an intact $RB1$ allele at initial surgery and loss at a subsequent operation. Thus, loss of $RB1$ may more accurately reflect tumors destined for recurrence or a predilection toward particularly aggressive behavior.

Because TSGs may be inactivated by a variety of genetic or epigenetic mechanisms, we determined IHC expression of pRB irrespective of chromosome 13q LOH status. Loss of pRB was detected at a significantly higher frequency in somatotrophinomas (27%, $P = 0.002$) than in nonfunctional tumors (4%). Although two earlier studies (12, 33)
have failed to detect loss of pRB in pituitary tumors, neither study included somatotrophinomas, suggesting to us that this may indeed represent a subtype-specific phenomenon. Indeed, our studies of the TSG p16 have shown that methylation and loss of protein to be significantly associated with nonfunctional tumors but not somatotrophinomas (32). The loss of pRB and retention of p16 in somatotrophinomas may well represent mutually exclusive phenomena that are also apparent in several other tumor types. The lack of any clear association between allelic loss at RB1 and absent pRB in these and earlier studies further supports the suggestion of another or other TSGs in this region (12, 13). In addition to pituitary tumors, a lack of concordance between loss of RB1 and absent pRB in several different tumor types has led to a similar conclusion (14, 15, 17, 18, 20, 23) and in some cases identified novel regions of deletion and candidate TSG loci. Although the mechanism responsible for loss of pRB in our tumor cohorts remains to be determined, two other recent studies have described loss of pRB in pituitary tumors. The first report (34) showed that 2 of 28 adenomas had undetectable pRB, and in a subsequent study (35) of an adrenocorticotrophic hormone-secreting tumor, the benign adenoma showed strong immunoreactivity for pRB, whereas an adjacent sellar carcinoma and its metastases were pRB negative.

In general, in those tumors showing loss at two or more markers, deletions were large, frequently involving juxtaposed markers. In our own previous study, we also found that some tumors had evidence of codeletion involving RB1 and a more centromeric marker (D13S155). Pei et al. (12) also reported codeletion of RB1, in this case, with a marker either centromeric or telomeric to this locus in 2 of 13 tumors studied. Although the size of the deletions and the overall pattern of loss do not readily allow us to draw firm conclusions regarding a specific target(s), several regions are worthy of note. In particular, a region 3 cM telomeric to RB1 (D13S1319) was the most frequently lost locus in invasive tumors, regardless of subtype. In contrast, a closely spaced marker, 2 cM telomeric to RB1 (D13S272) and linked to the putative TSG termed DBM (20–22) was lost at low frequency (in this case, a low frequency was arbitrarily defined as 15% or less). An additional region extending from the marker D13S1250 to D13S253 showed frequent deletion, principally in invasive nonfunctional tumors. This region, centromeric to RB1, harbors the characterized TSG BRCA2 (19) and the recently described putative TSG BRUSHI (23). Because the markers used in this study are closely linked to these genes, more detailed investigations may define a role for these genes in pituitary tumorigenesis.

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