Advances in Brief

Frequent Loss of Heterozygosity at the Retinoblastoma Susceptibility Gene (RB) Locus in Aggressive Pituitary Tumors: Evidence for a Chromosome 13 Tumor Suppressor Gene Other Than RB

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Abstract

Mice bearing retinoblastoma susceptibility gene (RB) germ-line mutations almost invariably develop pituitary neoplasms. We therefore tested 17 patients with pituitary tumors for loss of heterozygosity (LOH) using an RB sequence polymorphism and 5 polymorphic microsatellite markers surrounding the RB gene on the long arm of chromosome 13. In all of the 13 malignant or highly invasive pituitary tumor cases, and in 4 of their respective metastases, a RB allele was lost. In contrast, no LOH at the RB locus was detected in 4 benign pituitary adenoma cases. Three invasive tumors also lost a portion of 13q, which included D13s137, D13s133, and D13s118 telomeric and centromeric to RB, respectively. Immunohistochemical analysis, however, revealed the presence of RB protein in tumors with LOH and the RB locus. Therefore, although inactivation of RB may play a role in the development of invasive pituitary adenomas and carcinomas in mice, another tumor suppressor gene on 13q is likely involved in human pituitary tumor progression. LOH of 13q markers may also be of predictive value in determining the biological behavior of pituitary macroadenomas and their progression to invasiveness and frank malignancy.

Introduction

Pituitary tumors are most often benign monolocular adenomas that are either hormonally functional or nonfunctional (1). They may become invasive, especially to the parasellar sinuses, impinging on cranial nerves and brain structures; very rarely, true de novo pituitary malignancy with extracranial metastases occurs (2). The pathophysiological diversity of these tumors, including hyperplasia, adenoma formation, invasiveness, and carcinoma with metastases, represents an opportunity to identify markers of tumor progression in a spectrum of patients exhibiting characteristic biochemical, imaging, and clinical features.

The RB gene located at chromosomal region 13q14 is an important regulator of cell proliferation (3). Inactivation of both RB alleles by somatic mutation leads to sporadic retinoblastomas (3–5). LOH on chromosome 13q is associated with RB functional loss in bladder, parathyroid, and liver carcinoma (6–8), and astrocytoma (9) but not ovarian (10, 11) or head and neck (12). The lack of concordance between LOH at the RB locus and RB protein status in ovarian and head and neck cancer strongly suggests that another tumor suppressor gene on 13q other than the RB gene plays an important role in these tumor types. Since mice heterozygous for an RB mutation do not develop retinoblastomas but demonstrate a 100% predisposition to pituitary tumor development with increasing age (13), we investigated LOH on chromosome 13 and RB immunostaining in human pituitary malignancies.

True pituitary carcinomas fulfilling the strict criteria of cerebrosplinal or extracranial metastases are rare, and fewer than 40 patients have been documented (14–18). Delineation of mutations in pituitary carcinomas and invasive adenomas would characterize one end of the biological spectrum of pituitary neoplasms. To identify potential markers of invasive adenomas and pituitary carcinomas, we therefore used a series of polymorphic microsatellite markers on chromosome 13q to determine the minimal area of loss in these tumors. Despite the fact that we observed LOH at the RB locus in all of the aggressive tumors studied, RB protein was identified by immunohistochemistry in these specimens, suggesting that another tumor suppressor gene on 13q is involved in pituitary tumor invasiveness.

Materials and Methods

Preparation of Genomic DNA from Pituitary Tumors. Tumor tissue was obtained from 7 patients with pituitary carcinomas, 3 of whom have been described previously (14, 17, 18). Four of the malignant tumors hypersecreted ACTH (Cushing’s syndrome), and three were prolactin-secreting tumors. Six invasive adenomas, including two plurihormonal adenomas, two ACTH secreting tumors, two gonadotroph adenomas, and four benign adenomas, were analyzed similarly (summarized in Table 1). DNA was extracted from tumor tissue either embedded on glass slides or frozen at time of surgery. To prepare genomic DNA, tumor tissues were suspended in 400 µl SET (50 mM NaCl-40 mM Tris, pH 7.4-0.5% SDS) containing 25 µl proteinase K (20 mg/ml). They were incubated at 55°C overnight, and phenol chloroform was extracted, precipitated with ethanol, and resuspended in Tris-EDTA.

RB Sequence Polymorphisms. A DNA sequence polymorphism near the 3’ end of exon 20 (1.20) in the RB gene was utilized because 94% of unrelated individuals are heterozygous for this polymorphism (19). Primers for PCR included 5’-TGATACGGTACCTGTCGTAACAGGTGG-3’ and 5’-AATGTAACAAGGTGGTGGGT-3’. PCR reactions were performed with the use of 20 pmol concentrations of each primer, 1.25 mM MgCl2, 0.2 mM concentrations of each dNTP, 100 ng genomic DNA, 10 mM Tris (pH 8.3), 50 mM KCl, and 2.5 units Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) in a total volume of 50 µl. DNA was amplified by denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min for 35 cycles. A 10-min extension at 72°C was done after the last cycle. Two µl of the above PCR reaction were combined with 2 µl concentrations of 32P-end-labeled primer, and the mixture was denatured at 94°C for 5 min. A buffer containing 2.5 mM MgCl2, 5 mM Tris (pH 7.5), 3 mM DTT, 60 mM concentrations of each dNTP, and 2 units of Sequenase (United States Biochemical Corp., Cleveland, OH) was then added to a total volume of 10 µl. The reaction was performed at 37°C for 10 min, the product was denatured at 94°C and separated on 5% denaturing polyacrylamide gels. The gels were dried and exposed to X-ray film for 90 min.

Heterozygosity of Microsatellite Polymorphic Markers Surrounding RB Alleles on Chromosome 13. Primer pairs for amplifying polymorphic microsatellite DNA fragments were obtained from Research Genetics. These included: D13s118, D13s119, D13s133, D13s137, and D13s138. The reverse primer to the 3' end of primer D13s118 was 3' end of exon 20 (1.20) in the RB gene was utilized because 94% of unrelated individuals are heterozygous for this polymorphism (19). Primers for PCR included 5’-TGATACGGTACCTGTCGTAACAGGTGG-3’ and 5’-AATGTAACAAGGTGGTGGGT-3’. PCR reactions were performed with the use of 20 pmol concentrations of each primer, 1.25 mM MgCl2, 0.2 mM concentrations of each dNTP, 100 ng genomic DNA, 10 mM Tris (pH 8.3), 50 mM KCl, and 2.5 units Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) in a total volume of 50 µl. DNA was amplified by denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min for 35 cycles. A 10-min extension at 72°C was done after the last cycle. Two µl of the above PCR reaction were combined with 2 µl concentrations of 32P-end-labeled primer, and the mixture was denatured at 94°C for 5 min. A buffer containing 2.5 mM MgCl2, 5 mM Tris (pH 7.5), 3 mM DTT, 60 mM concentrations of each dNTP, and 2 units of Sequenase (United States Biochemical Corp., Cleveland, OH) was then added to a total volume of 10 µl. The reaction was performed at 37°C for 10 min, the product was denatured at 94°C and separated on 5% denaturing polyacrylamide gels. The gels were dried and exposed to X-ray film for 90 min.

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3 The abbreviations used are: RB, retinoblastoma susceptibility gene; LOH, loss of heterozygosity; ACTH, adrenocorticotropic hormone; GH, growth hormone; dNTP, deoxyribonucleotide triphosphate.
primer of each primer pair was end labeled by [γ-32P]ATP using T4 DNA polynucleotide kinase (Promega, Madison, WI). The PCR reaction mix contains 50 ng of genomic DNA, 70 mM dNTPs, 10 pmol of each primer, 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, and 0.5 unit Taq polymerase (Perkin-Elmer Cetus) in a total volume of 25 μL. DNA was amplified by denaturing at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min for 35 cycles. At the end of the reaction, an equal volume of loading buffer (98% formamide, 1 mM EDTA, 0.1% phenol blue, and 0.1% xylene) was added to each tube. Five μL of the PCR product were analyzed on 7% denaturing polyacrylamide gel containing 32% (v/v) formamide. Gels were run in 1x Tris-borate EDTA at 90 W for 2–4 h and then exposed to X-ray film for 10–30 min.

**Immunohistochemical Staining.** The method for staining RB nuclear protein in paraffin-embedded sections has been described previously (6–12). Briefly, the highly specific, affinity-purified, polyclonal anti-RB antibody RB-WL-1 was used for these studies. A tumor was considered to be RB positive if the RB staining pattern was heterogeneous, with a portion of the tumor cells showing typical RB nuclear staining. Tumors are scored as RB negative only if all malignant cells showed no RB nuclear protein staining and there is nuclear RB staining in some normal cells as a positive control.

**Results**

**RB Allele Analysis.** Recently, Yandell and Dryja (19) developed a method to identify DNA polymorphisms of the RB gene allowing resolution of allelic fragments which may differ in length by 1 base pair. To determine whether pituitary tumors exhibit LOH for RB, we screened pituitary tumors for allelic polymorphisms in exon 20 of the RB gene. As shown in Figs. 1 and 2A, benign pituitary adenomas and their matched lymphocyte DNA contain both RB alleles. Similarly, control lymphocyte DNA (from five healthy volunteers) yielded polymorphic allelic patterns indicating an intact RB gene. However, all pituitary carcinomas, as well as their respective metastatic deposits, uniformly exhibited loss of one RB allele. Both alleles of RB were present in the lymphocyte DNA of a patient with a prolactin cell carcinoma (Fig. 2A) whereas the pituitary DNA demonstrates loss of an RB allele.

In a plurihormonal invasive adenoma and a recurrent gonadotroph cell tumor, the RB alleles were intact in tumor specimens derived from the initial surgery, while allelic RB loss was detected in subsequent surgical specimens (Fig. 1B, Lanes 7, 8, 12, 13). Furthermore, allelic loss of RB was also seen in four other highly invasive adenomas, derived from patients requiring repeated surgery.

Because matched normal tissue was not available for most of the specimens, in some tumors the RB DNA bands were excised from the gel, eluted, and subcloned. Dideoxy-DNA sequencing confirmed that these bands consist of exon 20 of the RB gene albeit with an increase in the number of TTTC repeat sequences (data not shown).

**Heterozygosity of Microsatellite Polymorphic Markers on Chromosome 13.** To further map the deleted region on chromosome 13q, 5 polymorphic satellite markers surrounding RB were tested for LOH. As shown in Fig. 2, a prolactin cell carcinoma, which shows loss of one RB allele (Fig. 2A), retained all the polymorphic microsatellite DNA markers examined (Fig. 2B).

Fig. 3 summarizes the results of the analysis of polymorphic microsatellite DNA markers surrounding the RB allele. An ACTH carcinoma showed loss of heterozygosity on markers D13s133 and D13s137 telomeric to RB (Fig. 3, Lane 5). An invasive gonadotroph adenoma resected at a second surgery (Fig. 3, Lane 13), and an ACTH carcinoma (Fig. 3, Lane 16) displayed LOH for D13s118 centromeric to RB.

Because the RB gene was located within the minimal area of loss, immunostaining of tumor specimens for RB protein was performed (10). Diffuse positive nuclear RB immunoreactivity was detected in 2 of 2 benign pituitary adenomas, while heterogeneous RB nuclear staining was detected in 2 pituitary carcinoma specimens, and in all the recurrent tumors (summarized in Fig. 3). Therefore all tumors

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**Table 1 Summary of tumors analyzed in this study**

<table>
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<tr>
<th>Case</th>
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<td>ACTH</td>
<td>Metastatic carcinoma</td>
</tr>
<tr>
<td>2</td>
<td>ACTH</td>
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<tr>
<td>3</td>
<td>ACTH</td>
<td>Metastatic carcinoma</td>
</tr>
<tr>
<td>4</td>
<td>PRL</td>
<td>Metastatic carcinoma</td>
</tr>
<tr>
<td>5</td>
<td>ACTH</td>
<td>Highly invasive adenoma</td>
</tr>
<tr>
<td>6</td>
<td>Plurihormonal</td>
<td>Highly invasive adenoma</td>
</tr>
<tr>
<td>7</td>
<td>Plurihormonal</td>
<td>Highly invasive adenoma</td>
</tr>
<tr>
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<td>ACTH</td>
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</tr>
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<td>PRL</td>
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</table>

* All patient samples analyzed in this study are listed, with their immunostaining and tumor status indicated. Matching lymphocyte DNA samples were available from cases 12, 14, 15, and 16. For cases 8 and 10, DNA samples from both the first and second surgery were also available.

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Fig. 2. RB polymorphisms and microsatellite markers on chromosome 13. DNA sequence polymorphisms in exon 20 of the RB gene and microsatellite markers were analyzed by electrophoresis of the PCR products through polyacrylamide gels. A, RB polymorphisms; Lane 1, DNA-derived prolactinoma cell carcinoma (patient 12) (T) and from paired lymphocytes (WBC); Lane 2, DNA derived from a noninvasive prolactinoma (patient 15) (T) and from paired lymphocyte (WBC). B, microsatellite markers on chromosome 13. Microsatellite markers include D13s118, D13s119, D13s133, D13s137, and D13s138. Their relative position to the RB allele on chromosome 13 is shown in Fig. 3. WBC, lymphocyte DNA; T, tumor DNA. DNA was isolated from patient 12. Lighter exposures of B demonstrated both alleles for all microsatellite markers.

were considered to have normal RB function, suggesting that another tumor suppressor gene lies close to the RB gene.

Discussion

This study demonstrates that invasive pituitary tumors exhibit a sequential loss of an RB allele and that pituitary carcinoma is also associated with loss of an RB allele. Because over 94% of unrelated individuals appear to be heterozygous for the 1.20 RB polymorphism utilized (19), it is highly unlikely that five of five patients with pituitary carcinomas and six of six patients with aggressive tumors are not heterozygous. The intact RB gene in noninvasive pituitary adenomas is consistent with similar findings in other studies (20, 21).

Because few mutations have been detected in benign pituitary adenomas, elucidating the molecular patterns of DNA mutations in pituitary carcinomas may provide insight into the molecular pathogenesis of these tumors. Although intrinsic genetic mutations have been invoked in the pathogenesis of pituitary adenomas, few mutations have been detected in benign pituitary adenomas. A single mutation in the α-subunit of the Gs protein has been identified in about a one-third of GH-secreting pituitary tumors. This mutation causes constitutive activation of the Gs α-subunit by inhibition of intrinsic GTPase activity, which results in GH cell proliferation and constitutive GH hypersecretion (22). Only a single invasive prolactinoma has been shown to harbor a ras mutation (23), while no p53 (24) or RB (20, 21) mutations have been detected in benign pituitary adenomas. ras mutations have been detected in extrapituitary metastases of pituitary carcinomas but not in primary malignant pituitary tumors (25). True pituitary carcinomas with extrapituitary metastases comprise a small spectrum of pituitary tumors exhibiting aggressive biological behavior. There are currently no reliable distinguishing histological or biochemical characteristics of aggressive pituitary tumor, and this study identifies a potential molecular marker of aggressive pituitary behavior and carcinoma.

Development of pituitary tumors in 5 of 20 mice heterozygous for an RB mutation, as well as in 4 chimeras originated from heterozygous embryonic stem cells, is consistent with the two-hit tumorigenesis hypothesis proposed by Knudson (26), whereby inactivation of one allele is followed by loss of function of the remaining allele (26). In a subsequent study, germ-line mutation of the RB gene was associated with almost 100% penetrance of a pituitary tumor arising from the intermediate lobe and expressing a melanocyte-stimulating hormone (27). The loss of one RB allele in invasive but not in benign adenomas suggests that the LOH represents a propensity specifically associated with pituitary tumor invasiveness.

In 3 aggressive tumors, microsatellite analysis revealed a minimal area of chromosomal loss close to the RB locus. However, tumors with LOH at the RB locus have normal RB nuclear staining. This discrepancy suggests the existence of another tumor suppressor locus at 13q14 which is likely involved in pituitary tumor progression. Characterization of the role of chromosome 13 LOH in macroadenomas will potentially help predict invasiveness and metastatic potential of pituitary tumors in an attempt to define their biological spectrum. Screening for chromosome 13 LOH at the RB locus will assist in defining tumor aggression, will stimulate the search for unsuspected extrapituitary metastases, and may predict propensity for tumor recurrence.
Acknowledgments

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References

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