Clonal origin of pituitary adenomas

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Benign pituitary adenomas are among the most common neurosurgical tumors and account for a diversity of clinical syndromes due to their hormone content and release. To determine whether these tumors arise from a single cell or multiple cells, the authors studied X chromosome inactivation in deoxyribonucleic acid (DNA) isolated from pituitary adenomas in women. Tumors of three different hormonal subtypes were examined. One tumor contained cells immunoreactive for prolactin and human growth hormone; one tumor contained foci immunoreactive for the β-subunits of luteinizing hormone and follicle-stimulating hormone; and the third tumor had no immunoreactive prolactin, human growth hormone, or thyroid-stimulating hormone, or the α-subunit. Analysis of the DNA revealed that, in each of the three pituitary tumors, one X chromosome was active in all cells and one X chromosome was inactive, indicating that each of these tumors was monoclonal in origin. It is concluded that clinically evident pituitary tumors arise from a genetic mutation in a single cell.

KEY WORDS · pituitary adenoma · clonal analysis · chromosome, X · immunohistochemistry · glycoprotein hormone

Benign pituitary adenomas are among the most common neurosurgical tumors and account for a diversity of clinical syndromes due to their hormone content and release. Pituitary adenomas may secrete one, several, or no known hormones. While these end-products of pituitary tumors have been extensively studied, the initiating events of tumorigenesis have not. It is not known if most pituitary adenomas arise from single mutations or if most are formed from multiple cells simultaneously stimulated by factors released from the hypothalamus. In the first instance, one might expect them to be clonal in origin; in the second, they might be polyclonal. Both mechanisms are possible and either could account for a subset of pituitary tumors.

Recently, Vogelstein and coworkers1 described a molecular genetic approach for determining the clonal origin of tumors using deoxyribonucleic acid (DNA) restriction fragment length polymorphisms of the X chromosome. This technique is based on three premises. First, according to the Lyon hypothesis, one of the X chromosomes in each cell is randomly inactivated early in the development of the female embryo and this inactivation pattern is inherited in a stable manner by the progeny cells. Second, genes on the active and inactive X chromosomes differ in the methylation of cytosine residues and these methylated cytosines are readily detected by certain restriction endonucleases. Third, the maternal and paternal X chromosomes contain normal variations that can be detected using DNA polymorphisms.

To carry out the clonal analysis, we used a probe that detects a polymorphism in the 5' region of the X chromosomal gene hypoxanthine phosphoribosyltransferase (HPRT). Cellular DNA's were digested with one endonuclease that distinguishes the maternal and paternal copies of the X-linked gene through a restriction fragment length polymorphism. The DNA's were also digested with the first enzyme plus a second endonuclease that distinguishes inactive from active copies of the gene based on changes in methylation. If a tumor is monoclonal, the paternal copy of the gene will be cleaved by the second enzyme in a different manner from the maternal copy, since the paternal gene will be active in all cells or inactive in all cells. If the tumor is polyclonal, one-half of the cells will have an active paternal gene and one-half an active maternal gene, so that both gene copies will be equally affected by the second enzyme.

We have examined three pituitary adenomas of different hormonal subtypes from female patients using the X chromosome inactivation approach, and in this
report present evidence that all three tumors are monoclonal in origin.

Materials and Methods

Tissue Specimens

Pituitary adenomas from 12 unselected female patients were studied. At surgery, portions of each tumor were fixed in formalin and embedded in paraffin for diagnosis and immunohistochemical staining. Each tumor was stained for some or all of the following pituitary hormones: prolactin, human growth hormone (HGH), adrenocorticotropic hormone (ACTH), the β-subunits of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and thyroid-stimulating hormone (TSH), and the common α-subunit of the glycoprotein hormones, as previously described. The remaining tumor fragments were frozen for later DNA analysis. Blood samples were also obtained at surgery to serve as normal tissue controls. Normal pituitaries and muscle tissue were also obtained from six female autopsies.

DNA Analyses

High-molecular-weight DNA was extracted from peripheral blood leukocytes and from frozen pulverized tumor tissue by sodium dodecyl sulfate (SDS)-proteinase K digestion followed by phenol and chloroform extraction. The DNA’s were then digested with restriction endonucleases according to the recommendations of the supplier* or of Vogelstein, et al. The digested DNA’s were resolved by agarose gel electrophoresis and transferred to nylon membrane filters† by Southern blotting. The DNA probes were labeled with 32P-deoxyadenosine triphosphate by random oligonucleotide priming  and hybridized to filters for 48 hours at 65°C in 6× standard saline citrate (0.9 M sodium chloride and 0.09 M sodium citrate), 1× Denhardt’s solution (10 mg/liter Ficoll-400, 10 mg/liter polyvinylpyrrolidone, and 10 mg/liter bovine serum albumin), 0.3% SDS, and 0.1 mg/ml salmon testis DNA. The filters were washed from 3× to 0.5× standard saline citrate at 65°C and exposed to Kodak XAR-5 x-ray film with a Dupont Cronex intensifying screen at ~80°C for 48 hours. The HPRT probe DNA (HPRT700) was a 700-base pair BamHI/XhoI fragment derived from pHPRT800, generously provided by B. Vogelstein. The intensities of DNA fragment bands were quantitated with a densitometer.‡

Leukocyte DNA’s from all 12 female patients were digested with BamHI. Three patients proved heterozygous for the HPRT/BamHI polymorphism (discussed below) and were therefore informative for the X chromosome inactivation analysis. As normal control specimens, pituitary and muscle tissue was obtained from six females at autopsy. Two of these individuals were heterozygous for the HPRT/BamHI polymorphism, and clonal analysis was performed on the anterior lobes of the autopsied pituitaries.

Results

Histological Findings

The three pituitary adenomas informative for the clonal analysis had three different histological patterns. Tumor 1 was large with a papillary pattern, and consisted of a mixed population of sparsely and densely granulated tumor cells with occasional oncocyes (Fig. 1). The sparsely granulated cells had prominent Golgi apparatus (G). The cells are arranged around an apparent lumen (L). Some of the cells have accumulated lipid in them. An occasional cell has a coarse chromatin pattern, but the majority of cells have a finely distributed euchromatin. × 3600.

* Restriction endonuclease supplied by New England Biolabs, Beverly, Massachusetts, or Boehringer Mannheim, Indianapolis, Indiana.
† Hybond-N manufactured by Amersham, Arlington Heights, Illinois.
‡ Ultrascan XL densitometer manufactured by LKB Instruments, Gaithersburg, Maryland.
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Clonal Analysis

The three pituitary adenoma DNA's heterozygous for the HPRT/BamHI polymorphism were subjected to X chromosome inactivation analysis. Blood leukocyte DNA's from all three patients served as normal-tissue controls and demonstrated the pattern expected for normal polyclonal tissue (Fig. 4 lanes a and b). In all three a lanes, leukocyte DNA was digested with BamHI and PvuII and probed with the labeled HPRT700 fragment. Hybridization to two bands (approximately 12 and 18 kb) was observed in each case, indicating that one X chromosome contained the polymorphic BamHI site while the other does not (see restriction endonuclease cleavage map in Fig. 5). There are nine HpaII sites within the relevant region of the HPRT gene (Fig. 5). Since HpaII only cleaves the unmethylated recognition sites, it will preferentially cleave the inactive HPRT allele. In the three b lanes, aliquots of leukocyte DNA were digested with BamHI and PvuII and then further digested with HpaII. The relative intensity of both the 12- and 18-kb fragments decreased by 33% to 75% compared to lanes a. This is the expected pattern in a polyclonal tissue where approximately one-half of the cells have one X chromosome inactive and the others have the other X chromosome inactive.

The DNA's from the three pituitary adenomas were similarly analyzed (Fig. 4 lanes c and d). Digestion with BamHI and PvuII followed by HpaII digestion (lanes d) resulted in selective degradation of only one fragment from lanes c. In one tumor (Case 1) the 18-kb fragment nm in diameter). No immunoreactive prolactin, HGH, β-subunits of TSH, LH, or FSH, or the α-subunit was detected. Tumor 2 consisted of mostly sparsely granulated cells with prominent rough endoplasmic reticulum and a coarse nuclear chromatin (Fig. 2 left). The secretory granules were quite large (100 to 500 nm). An occasional cell contained numerous granules and a more finely stippled nuclear chromatin. Most of the tumor cells stained for both HGH and prolactin (Fig. 2 center and right). Tumor 3 consisted of a population of cells with uniform-appearing nuclei and prominent rough endoplasmic reticulum (Fig. 3 left). Slightly less than one-half of the cells contained numerous small (40 to 120 nm) secretory granules. This appearance is consistent with gonadotropin-associated adenomas. The tumor contained focal groups of cells immunoreactive for β-LH and β-FSH (Fig. 3 center and right). Alpha-subunit staining was also present, but in a lesser amount. No immunoreactive β-TSH, HGH, or prolactin was seen.

Fig. 2. Left: Electron micrograph of Tumor 2, revealing cells with prominent rough endoplasmic reticulum (R). Some of the cells have numerous coarse granules (D) and other cells are sparsely granulated (S). The cells have a uniform type of nuclear chromatin pattern. × 3600. Center: Photomicrograph of Tumor 2 stained immunocytochemically for growth hormone. × 200. Right: Photomicrograph of paraffin-embedded sections immunostained for prolactin. × 200. In the two photomicrographs, most of the cells are positive for both growth hormone and prolactin.
was digested and in the other two (Cases 2 and 3) the 12-kb fragment was digested, strongly suggesting the clonal origin of each of these tumors. To provide further quantitative evidence of clonality, densitometry was performed and we expressed the decrease in intensities of the two alleles following HpaII digestion relative to one another. The polyclonal leukocyte DNA digestion patterns showed relative “cleavage ratios” of 1.2, 1.3, and 1.1, respectively, close to the expected value of 1.0. In contrast, the HpaII cleavage ratios of the pituitary tumor DNA’s were 10, 3.0, and 6.2, respectively, consistent with a clonal origin of these tumors.

Normal pituitary tissue obtained from two females at autopsy proved informative and was examined to determine if small specimens of normal pituitary tissue are polyclonal by X chromosome inactivation analysis. Digestion of the pituitary DNA’s with BamHI and PvuII produced the 18- and 12-kb alleles (data not shown). Further digestion with HpaII resulted in a 35% to 65% reduction in the intensities of both alleles, indicating that these normal pituitary anterior lobes were polyclonal in origin.

Discussion

Pituitary tumors are among the most common neoplasms in neurosurgery, but little is known of their cellular or molecular origins. Many adenomas consist of more than one immunoreactive cell type. For example, Kovacs and Horvath noted that the mixed growth hormone/prolactin-producing adenomas tend to have two morphological types of cells and all of the acromegaly-associated adenomas that we have seen at

FIG. 3. Left: Electron micrograph of a representative area of Tumor 3. The cells have uniform-appearing nuclei. Some of the cells have dilated endoplasmic reticulum (E). Most of the cells are sparsely granulated. x 3600. Center and Right: Immunocytochemically stained adjacent paraffin-embedded sections stained for the β-subunits of luteinizing hormone (center) and follicle-stimulating hormone (right). These pictures are from the same area of adjacent sections. Note that positive immunoreactive cells are present in the same area of the tumor for both hormones. x 200.

FIG. 4. Clonal analysis of pituitary adenomas from three patients (Cases 1 to 3) using HPRT700 as a probe. The DNA’s from blood leukocytes (lanes a and b) and tumors (lanes c and d) were digested with BamHI and PvuII. One aliquot was not digested further (lanes a and c) while the other was digested with HpaII (lanes b and d). Comparison of each lane b with its lane a counterpart demonstrates an equal loss of each DNA band, showing the polyclonal nature of normal blood leukocytes. In contrast, comparison of each lane d with its lane c counterpart demonstrates loss of only one band, revealing the clonal nature of each pituitary adenoma.
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the Massachusetts General Hospital have also had at least some prolactin-positive cells (ET Hedley-Whyte, unpublished data). This might imply that such a tumor arises from more than one cell. One cause of polyclonality would be if the tumor were caused by stimulation of two pituitary cell populations by a hypothalamic hormone or growth factor. However, our study demonstrates that such tumors are clonal in origin and suggests that they arise from a single genetic mutation. The cause of the different hormone expression in subpopulations of tumor cells has not yet been determined but could represent a later secondary mutation or other cellular changes at a transcriptional level. Whatever the cause of the differing hormonal expressions, our data demonstrating clonality for these three different adenomas suggest that an early event in tumorigenesis occurs in a single cell leading to a clonal population of tumor cells. These findings now need to be extended to a larger number of pituitary tumors of diverse nature.

The same techniques are also applicable to other brain tumors. This is especially true for gliomas and neurofibromas where, analogously, mixed cell populations exist in the tumor. Gliomas often consist of astrocytes, oligodendrocytes, and neurofibromas of Schwann cells, fibroblasts, and mast cells. In either instance, tumorigenesis could be caused by an exogenous factor stimulating more than one cell population or, alternatively, by a clonal population of cells caused to proliferate by a single clonal genetic event. For these tumors, it may be necessary that these molecular techniques be applied to the tumor as a whole as well as to the purified subpopulations of each cell type. Such information is important not only for understanding the origins of nervous system tumors but also for understanding tumor heterogeneity and thus for planning appropriate therapeutic strategies.

Acknowledgments

We are indebted to Dr. B. Vogelstein for the HPRT probe, Dr. A. Arnold for helpful advice, and Dr. N. Zervas and members of the neurosurgical staff for providing tumor specimens.

References


Manuscript received January 30, 1990.
This work was supported by United States Public Health Service Grant PO1 NS24279 and a grant from Neurofibromatosis, Inc.-Mass. Bay Area to Drs. Jacoby and Martuza and a Faculty Research Award from the American Cancer Society to Dr. Seizinger.

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