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Clonal Analysis of Solitary Follicular Nodules in the Thyroid

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Accumulated data using functional, morphologic, and histochemical analysis suggests that follicular proliferations in the thyroid include polyclonal and monoclonal patterns with encapsulated follicular adenomas most frequently monoclonal, and other nodules generally polyclonal. However, examples of polyclonal carcinomas or adenomas raise the possibility that histologically similar lesions may arise through different pathogenetic mechanisms. The authors have performed a clonal analysis of histologically benign and malignant thyroid nodules in seven women using HPRT (hypoxanthine phosphoribosyl transferase) and PGK (phosphoglycerate kinase) restriction fragment length polymorphisms (RFLPs) on the X chromosome. These RFLPs used in concert with methylation-sensitive restriction endonucleases HpaII and HhaI permit distinction of active and inactive X chromosomes. DNA from a multinodular goiter showed equal sensitivity of both X chromosome RFLP alleles to a methylation-sensitive restriction endonuclease, consistent with a polyclonal origin. In contrast, three solitary follicular nodules and three carcinomas displayed predominant sensitivity of a single RFLP allele, consistent with a monoclonal origin. Although further detailed studies will be necessary to understand polyclonal origins reported for some adenomas, our data from a limited number of samples supports a predominantly monoclonal origin, and possible neoplastic pathogenesis, for many solitary adenomatous nodules in the thyroid. (Am J Pathol 1990, 137:553–562)

Follicular nodules of the thyroid gland, both single and multiple, are commonly encountered lesions in females. Thyroid glands from patients with multiple follicular nodules are referred to as multinodular goiter.[1–3] Glands from patients with a single follicular nodule are referred to by several terms, including follicular adenoma, adenomatoid nodule, or adenomatous follicular nodule, reflecting uncertainty about whether these lesions are true neoplasms or local hyperplasias.[4–7]

Information to date bearing on the pathogenesis of follicular nodules of the thyroid has been interpreted to support both neoplastic and hyperplastic modes of origin. Studer's group[5,8–12] found differential iodine uptake and proliferation in different areas of individual thyroid nodules, suggesting that multinodular goiters from patients in an endemic region may be polyclonal proliferations. These investigators suggested that nodular lesions of the thyroid arise from subpopulations of follicular cells that maintain the growth potential of fetal cells and proliferate more rapidly than neighboring cells to form locally expanding mass lesions. Studer et al further postulated that proliferation of cells with the shortest growth cycle might give rise to true monoclonal proliferations, but this would be a rare occurrence. Studer's group did not speculate on how these results might apply to sporadic nodular goiter (nonendemic) or solitary follicular nodules of the thyroid.[5] In other studies, Fialkow[13] found a monoclonal pattern of glucose 6-phosphate dehydrogenase (G6PD) isoenzymes in a number of solitary follicular nodules. Thomas, Williams, and Williams[14] also have demonstrated by isoenzyme histochemical techniques that encapsulated adenomatous nodules induced by long-term goitrogenic treatment of mice with ^{131}I are largely monoclonal, whereas other nodules are predominantly polyclonal.[14]

An analysis of clonality in spontaneous nodular lesions of the human thyroid could provide important insights into the causes and origins of these lesions. We have examined the question of clonality in these lesions using a molecular genetic analysis of X chromosome inactivation that

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uses restriction fragment length polymorphisms (RFLPs) to distinguish each X chromosome and methylation-sensitive restriction endonucleases (MSRE) to identify whether the same X chromosome is inactivated in all cells of the lesion. If proliferation in solitary follicular nodules was caused by stimulation by soluble mediators (hormones) or to subpopulations of follicular cells with higher intrinsic proliferative rates, we would expect these lesions to be polyclonal and contain cells with each of the two X chromosomes active. However, if an intrinsic abnormality in a single cell resulted in a proliferative advantage, then all the cells in the resulting lesion would be descended from a single cell and therefore would have the same active X chromosome. Although there are alternative explanations for clonal patterns (see Discussion), this methodologic approach has been used successfully to demonstrate clonality in other cellular proliferations of uncertain histogenesis, including: acute myeloid leukemia in apparent remission,[15] parathyroid adenomas,[16] and adenomatous polyps of the colon.[17] We were particularly interested in solitary follicular nodules of the thyroid, but also examined multinodular goiter and carcinomas for comparison.

Materials and Methods

Patients and Samples

Fresh thyroid tissue was obtained from 32 female patients undergoing surgery for nodular thyroid disease at the Hospital of the University of Pennsylvania in 1988. Patients ranged in age from 19 to 76 years (average, 40), and overall exhibited a spectrum of lesions, including nodular goiter, solitary follicular nodules, chronic lymphocytic thyroiditis, and carcinomas, both papillary and follicular. The central portions of nodular lesions were carefully dissected out (preserving and avoiding capsular areas), and the tissue was snap-frozen in liquid nitrogen. A portion of surrounding normal thyroid was separately sampled. Peripheral blood from each patient was screened for the polymorphic X chromosome markers hypoxanthine phosphoribosyl transferase (HPRT)[18] and phosphoglycerate kinase (PGK1)[19]—see below; normal thyroid was used if peripheral blood was unavailable. A patient was informative for our studies if her normal tissue was heterozygous for RFLP markers for the HPRT and/or the PGK genes.

Frozen sections were examined from the thyroid lesions of informative patients to confirm that normal thyroid was lacking, and there was no appreciable percentage of connective tissue cells. The histologic diagnosis was confirmed without knowledge of the molecular studies by two of us (DGH and VAL). DNA was extracted from each thyroid lesion and analyzed for methylation near informative RFLP markers.

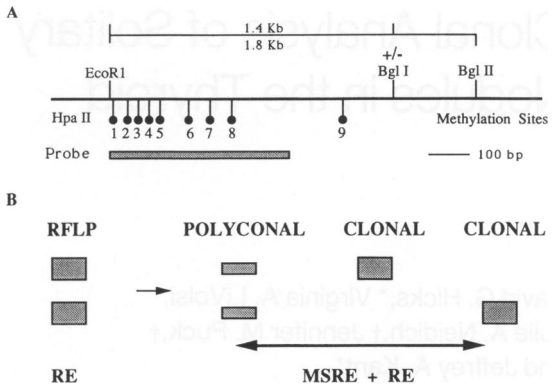

Figure 1. *Clonal analysis using X chromosome RFLPs, which are sensitive to methylation-sensitive restriction endonucleases (MSRE).* **(A)** *Genetic map of the 5′ portion of the PGK1 locus on the X chromosome (adapted from Vogelstein et al.[21]) The hatchmarks above the line indicate restriction endonuclease sites. BglI (+/−) marks a polymorphic site; EcoRI and Bgl II are trimming sites. The presence or absence of the BglI polymorphic site would give rise to 1.4 or 1.8 kb DNA restriction fragments on Southern blot analysis. The "lollipops" numbered 1 through 9 indicate the positions of Hpa II restriction endonuclease sites that are methylated on the inactive chromosome. Hpa II will not cut methylated DNA and therefore digests this region of PGK1 only on the active X chromosome.* **(B)** *Predicted southern blot patterns for a methylation-sensitive X chromosome RFLP site in polyclonal and monoclonal proliferations. Heterozygous patients display both RFLP alleles when cut with appropriate restriction endonucleases (RE). Polyclonal proliferation should demonstrate a mixture of active X chromosomes with both RFLP alleles; digestion of both restriction fragments by a methylation-sensitive restriction endonuclease (MSRE) such as HpaII leads to a decrease in hybridization intensity of each allele. Each cell of a clonal proliferation contains the same active X chromosome with a single RFLP allele. Digestion of the region surrounding this allele by a MSRE leaves only a single allele.*

Study Rationale

The general approach we used to assess clonality in thyroid nodules rests on two principles: 1) RFLPs associated with the X chromosome genes, HPRT and PGK, can be used to distinguish maternal and paternal X chromosomes in cells from approximately 25% of women for PGK and 20% to 30% of women for HPRT,[20,21] and 2) one X chromosome is randomly inactivated in each cell of a female early in embryogenesis; this is commonly referred to as Lyonization.[22] The inactivation pattern of a specific X chromosome is a heritable attribute passed to progeny of these cells[22] and is usually associated with hypermethylation of genes[23]; paradoxically, the HPRT gene on the *active* X chromosome is hypermethylated.[20] Methylation-sensitive restriction endonucleases such as HpaII and HhaI, which will not cut methylated sequences, do not digest these regions.[24–26] Therefore, in each cell of an informative female, one RFLP fragment will remain intact while the other is degraded, depending on which X chromosome is inactive.

For example, in a patient informative for the polymorphic PGK BglI site, DNA digested with BglI followed by

Table 1. *X Chromosome RFLP LOCI**

Gene	Restriction enzyme	RFLP sizes	MSRE
HPRT	(BamHI)†	24, 12 kb	HhaI
PGK1	(BglI)† + (BglII + EcoR1)‡	1.8, 1.4 kb	HpaII

* X chromosome RFLP loci, HPRT, and PGK1 that were examined in the thyroid lesions. The restriction endonucleases and methylation-sensitive restriction endonucleases (MSRE) used are shown. The size of the restriction fragments expected from informative patients is indicated. All restriction digestions contained 60 μl final volume.

[a] For RFLP analysis alone, DNA was digested with BamHI for 3 to 6 hours at 37°C. For studies of methylation sensitivity, DNA was first digested with HhaI for 4 hours; after adjusting conditions by adding appropriate amounts of NaCl, TRIS-HCl (pH 8.0), and $MgCl_2$, digestion was continued for 2 to 4 hours with 30 units of BamHI before electrophoresis and Southern blot analysis. The HPRT gene probe was a 1.8-kb genomic DNA fragment.[18]

[b] For RFLP analysis alone, DNA was digested first with BglII for 4 to 8 hours. After adjusting conditions by adding appropriate amounts of NaCl, Tris-HCl (pH 8.0), and $MgCl_2$, 20 units each of BglII and EcoR1 were added and digestions continued an additional 2 hours. For studies of methylation sensitivity, DNA was first digested for 2 to 3 hours with HpaII in 0.02 mol/l TRIS-HCl (pH 7.4), 0.01 mol/l $MgCl_2$, with conditions adjusted to 0.05 mol/l TRIS-HCl (pH 8.0), 0.01 mol/l $MgCl_2$, and 0.05 mol/l NaCl for digestion with 20 units of BglII and EcoR1 as noted above. The PGK1 gene probe was a 0.8-kb genomic DNA fragment.

† Polymorphic site RE.

‡ Trimming site RE.

Bgl II and EcoRI (to yield reasonably sized pieces for Southern analysis), produces 1.4- and 1.8-kb fragments that hybridize to a PGk gene probe (Figure 1A). A number of HpaII sites are methylated on the inactive X chromosome. HpaII cuts unmethylated DNA at these sites; thus, this region of the PGK locus is digested on the active X chromosome resulting in loss of the 1.4- or 1.8-kb allele, depending on which X chromosome has been inactivated. Conversely, the allele from the inactive X chromosome that is methylated at these sites remains intact. DNA extracted from a clonal population in which each cell and its pattern of X chromosome inactivation is derived from a common progenitor will demonstrate complete loss of one polymorphic fragment and retention of the other (Figure 1B). DNA from a polyclonal population still would demonstrate both alleles, but at reduced hybridization intensity, reflecting a mixture of cells in which either X chromosome may be the inactive one.

DNA RFLP Analysis and X Chromosome Methylation Studies

Peripheral blood was lysed with a triton–sucrose solution, and frozen tissue was ground with a mortar and pestle before DNA purification. DNA was purified with sodium dodecyl sulfate proteinase K (100 μg/ml) digestion, and phenol/chloroform extraction × 4.[27]

All restriction enzymes and restriction enzyme buffers were from Bethesda Research Laboratories (Gaithersburg, MD). Control DNA (8 to 10 μg) from peripheral blood or normal thyroid tissue was initially digested with restriction endonucleases as described in Table 1. Digestion products were electrophoretically separated on a 0.8% agarose gel and transferred to nylon membranes (Hybond-N, Amersham, Arlington Heights, IL) by the method of Southern.[28] The membranes were probed with 1.8 (HPRT) or 0.8 (PGK) kb genomic probes that detect allelic fragments in informative (heterozygous) patients, as described in Table 1. DNA from thyroid lesions in heterozygous patients then was restudied in parallel with control tissue from the same patient with or without predigestion, using the methylation-sensitive restriction endonucleases (MSRE) HhaI or HpaII. The resulting digests were electrophoresed, blotted, and hybridized with the appropriate gene probe to examine methylation sensitivity of individual RFLP bands.

Results

Of the patients studied, nine were informative for PGK (six patients) or HPRT (three patients) polymorphisms. None of the patients studied were informative for both RFLPs. Seven of the informative patients had sufficient material available for detailed study of the pattern of X chromosome inactivation in their thyroid lesions.

The results of clonality studies in the thyroid lesions from these seven patients are summarized in Table 2 and discussed below in greater detail with examples. Three

Table 2. *Clonality Assessment of Thyroid Lesions**

Histologic diagnosis	Informative RFLP	Methylation analysis
Multiple follicular nodules		
Nodular goiter	PGK	Polyclonal
Solitary follicular nodules		
Follicular adenoma	HPRT	Monoclonal
Follicular adenoma	PGK	Monoclonal
Follicular adenoma	HPRT	Loss of heterozygosity consistent with monoclonal pattern
Carcinomas		
Follicular carcinoma	PGK	Monoclonal
Papillary carcinoma	PGK	Monoclonal
Papillary carcinoma	PGK	Monoclonal with aberrant new band

* Diagnosis and clonal analysis of seven patients with nodular lesions of the thyroid who were heterozygous for HPRT and PGK RFLPs. Clonality was assessed using the differential sensitivity to methylation-sensitive restriction endonucleases of X chromosome RFLP alleles in cells from the thyroid lesion compared with the patient's peripheral blood as a control.

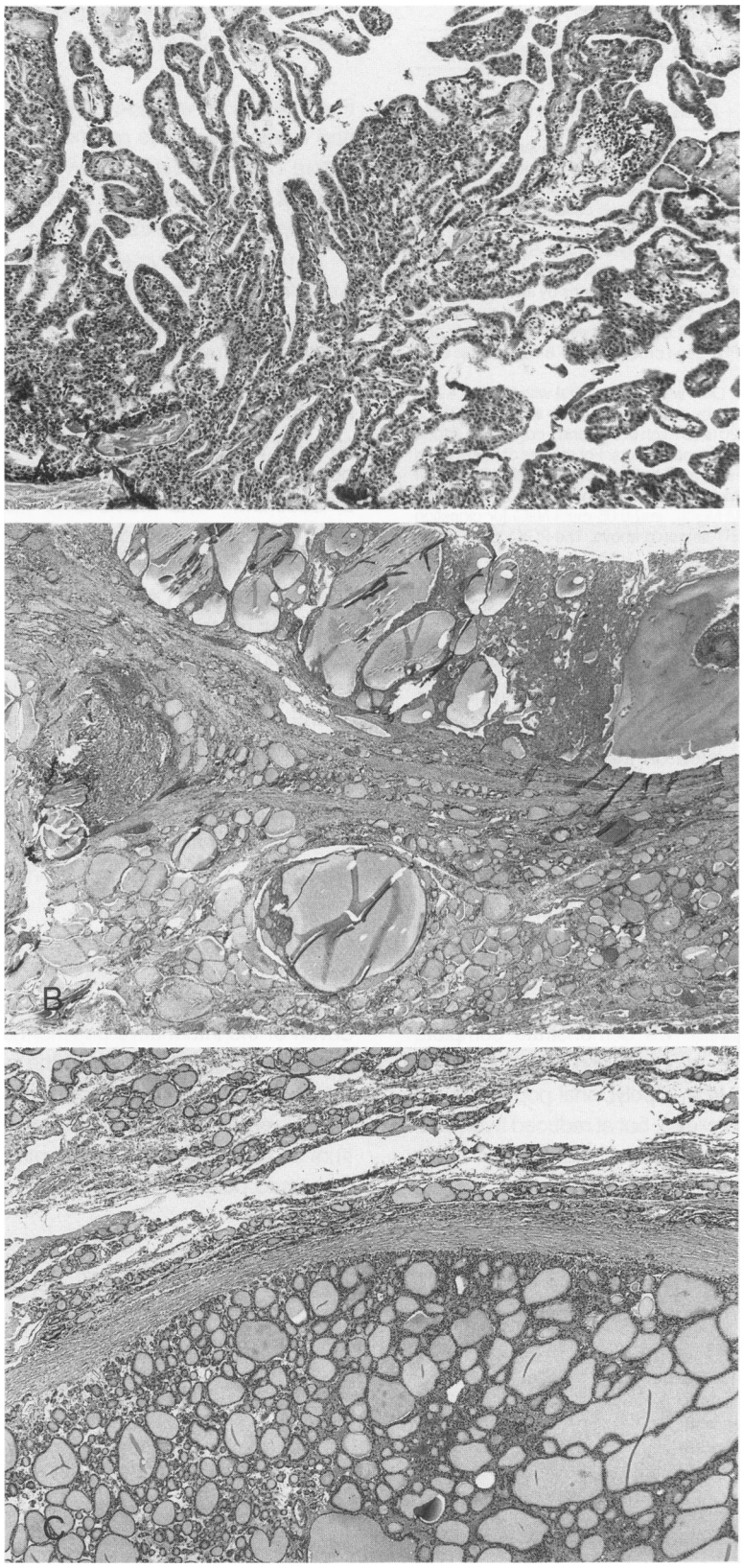

Figure 2.

carcinomas proved to be clonal. A single patient with multinodular goiter showed a polyclonal pattern. Three solitary follicular nodules, the focus of our study, all demonstrated a clonal origin by molecular genetic analysis.

Thyroid Carcinoma

Three thyroid carcinomas were studied (two papillary carcinomas and one follicular carcinoma). Histologically, the follicular carcinoma showed a uniformity of follicular size with capsular invasion. Both papillary carcinomas demonstrated papillary architecture with overlapping grooved nuclei and nuclear clearing (Figure 2A).

Peripheral blood from each carcinoma patient demonstrated informative PGK RFLPs, each with equal methylation sensitivity to HpaII digestion (Figure 3B and C, lanes a and b). In contrast, DNA from the follicular carcinoma showed a monoclonal pattern of HpaII degradation with loss of only the lower 1.4-kb fragment, indicating that virtually all cells from which DNA had been prepared shared the same inactive X chromosome, (Figure 3C, lanes c and d). Two patterns were observed in the papillary carcinomas. One (not shown) was identical to that described for the follicular carcinoma. The other showed loss of the 1.4-kb RFLP band, diminution of the 1.8-kb band, and the appearance of a new band at approximately 1.6 kb (Figure 3B, lanes c and d). The new band presumably reflects hypomethylation of site 4 in the PGK gene (Figure 1A; site 3 or 5 are also possibilities) normally methylated on the inactive (1.8 kb allele) X chromosome. Resulting digestion at this site by HpaII results in a shift of the upper RFLP band to a slightly lower position.

Multinodular Goiter

This thyroid displayed multiple circumscribed follicular nodules with varying sized follicles, focal fibrosis, and

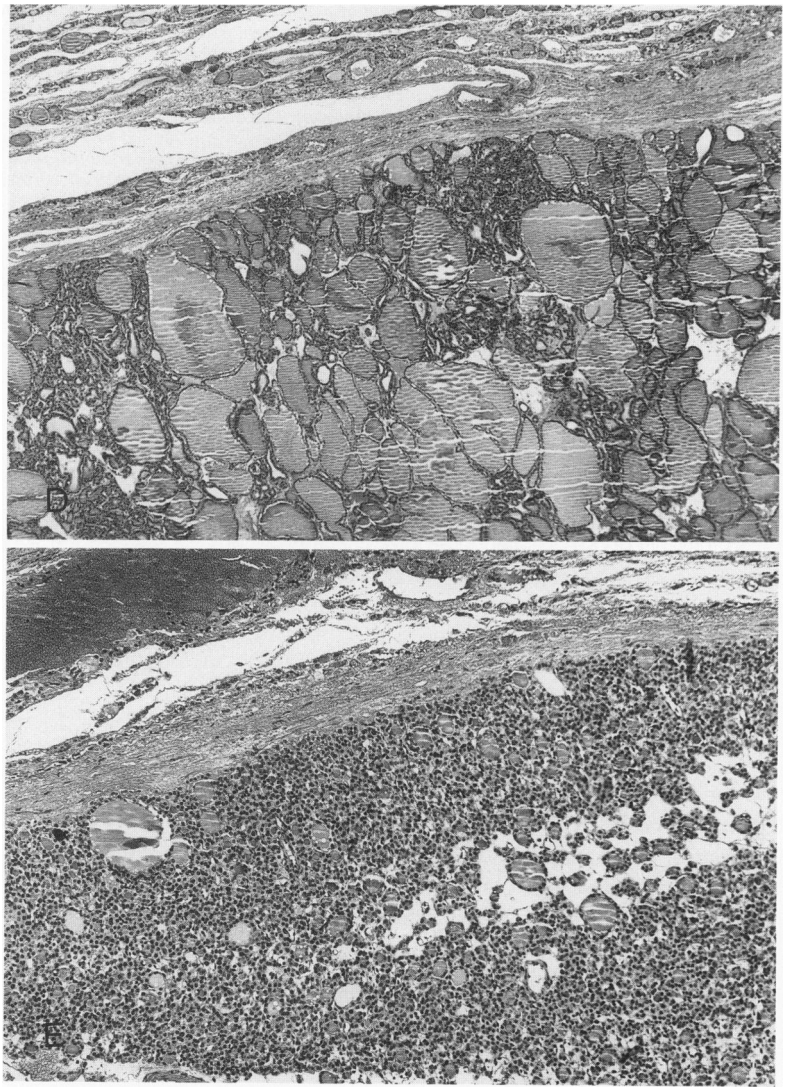

Figure 2. *Histology of thyroid lesions. Papillary carcinoma demonstrating papillary architecture, nuclear crowding, and nuclear clearing.* **(A)** *Multinodular goiter demonstrating multiple follicle nodules, with variation in size of individual follicles and intervening fibrosis.* **(B)** *Three solitary follicular nodules all showed a well-circumscribed border and at least partial encapsulation with compression of the surrounding thyroid parenchyma. Follicular size varied among the lesions: two showed microfollicles and macrofollicles* **(C** *and* **D),** *whereas the third was predominantly microfollicular* **(E).**

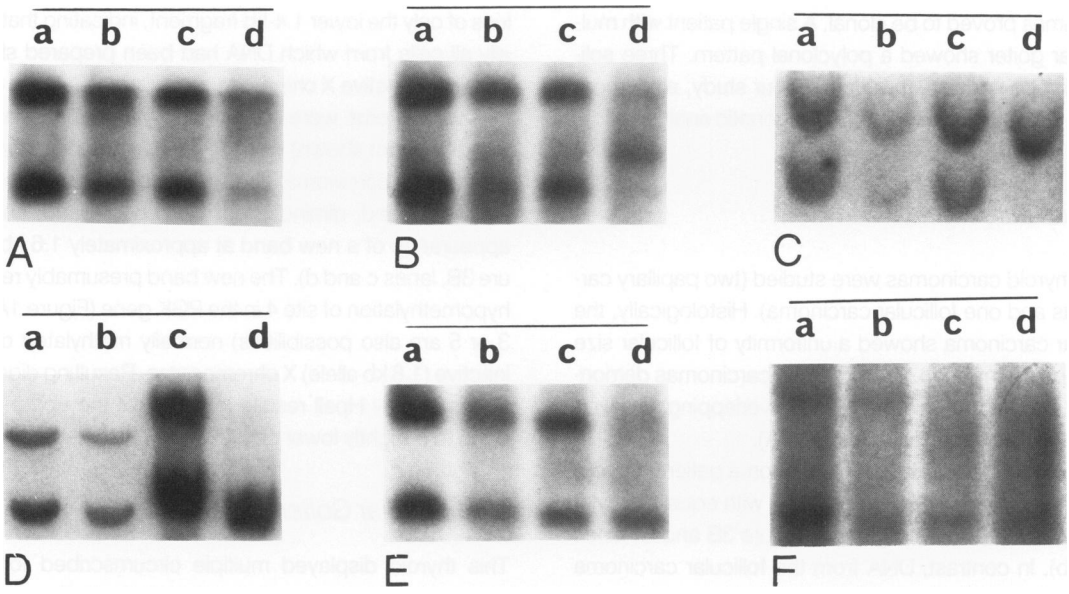

Figure 3. *Clonal analysis of thyroid lesions using methylation-sensitive RFLPs. For all specimens* (A–F), *lane a contains DNA from peripheral blood (PB) digested with restriction enzymes (RE) demonstrating the polymorphic site; lane b contains PB DNA digested with RE + methylation-sensitive restriction endonuclease (HpaII or HhaI); lane c contains thyroid lesional DNA treated with RE; and lane d contains thyroid lesional DNA treated with RE + HpaI or HhaI. RFLP sizes for PGK and HPRT are as indicated in Table 1.* **A**: *Nodular goiter, PGK1 RFLP. In a single sampled nodule from the thyroid, both RFLP bands showed partial sensitivity to MSRE (lane c + d).* **B**: *Papillary carcinoma, PGK1 RFLP. The lower band from tumor DNA shows complete methylation sensitivity; the upper band is diminished, but there is a new band above the 1.4-kb RFLP that reflects abnormal hypomethylation of a site normally methylated on the inactive X chromosome.* **C**: *Follicular carcinoma, PGK1 RFLP. The lower RFLP band from the thyroid tumor shows complete methylation sensitivity indicating a clonal proliferation.* **D**: *Solitary follicular nodule, HPRT RFLP. Selective and complete loss of the upper RFLP band from the thyroid nodule indicates a clonal proliferation.* **E**: *Solitary follicular nodule, PGK1 RFLP. Selective loss of the upper RFLP band from the thyroid nodule indicates a clonal proliferation.* **F**: *Solitary follicular nodule, HPRT RFLP. Lesional DNA lacks the upper RFLP band even in the absence of HhaI, indicating loss of this region on one X chromosome.*

hemorrhage (Figure 2B). Southern blot analysis using the PGK probe on DNA from the peripheral blood showed equal methylation of 1.4 and 1.8 kb alleles (Figure 3A, lanes a and b). Unlike carcinoma (above) or follicular nodule samples (below), DNA from the single nodule sampled for analysis from the multinodular goiter demonstrated approximately equal sensitivity of both the 1.4- and 1.8-kb alleles to HpaII digestion (Figure 3A, lanes c and d), indicating that this nodule contained a polyclonal mixture of cells, some with an inactive paternal X chromosome and others with an inactive maternal X chromosome.

Adenomatoid Nodules

The three solitary nodules studied measured 4.0, 4.5, and 5.0 cm in greatest dimension. Histologically, these lesions were single, well circumscribed, at least partially encapsulated, and compressed surrounding normal thyroid parenchyma near their borders (Figure 2C through E). Follicular size varied among the three lesions; one demonstrated predominantly microfollicles, whereas the others showed a mixture of microfollicles and macrofollicles (Figure 2C through E). Follicular epithelial cells in all three lesions were cuboidal with eosinophilic cytoplasm and cytologically bland oval nuclei.

Two of the patients with adenomatoid nodules were informative for the HPRT and one for the PGK X chromosome polymorphism. Peripheral blood from each patient demonstrated equal sensitivity of both RFLP alleles to methylation-sensitive restriction endonucleases HhaI or HpaII (Figure 3D–F, lanes a and b). In contrast, DNA prepared from each of two solitary thyroid nodules showed sensitivity solely of the larger RFLP allele (Figure 3D and E, lanes c and d). The third patient displayed loss of the larger HPRT allele only in tumor tissue, both with and without prior digestion by HhaI (Figure 3F, lanes c and d).

Discussion

We present molecular genetic evidence from a limited sample of thyroid lesions that all cells composing both solitary follicular nodules and carcinomas contain the same active and inactive X chromosome and are thus of monoclonal origin. Three of three carcinomas and three of three adenomas demonstrated RFLP methylation sensitivity patterns that were consistent with monoclonal deri-

vation. Our results support a previous report of monoclonality in four human thyroid adenomas using a biochemically detectable protein polymorphism of the enzyme glucose 6-phosphate dehydrogenase.[13] Adenomas induced by ionizing radiation in experimental animals also have been interpreted as clonal by isoenzyme histochemistry; rare polyclonal patterns were interpreted as artifactual.[14] The relevance of this experimental system to naturally occurring solitary follicular nodules is unclear. Namba and Fagin[29] have reported in abstract both monoclonal and polyclonal thyroid adenomas and carcinomas using methylation-sensitive restriction endonucleases and X chromosome RFLPs. We detected no polyclonal adenomas or carcinomas in our limited sample, and possible reasons for differences among studies are discussed below.

The study of clonality in frank tumors and proliferative lesions of various organs has led to an increased understanding of the origin and pathogenesis of neoplastic proliferations. The demonstration of monoclonality in proliferative lesions suggests that changes such as a somatic mutation or loss of normal regulatory mechanisms can result in uncontrolled proliferation of a single progenitor cell, which then may expand into a tumor.[30] The alternative demonstration of polyclonality suggests a response to exogenous stimuli by otherwise normal cells[16] or, as suggested by Studer,[6] the possible persistence in adult organs of limited groups of cells that maintain enhanced growth potential.

Studer's group has extensively studied nodular lesions of the thyroid in Switzerland, showing that there is significant heterogeneity with respect to iodine metabolism, endocytotic response, thyroglobulin synthesis, and growth among follicular epithelial cells in the majority of these lesions.[5,9,10,31] They interpret their data to suggest that thyroid nodules should be polyclonal in origin; these authors admit they have not applied their techniques yet to goiters or adenomas from nonendemic regions.[5] Studer's group also points out that functional or growth heterogeneity within a tumor does not necessarily exclude a monoclonal origin,[7] and there is support for the concept of heterogeneity among the cells of clonal lesions in other tumor systems.[32] Our study of a single nodule from a patient with multinodular goiter supports the concept of this lesion as a polyclonal proliferation. By contrast, all solitary adenomas we studied were monoclonal.

Several tools have proved particularly useful for clonal analysis in lesions from different organs. The demonstration of specific chromosomal abnormalities in tumors has provided clear indications of clonality in a number of different disease states, especially in the hematopoietic system.[33] Immunoglobulin or T cell antigen receptor gene rearrangements unique to the normal development of lymphocytes have offered a powerful and sensitive way to detect even small numbers of clonal cells within a large population of cells.[34–36] Technical difficulties associated with cytogenetic analysis and the absence of rearranging genes has hindered studies of clonality in proliferative lesions of many other organs.

A major alternative approach in such tissues has capitalized on the Lyonization of X chromosomes in the somatic cells of females.[22] A large number of studies have looked within tumors at the distribution of different isoenzymes of G6PD, which resides on the X chromosome. Such studies assume that normal and neoplastic cells within a lesion all contribute equal amounts of G6PD, which is difficult to assess.[13] Williams and co-workers[14] have recently reported a direct histochemical method of evaluating G6PD isoenzyme phenotype in individual tissue cells that circumvents uncertainties associated with tissue homogenization. Using this technique, they have shown that experimentally induced thyroid adenomas in mice are monoclonal proliferations.

Molecular genetic approaches that exploit RFLP polymorphisms associated with X chromosome genes lack many of the disadvantages associated with G6PD protein polymorphisms. For one, they are applicable to a greater percentage of the female population (In particular, they are applicable to a greater percentage of Caucasian populations). Moreover, RFLP markers are easily detectable, and each cell contributes the same amount of DNA. Parathyroid[37] and colonic[38] adenomas were previously judged to be polyclonal proliferations by G6PD polymorphisms, but have subsequently been shown to be monoclonal by methylation-sensitive DNA polymorphism studies,[16,17] suggesting that molecular methods may provide a more accurate method of assessing clonality.

A disadvantage associated with both DNA and protein polymorphism studies of clonality is the problem of "contaminating" normal cells such as fibroblasts, endothelial cells, and inflammatory cells sampled with the proliferation under study. Depending on the type of lesion, these cells may complicate the assessment of methylation sensitivity of X chromosome RFLPs by contributing an unknown but potentially significant amount of DNA, which would be expected to display random X chromosome inactivation. Great care must be taken in these types of studies to minimize or control for nonlesional cell contamination to avoid confusing results or incorrect interpretations of methylation sensitivity data. Fortunately, in the solitary follicular thyroid nodules we studied, minimal amounts of fibrous stroma or inflammatory cells were present, as confirmed by the monoclonal patterns observed. The selection of tissue from noncapsular areas of the lesions also minimized the likelihood of contamination by normal surrounding polyclonal thyroid, and we confirmed the extent of nonlesional tissue in all samples by frozen section analysis.

The concept of "patch size" within a normal organ has important implications for the interpretation of clonality studies on proliferations of cells constituting that organ. A tumor that appears monoclonal theoretically could have arisen from two, three, or many adjacent cells in a "patch" in which all cells share identical inactivated X chromosomes because of embryogenic growth patterns that give rise to the adult organ.[13] Exogenous stimuli causing proliferation of more than one cell within a homogeneous patch would yield the same monoclonal pattern as seen with origin from a single progenitor cell, using the method we have employed. The study of mosaic patterns in different organs from chimeric animals has suggested that cells are not randomly distributed in all tissues.[39,40] Although studies of rat liver have shown a fine pattern of mosaicism that appears to be random with respect to the lobular and the acinar architecture of the liver,[39] mouse small intestine shows a nonrandom distribution of patch sizes within the mucosa.[40] To our knowledge, detailed studies of patch size have not been performed for the normal thyroid, although immunohistochemical data on phenotypes of adjacent cell pairs demonstrate a modest nonrandom distribution of 3 to 1.[14] Although we can not exclude multicellular origin from "patches" of follicular cells in the thyroid as an explanation for the monoclonal patterns we observed, data on patch size in the mouse, a polyclonal pattern in the goiter nodule studied, and a consistent monoclonal pattern in carcinomas and solitary follicular adenomas suggests that those latter lesions most likely arose from a single progenitor cell. A portion of adjacent normal thyroid was available for study in one of our patients with a solitary follicular nodule. In contrast to the pattern from the solitary follicular nodule, DNA from this region showed equal methylation sensitivity for both RFLP alleles, consistent with the absence of very large patches of identical follicular cells in normal thyroid (data not shown).

In a similar vein, we cannot exclude polyclonal cellular origin of a nodule with subsequent selection of a small number of cells that share the same inactivated X chromosome and maintain an enhanced growth potential, thus yielding an overall monoclonal pattern (see Studer[6,31]). The lesions we studied were large, clinically apparent, and presumably represent a well-developed stage in the pathogenesis of solitary nodules and carcinomas. Apparent monoclonality at this stage of the disease process might not necessarily reflect initiating or even early stages in the pathogenesis of these lesions.[41] In this respect, the study of small clinically inapparent lesions might yield insights into the pathogenesis and earlier stages of disease progression.

An amalgamation of studies to date favors a monoclonal origin for many adenomatoid nodules in the thyroid. Less common polyclonal carcinomas or adenomas such as those reported by Namba and Fagin[29] presumably represent proliferations derived from two or more follicular cells, assuming the absence of contaminating stromal cells. These lesions raise interesting questions regarding pathogenetic mechanisms compared with their monoclonal counterparts.

It has long been recognized that it can be difficult to distinguish by clinical and histopathologic criteria between the early stages of a nodular goiter containing a single nodule and a solitary follicular adenoma of similar size.[42] It is possible that clonal assessment by molecular methods might be useful clinically to distinguish an early nodular goiter from a true follicular adenoma.

Both aberrant methylation and loss of heterozygosity that we observed have been described in other tumors.[43–45] Abnormal PGK gene methylation was seen in one of our papillary carcinomas.[43,44] The demonstration of tumor-specific loss of chromosomal material in a benign tumor has only rarely been described,[45] and, to our knowledge, this is the first report of the loss of heterozygosity in a benign lesion of the thyroid. A lack of material did not permit us to more definitively explore the extent of X chromosome deletion with informative RFLP markers close to and further from the HPRT locus in this patient. However, the observed methylation pattern strongly suggests a monoclonal proliferation, as it was unique to the lesion.

Our data indicates that the solitary follicular nodules we studied are monoclonal proliferations. In this respect they differ from benign multinodular lesions and are similar to histologically malignant carcinomas. Whereas a consistent pattern was observed for each histologic group, we acknowledge the relatively small number of samples examined to date and the necessity by the analytic method chosen that only women heterozygous for HPRT or PGK RFLPs were studied. Thus, it is not proven these results can be extrapolated to all solitary follicular nodules in the thyroid, and other studies have reported that solitary nodules may be either monoclonal or polyclonal.[13,14,29] Our results do lend support to the concept that some, perhaps many or most, solitary follicular nodules of the thyroid are neoplastic in origin. From a clinical standpoint, solitary follicular nodules behave as benign lesions. Whether by further genetic or other changes they evolve to biologically more aggressive malignant lesions is at this point unknown, and further studies need to be undertaken to better define their place in the pathogenesis of thyroid neoplasia.

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