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Clonality of the Parathyroid Nodules with Uremic Parathyroid Hyperplasia

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Clonal assessment suggests that most parathyroid adenomas and a subset of uremic parathyroid hyperplasia are monoclonal. A weakness that remains in the prior clonal studies is assessing the clonal status of the tissue fragments containing multiple nodules rather than a single nodule emerging in the uremic parathyroid hyperplasia. We applied the X chromosome-linked phosphoglycerate kinase (PGK) gene inactivation assay method for clonality to study individual nodules. Materials were obtained from 31 cases with parathyroid adenoma and 16 with uremic parathyroid hyperplasia. 17 cases were heterozygous in the PGK-1 locus. We were able to assess the clonality of 10 parathyroid adenomas and 7 hyperplastic glands. Monoclonality was demonstrable in 9 of the 10 parathyroid adenomas and in 4 of the 7 hyperplastic glands. Further analysis of 11 individ-

ual nodules microdissected from 3 monoclonal and 1 polyclonal hyperplastic glands revealed that 6 nodules were monoclonal and 5 were polyclonal. Nodules arising in a hyperplastic gland could be of monoclonal or polyclonal origin. Polyclonal and monoclonal nodules coexisted within single glands. Our findings indicate a progression from generalized hyperplasia to a monoclonal tumor in uremic parathyroid hyperplasia. Comparing clonality with the parathyroid hormone (PTH) immunoreactivity and histological features, we found that monoclonal nodules showed a homogeneous immunoreactivity against PTH antibody, whereas most of the polyclonal nodules showed a heterogeneous staining. Classic morphological criteria alone was inadequate to distinguish a monoclonal from a polyclonal nodule. (Pathology Oncology Research Vol 3, No 3, 198-203, 1997)

Key words: parathyroid adenoma, uremic parathyroid hyperplasia, clonality, PGK gene

Introduction

Hyperparathyroidism has long been appreciated to be expressed as hypercalcemia in association with excessive secretion of the PTH, but the molecular pathophysiology is poorly characterized until now.^{1,2} Differentiation between parathyroid adenoma and hyperplasia is still difficult both for clinicians and pathologists.³⁻⁵ Recently, some authors have applied X-inactivation analysis to the clonal assessment of parathyroid adenomas, and they have

found that most parathyroid adenomas were monoclonal. This finding also extended to the uremic parathyroid hyperplasia and multiple endocrine neoplasia type 1 (MEN-1).⁶⁻⁷ Moreover, loss of heterozygosity involving the 11q13 region has been detected in the majority of parathyroid tumors from MEN-1 patients and a subset of uremic parathyroid hyperplasia, which provided indirect evidence for the clonal origin of these lesions.^{8,9} These observations lessen the distinction between parathyroid hyperplasia and adenoma and may in part explain the inadequacy of histopathological criteria used to distinguish between these two different lesions.

However, these studies are based on the analysis of tissue fragments containing multiple nodules emerging in the uremic parathyroid hyperplasia. The assessment

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may reflect the clonal status of a single nodular tumor, such as parathyroid adenomas. It is apparent that the clonal assessment is inadequate for a gland with a multinodular structure and it is also not enough for the understanding of the pathogenesis of the uremic parathyroid hyperplasia.

In the present study, we examined the clonality of parathyroid adenomas and uremic parathyroid hyperplasia using X-chromosome-linked PGK gene inactivation assay. We further analyzed the clonality of individual nodules arising in uremic parathyroid hyperplasia and compared the clonality with the histopathological features.

Materials and Methods

Cases and parathyroid specimens

Forty seven female cases were included in our analysis, who underwent surgical treatment in four separate hospitals because of primary or secondary hyperparathyroidism and resistance to medical treatment. These cases were categorized as having parathyroid adenoma (31 cases) or uremic parathyroid hyperplasia (16 cases) according to the accepted clinicopathological criteria. None of the patients had a history of neck irradiation, renal transplantation or a family history of hyperparathyroidism. The parathyroid specimens were formalin-fixed, paraffin-embedded or frozen tissue.

Microdissection

Six consecutive sections with a thickness of 10 μ m were cut from the parathyroid glands. For the parathyroid adenomas, the tumor mass was microdissected from the sections for clonal analysis. For the uremic parathyroid hyperplasia, first, the tissue fragments containing all the nodules were taken from 3 sections and subjected to clonal analysis. Then, individual nodules were microdissected separately from 3 or 4 sections using a microcapillary tube. This microcapillary tube was pulled to a fine tip and was cleaned with 100% ethanol to avoid contamination among samples. These nodules were separately subjected to clonal analysis. In addition, we further subdivided a polyclonal nodule into 3 regions, and the 3 regions were separately microdissected and subjected to analysis. The thyroid tissue, lymph nodes or thymus from the same patient was used as a normal control.

Clonal analysis

DNA was extracted using modified Goelz's method¹⁰ or QIAamp tissue kit (QIAGEN GmbH, Germany), according to the manufacture's protocol. Genomic DNA with or without pretreatment was amplified in pairs using

primers 1A, 1B and internal primers 2A, 2B as reported by D. Gary Gilliland, et al.¹¹ The pretreatment of DNA (0.1–0.4 μ g) was carried out using 15 U of Hpa II methylation-sensitive enzyme (Takara Shuzo Co. Ltd. Japan) for over 12 hours at 37 °C in a total of 50 μ l reaction solution. Eight μ l of the PCR products were digested for 4 hours at 45 °C with 15 U of Bst XI (Takara Shuzo Co. Ltd. Japan). Completeness of the digestion was controlled using the PCR product from a male DNA with only the inactive PGK allele. Following purification, the digested product was analyzed in agarose gel (2% Nusieve GTG and 1% Seakem, FMC, USA) and stained with ethidium bromide. Clonality was determined in comparison with the normal tissue.

Immunohistochemical study

Sections were cut at a thickness of 4 μ m. Avidin-biotin peroxidase complex (ABC) method was used to analyze the immunoreactivity against monoclonal PTH antibody (DAKO, optimal dilution 1:2560). Antigen was retrieved by microwave irradiation before applying the PTH antibody.¹² The period of irradiation was 5 minutes, repeated 3 times, with the sections immersed in the citric acid buffer (pH 6.0). Finally, the sections were counterstained with methyl green. Omission of the primary antibody was used as a negative control.

Results

Heterozygosity – Of the 47 female cases, 17 were heterozygous in the PGK-1 locus. We were able to assess the clonal status in 14 of the 17 cases. The 14 cases included 10 with parathyroid adenoma and 4 with uremic parathyroid hyperplasia.

Clonality of the parathyroid adenomas – 9 out of the 10 parathyroid adenomas showed a monoclonal pattern, representing either the uncut 530 base pairs (bp) or the cleaved 433 bp in the agarose gel. One parathyroid adenoma demonstrated a polyclonal pattern, which might be due to the contamination of the rich blood vessels in the tumor stroma (Fig. 1).

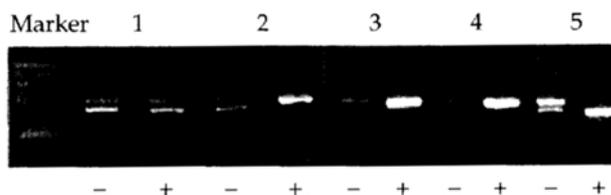


Figure 1. Representative analysis of the clonality of 5 parathyroid adenomas (lane 1-5). One parathyroid adenoma (lane 1) showed a polyclonal pattern. The others were all monoclonal. Marker: PhiX174 RF DNA Hae III digest. "+" and "-": with and without pretreatment of DNA by Hpa II.

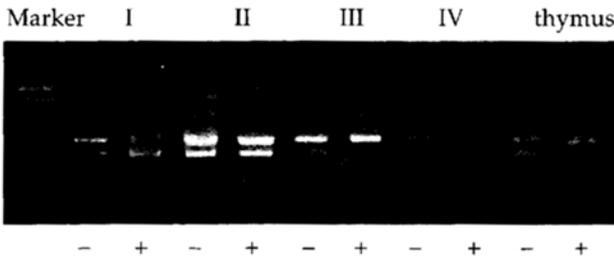


Figure 2. Clonal results of the hyperplastic glands from the patient presented in table 1. Glands I, II, IV show a polyclonal pattern and gland III shows a monoclonal pattern. Thymic tissue as a normal control shows a polyclonal pattern. Marker: PhiX174 RF DNA Hae III digest. "+" and "-": with and without pretreatment of DNA by Hpa II.

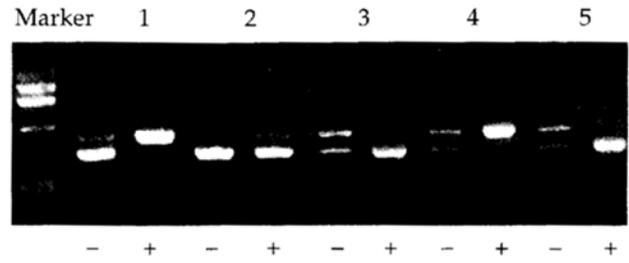


Figure 3. Representative clonality of the individual nodules microdissected from gland II in Figure 2. Monoclonal pattern is demonstrated in nodules 1, 4, 5. The remaining nodules showed a polyclonal pattern. Marker: PhiX174 RF DNA Hae III digest.

Clonality of the uremic parathyroid hyperplasia and individual nodules – Of the 4 cases, 7 affected glands were detected in their clonalities. In 3 cases, one gland was assessed respectively and all were monoclonal. In the additional case, 4 glands were separately analyzed; 1 gland showed a monoclonal and 3 showed a polyclonal pattern (Fig. 2). Tissue microdissection yielded 11 nodules (Table 1). Five nodules were obtained from one gland with a polyclonal pattern of which 3 demonstrated a monoclonal pattern and 2 were polyclonal (Fig. 3). The

other 4 nodules were obtained from an additional gland with a monoclonal pattern; 1 was monoclonal and 3 were polyclonal. The remaining two nodules were microdissected from two other separate glands with monoclonality and both showed a similar monoclonal pattern to that of the primary gland. Further analysis of a polyclonal nodule showed that the 3 regions from the nodule were all polyclonal, similar to the clonality of the nodule. Monoclonal nodules and polyclonal nodules coexisted within single glands. A polyclonal pattern based on the analysis of the tissue fragments containing multiple nodules did not indicate a polyclonal expansion of the gland. Similarly, polyclonal nodules could exist within a gland showing a monoclonal pattern.

Table 1. Representative analysis of one case with uremic parathyroid hyperplasia

Gland No	Nodularity	Size*	Staining pattern	Clonality
I	diffuse	3	homogenous	polyclonal
II	multinodular	23	heterogenous	polyclonal
	nodule 1	10	homogenous	monoclonal
	nodule 2	19	heterogenous	polyclonal
	nodule 3	4	heterogenous	polyclonal
	nodule 4	6	homogenous	monoclonal
III	multinodular	15	heterogenous	monoclonal
	nodule 1	2	heterogenous	polyclonal
	nodule 2	3	homogenous	polyclonal
	nodule 3	3	homogenous	polyclonal
IV	multinodular	24	heterogenous	polyclonal
	nodule 4	6	homogenous	monoclonal

* mm in diameter;

The patient, a 41 year-old female, had accepted dialysis for 40 years until the parathyroidectomy and autotransplantation 20 months ago because of chronic renal failure. The laboratory examination revealed a high serum PTH level (HS-PTH 398 pg/ml), hypercalcemia (10.5 mg/dl) and hyperphosphatemia (5.6 mg/dl). The physical examination revealed soft tissue calcification and multiple bone fractures. After the operation, the serum calcium level returned to normal until now.

The clonal inconsistency between the glands and the individual nodules may be common, although our sample was small. As we analyze the tissue fragments of a multinodular gland rather than single nodules, the clonal status of the gland would be the reflection of what kind

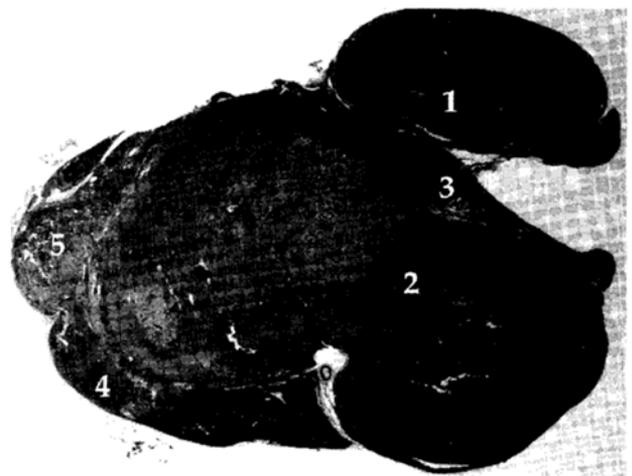


Figure 4. The parathyroid gland II in Table 1, showing the multinodular feature (H&E stain, X1.8). The number of the nodules is the same as that in Figure 3.

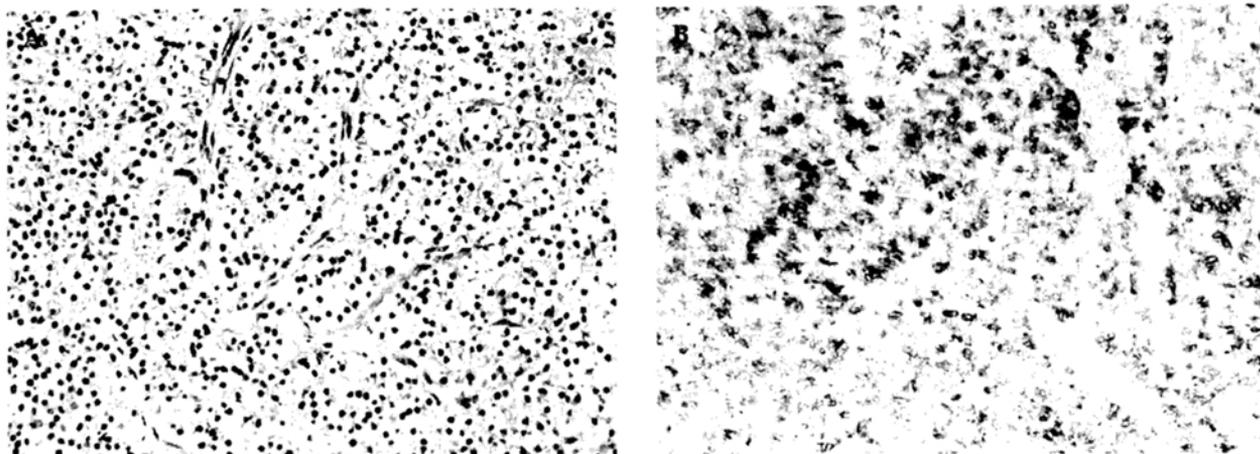


Figure 5. Nodule 1 in gland II, showing that histologically the nodule is composed of uniform chief cells (A) (H&E stain, X100). Immunohistochemically it shows a homogeneous staining against PTH antibody (B) (ABC, X100).

of nodules (monoclonal or polyclonal) have the advantage within the gland.

Immunohistochemical findings (Fig. 4–6) – According to the PTH immunoreactivity, we could divide the affected glands into two groups: one group showed a homogeneous and another showed a heterogeneous staining. Most of the parathyroid adenomas demonstrated a homogeneous staining, whereas in the parathyroid hyperplasia, different nodules showed different staining patterns or intensities.

Some nodules were stained homogeneously and some were stained heterogeneously. As we compared the immunohistochemical features with their clonalities, we noticed that the monoclonal nodules showed a homogeneous staining (6/6) and most of the polyclonal nodules showed a heterogeneous staining (3/5). In H&E staining, the morphological features alone were inadequate to distinguish monoclonal from polyclonal nodules.

Discussion

X-inactivation analysis is a powerful method that does not require information about a potentially defective gene. Several groups have reported that most of the parathyroid adenomas and a substantial uremic parathyroid hyperplasia are monoclonal.^{6,13,14} These findings provide new insights into the pathogenesis of the primary and secondary hyperparathyroidism. Our clonal results about the parathyroid adenomas and hyperplasias are consistent with their conclusions. However, to draw conclusions concerning the pathogenesis of the uremic parathyroid hyperplasia, it is essential to consider the feature that most of the affected glands are composed of multiple nodules. In the previous analysis of clonality, they have only analyzed the clonal status of the tissue fragments containing multiple nodules. The assessment of individual parathyroid nodules has not been carried out. Our analysis revealed that nodules arising

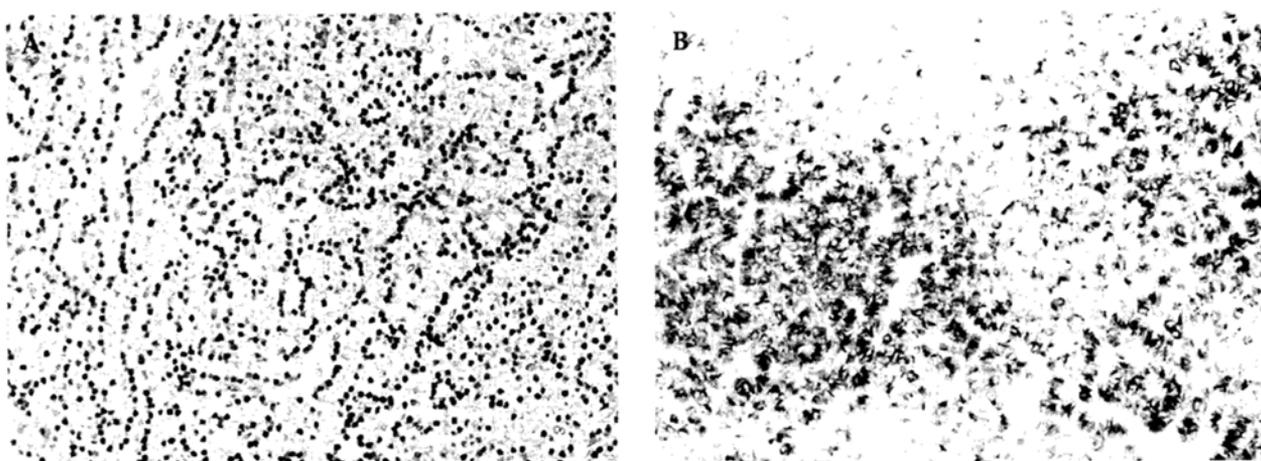


Figure 6. Nodule 2 in gland II. The nodule is also composed of uniform chief cells similar to nodule 1 (A) (H&E, X100), but it demonstrates a heterogeneous staining against PTH antibody (B) (ABC, X100).

in hyperplastic glands may be of polyclonal as well as clonal origin. Coexistence of polyclonal and monoclonal nodules is easily interpreted as polyclonality of the lesion by analysis of the tissue fragments containing all the nodules. Within a gland undergoing hyperplasia, one or more cells may undergo a mutation that confers a selective growth advantage on the progeny of those cells. Coexistence of both clonal and polyclonal nodules may reflect a progression from generalized hyperplasia to neoplastic lesion in the uremic parathyroid hyperplasia. Similar results are obtained and are becoming apparent in MEN-1 endocrine lesion with different allelic losses in the nodules.^{9,15} Our findings are more or less similar to those of the clonal analysis in the adenomatous goiter of the thyroid and in the liver cirrhosis. For both lesions, coexistence of clonal and polyclonal nodules and the neoplastic nature of the clonal nodules have been well documented.^{16,17,18}

For a polyclonal pattern, it could be misinterpreted for a variety of reasons, such as contamination of normal tissue in the stroma or hyperplastic parathyroid cells among the nodules.⁸ The most reasonable explanation may be that monoclonal and polyclonal nodules coexist within a single gland, as revealed by us. To interpret the monoclonality of individual nodules, it invokes the concept of "patch size."^{16,19} This theory suggests that an apparently monoclonal lesion may originate from a small number of cells in a "patch" with the same pattern of X-chromosome inactivation. Therefore, demonstrating molecular monoclonality among cells within the same patch would be uninformative. Unfortunately, we know little about the patch size in the parathyroid gland. We have checked the concept of "patch size" by dividing one polyclonal nodule into several regions. Similar polyclonal patterns were obtained from all 3 regions. Our results indicate that the parathyroid anlage is already multicellular at the time of X-inactivation as observed in thyroid anlage.¹⁶ We prefer to consider that the monoclonal nodule represents a monoclonal tumor rather than a simple increase of a patch size due to cellular hyperplasia. In a recent report by Lubensky et al,⁹ they concluded that a parathyroid lesion associated with MEN-1 could consist of more than one clone with different allelic losses. Their findings agree with our explanation of the "patch size."

Histopathological criteria appear inadequate to distinguish between parathyroid adenoma and hyperplasia.^{20,21,22} However, as we compared the immunohistochemical characteristics with clonality, we found that the parathyroid adenoma showed a homogeneous reaction against PTH antibody. Similarly, monoclonal nodules also showed a homogeneous staining. PTH immunoreactivity was consistent with the clonal status to a certain extent. In the histology, morphologically similar nodules were either monoclonal or polyclonal. It was inadequate to differentiate between them by morphological features alone.

Nodularity was not a diagnostic parameter for parathyroid hyperplasia.

In summary, we analyzed in detail the clonality of uremic parathyroid hyperplasia. Nodules arising in a hyperplastic gland could be of monoclonal or polyclonal origin. Our results indicate a progression from a generalized hyperplasia to a monoclonal tumor in the secondary hyperparathyroidism associated with chronic renal failure. PTH immunoreactivity rather than histological features may be of help to differentiate a monoclonal tumor from polyclonal hyperplasia to a certain extent.

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References

1. Arnold A: Genetic basis of endocrine diseases 5: Molecular genetics of parathyroid gland neoplasia. *J Clin Endocrinol Metab* 77:1108-1112, 1993.
2. Backdahl M, Howe JR, Lairmore TC, Wells SA: The molecular biology of parathyroid diseases. *World J Surg* 15:756-762 1991.
3. Grimelius L, Akerström G, Bondeson L, et al: The role of the pathologist in the diagnosis and surgical decision making in hyperparathyroidism. *World J Surg* 15:698-705 1991.
4. van Heerden JA, Grant CS: Surgical treatment of primary hyperparathyroidism: An institutional perspective. *World J Surg* 15:688-692 1991.
5. Wheeler MH: Hyperparathyroidism: A surgeon's view. *Endocrine Surg* 12:283-291 1995.
6. Arnold A, Brown MF, Cleflla P, et al: Monoclonality of parathyroid tumors in chronic renal failure and in primary parathyroid hyperplasia. *J Clin Invest* 95:2047-2053 1995.
7. Friedman E, Sakaguchi K, Bale AE, et al: Clonality of parathyroid tumors in familial multiple endocrine neoplasia type I. *N Engl J Med* 321:213-218 1989.
8. Falchetti A, Bale AE, Amorosi A, et al: Progression of uremic hyperparathyroidism involves allelic loss on chromosome 11. *J Clin Endocrinol Metab* 76:139-144 1993.
9. Lubensky IA, Debelenko LV, Zhuang ZP, et al: Allelic Deletions on chromosome 11q13 in multiple tumors from individual MEN 1 patients. *Cancer Res* 56:5272-5278 1996.
10. Goetz SE, Hamilton SR, Vogelstein B: Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. *Biochem Biophys Res Commun* 130:118-126 1985.
11. Gilliland DG, Blanchard KL, Levy J, et al: Clonality in myeloproliferative disorders: Analysis by means of the polymerase chain reaction. *Proc Natl Acad Sci USA* 88:6848-6852 1991.
12. Utsunomiya H, Shan L, Kawano I, et al: Immunolocalization of parathyroid hormone in human parathyroid glands with special references to microwave antigen retrieval. *Endocrine Pathol* 6:223-227 1995.
13. Noguchi S, Motomura K, Inaji H, et al: Clonal analysis of parathyroid adenomas by means of the polymerase chain reaction. *Cancer Lett* 78:93-97 1994.
14. Arnold A, Staunton CE, Kim HG, et al: Monoclonality and abnormal parathyroid hormone genes in parathyroid adenomas. *N Engl J Med* 318:658-662 1988.

15. *Brandi ML, Falchetti A, Tonelli F, Bordi C*: Editorial: are allelic losses at 11q13 universal in MEN 1 tumors? *J Clin Endocrinol Metab* 81:3162-3163, 1996.
16. *Apel RL, Ezzat S, Bapat BV, et al*: Clonality of thyroid nodules in sporadic goiter. *Diagn Mol Pathol* 4:113-121, 1995.
17. *Aeschmann S, Kopp PA, Kimura ED, et al*: Morphological and functional polymorphism within clonal thyroid nodules. *J Clin Endocrinol Metab* 77:846-851, 1993.
18. *Aihara T, Noguchi S, Sasaki Y, et al*: Clonal analysis of regenerative nodules in hepatitis C-induced liver disease. *Gastroenterology* 107:1805-1811, 1994.
19. *Fialkow PJ*: Clonal origin of human tumors. *Biochim Biophys Acta* 458:283-321, 1976.
20. *Shan L, Iwasaki A, Utsunomiya H, et al*: Immunoreactive characteristics and classification of hyperparathyroidism. *Endocrine Pathol* 2:145-152, 1995.
21. *Ghandur-Mnaymenh L, Kimura N*: The parathyroid adenoma, a histopathologic definition with a study of 172 cases of primary hyperparathyroidism. *Am J Pathol* 115:70-83, 1984.
22. *El-Deiry S, Levine MA*: Molecular overtones of primary hyperparathyroidism. *J Clin Endocrinol Metab* 80:3105-3106, 1995.