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Comparative analysis of clonality and pathology in primary and secondary hyperparathyroidism

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Abstract Parathyroid adenoma and hyperplasia are the most common causes for hyperparathyroidism, and distinction between them is controversial based on the current criteria for pathological diagnosis. We studied the clonality of hyperparathyroidism and its correlation with the pathological features, analysing 39 female patients with hyperparathyroidism. Clonality was successfully detected in 12 heterozygous cases by PCR amplification of *PGK-1* gene. The 12 cases yielded 14 hypercellular glands, 8 affected by primary and 6 by secondary hyperparathyroidism. The results revealed that 7 of the 8 glands with primary hyperparathyroidism showed monoclonal proliferation. Only 1 gland pathologically diagnosed as adenoma showed a polyclonal pattern. In the 4 cases with secondary hyperparathyroidism, at least one monoclonal tumour was detected in each case. Our data indicate that monoclonal tumours are more common than expected in both primary and secondary hyperparathyroidism. Monoclonal tumours and polyclonal hyperplasia can co-exist in the same patient. Comparative study of the clonality and the pathological features showed that the clonality was consistent with the diagnosis of parathyroid adenoma, whereas it was in conflict with the diagnosis of hyperplasia with multigland involvement. One of the reasons for this is that we are ignorant of the true natures of hyperparathyroidism with multigland involvement.

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Introduction

Hyperparathyroidism was once thought to be rare, but is now more commonly recognized, affecting 1 in 500 women over 40 years of age [22]. Causative parathyroid adenomas and hyperplasia are difficult to differentiate pathologically, and most cases with single gland involvement are diagnosed as adenoma, while most of those with multigland involvement are considered to be hyperplastic, particularly those related to dialysis.

Recent molecular analysis and clinical studies have revealed that the current criteria for pathological diagnosis do not reflect the biological or clinical differences among parathyroid diseases [2, 5]. Genetic abnormalities have been detected not only in cases affecting single glands but also in those with multigland involvement [3, 4, 6, 9]. Furthermore, a high rate of tumour-like recurrences of autograft parathyroid tissue and autonomous PTH secretion in secondary parathyroid nodules have often been observed on clinical examination [13, 21]. These findings have caused the diagnostic criteria to be called in question. We believe that some parathyroid diseases classed as hyperplasia on pathological examination may in fact be monoclonal tumours. There may also be a monoclonal component in the background of generalized hyperplasia, or a polyclonal cellular hyperplasia may evolve into a monoclonal tumour as genetic alternations occur.

A clonal analytic method using X-linked genes enables us to assess the clonality of most human tumours successfully. When the different methylation patterns and polymorphisms of X-linked genes are exploited, most human tumours are shown to be monoclonal [7, 16]. To verify our supposition, we analysed the clonality of hyperparathyroidism using X-linked *PGK-1* gene (phosphoglycerate kinase). The clonality was compared with the pathological features.

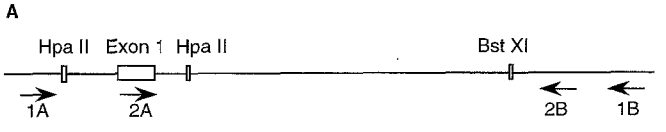
Materials and methods

In all, 29 cases of primary hyperparathyroidism and 10 of secondary hyperparathyroidism were analysed; all 39 patients concerned were female. We were able to assess the clonality in 12 heterozygous cases: 8 cases with primary hyperparathyroidism and 4 with secondary hyperparathyroidism related to dialysis. In 1 of the 4 cases with secondary hyperparathyroidism, 3 glands were available for analysis and in each of the others, 1 gland was studied. All patients had shown clinical-pathological features of hyperparathyroidism, and they had been operated on in four different hospitals. No patient had a history of neck irradiation or renal transplantation or any family history of hyperparathyroidism. The pathological features of these hypercellular glands were summarized according to reports published by Ghandur-Mnaymneh and Kimura [10] and others. A comparative study was carried out between the clonality and pathological features.

All the parathyroid tissues were formalin-fixed and paraffin-embedded (FFPE). Six sections with a thickness of 10 μm were cut from the FFPE tissues. The hypercellular parathyroid tissues only were dissected from the sections and subjected to the DNA preparation. The thyroid tissues or lymph nodes from the same case were used as controls. In addition, normal peripheral blood leucocytes from 2 female heterozygotes and 2 male, one normal parathyroid gland resected during a thyroid operation and the TT (medullary thyroid carcinoma) cell line were also analysed as technical controls. The TT cell line was generously donated by Dr. Shimaoka, K. (Rosewell Park Memorial Institute, Buffalo, USA). DNA from FFPE tissue was extracted using modified Goelz' s method [12] and DNA was extracted from peripheral blood leucocytes and TT cell line using IsoQuick kit (Microprobe, USA) according to the manufacturer's protocol.

For PCR amplification, a portion of *PGK-1* gene was amplified using primers 1A, 1B and internal primers 2A, 2B as reported by Gilliland et al. [11] with minor modifications (Fig. 1). Aliquots (0.5 μl) of DNA samples were amplified in a 50 μl reaction volume on a DNA thermal cycler (Perkin-Elmer Cetus) for 50 cycles using primers 1A and 1B, after which 5 μl of the amplified product was amplified using internal primers 2A and 2B over an additional 50 cycles. The PCR condition was 1 min at 94°C, 2 min at 58°C and 3 min at 72°C. Adequacy of amplification was assessed by agarose gel electrophoresis (2% Nusieve GTG and 1% Seakem, FMC, USA).

For heterozygosity determination, 8 μl of the PCR product was digested for 4 h at 45°C with 15 U of Bst XI (Takara Shuzo, Japan) in a total of 50 μl reactive mixture. Completeness of digestion was controlled using the PCR product from a male DNA with the inactive PGK allele only. Following incubation, 1.7 μl of 3 M sodium acetate, 1 μl of ethachinmate and 125 μl of 100% ethanol were added, mixed by vortexing, left to stand for over 10 min at –80°C and centrifuged at 15,000 rpm for 20 min at 4°C. The pellet was dissolved with 6 μl of H_2O and electrophoresed in agarose gel stained with ethidium bromide.



B

PCR primers
1A: 5'-CTGTTCCTGCCCGCGCGGTGTTCCGCATTC-3'
1B: 5'-ACGCCTGTTACGTAAGCTCTGCAGGCCTCC-3'
2A: 5'-AGCTGGACGTTAAAGGGAAGCGGGTCGTTA-3'
2B: 5'-TACTCCTGAAGTTAAATCAACATCCTCTTG-3'

Fig. 1 **A** Map of the *PGK* gene in the vicinity of methylated HpaII sites and polymorphic BstXI site. **B** Sequences of the primers used

For clonal analysis 5 μl of DNA (0.1–0.4 μg) from Bst XI site heterozygosity samples was predigested with 15 U of HpaII methylation-sensitive enzyme (Takara Shuzo, Japan) for over 12 h at 37°C in a total of 50 μl reaction solution. Then 5 μl (10–40 ng DNA) of the reaction solution was amplified as the template and the products were treated with Bst XI as described above.

Results

PCR amplification

PCR amplification of the *PGK-1* gene revealed 530-bp products as expected in all the cases. Of these, 16 cases were heterozygous in the Bst XI site and 12 were assessed for clonality successfully. The remaining 4 cases showed either no specific amplification product or inadequate products for analysis after pre-digestion of the DNA by HpaII.

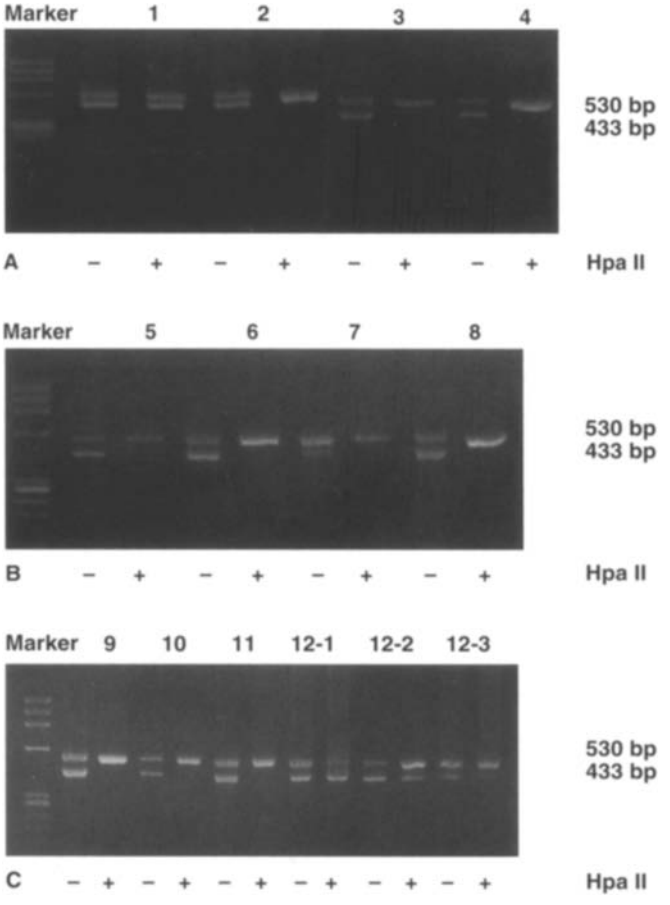


Fig. 2A–C Clonal analysis. Sample DNA was first treated with (+) or without (–) HpaII and then amplified and post-treated with BstXI. **A, B** Results of 8 cases with primary hyperparathyroidism. **C** Results of 4 cases with secondary hyperparathyroidism. Case 1 and two glands (12–1 and 12–2) of case 12 demonstrate polyclonal patterns representing 530-bp and 433-bp bands in the electrophoresis. Another gland (12–3) of case 12 shows a monoclonal pattern representing only a 530-bp band. The remaining cases show monoclonal origins despite the primary diagnosis. The results of normal control tissues in each case are not shown. Marker: PhiX174 RF DNA HaeIII digest

Clonality

The 8 glands with primary hyperparathyroidism (Fig. 2A,B) included 7 that demonstrated monoclonal patterns showing an uncleaved 530-bp band in the agarose gel. One gland with a pathological diagnosis of adenoma showed a polyclonal pattern presenting two bands (530 and 433 bp). Of the 4 cases with secondary hyperparathyroidism (Fig. 2C), only 1 hypercellular gland was assessed in 3 cases and all showed monoclonal patterns. In the remaining case, 3 glands were separately analysed; 1 gland demonstrated a monoclonal pattern and 2 glands were polyclonal.

All the normal control tissues showed polyclonal patterns, and the TT cell line showed a monoclonal pattern. For the males, only one allele of the two was detected. These findings indicate the validity of the technique and results (Fig. 3).

The pathological features were summarized according to the following four indices: (1) number of parathyroid glands involved, (2) whether the hypercellular gland was composed of single or multiple nodules; (3) presence or absence of a rim of normal parathyroid tissue; (4) presence or absence of fat cells within or among the nodules. From the table, we note that the results of clonal analysis were consistent with the diagnosis of adenoma in most instances. In other words, if the hypercellular gland had single gland involvement and was composed of a single nodule, with the presence of a rim of normal parathyroid tissue and an absence of fat cells within the nodule, it was monoclonal in origin. In contrast, the diagnostic indices of secondary hyperplasia were inconsistent with a clonal origin in most cases. Correlative patterns were not obtained between pathological features and clonal origins. Pathological criteria were inadequate for distinguishing monoclonal tumours from polyclonal hyperplasia.

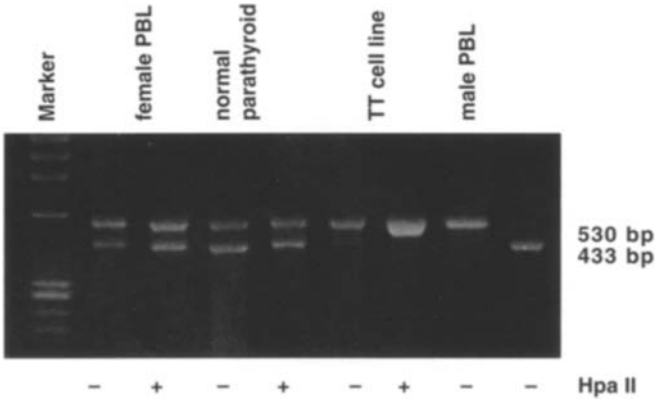


Fig. 3 Normal controls. A polyclonal pattern of PBL (peripheral blood leukocytes) DNA from a heterozygous female and of DNA from a female normal parathyroid. DNA from female TT cell line presents a monoclonal origin. Only one allele (433 bp) is detected in the male PBL DNA

Discussion

Parathyroid diseases develop in various types: hyperplastic/neoplastic and sporadic/familial and hereditary. Distinction among these forms is largely descriptive, and the inconsistency between pathological diagnosis and molecular analysis may reflect our ignorance of their pathogenesis. It is reasonably presumed that clonal expansion occurs against the background of hyperplasia because of mitotic errors in DNA, mutation or deletion. Our results have shown that monoclonal proliferation and polyclonal hyperplasia may coexist in the same patient and monoclonal tumours are common not only in primary but also in secondary hyperparathyroidism. These findings support our supposition. A similar conclusion was reported by Arnold [4] and Noguchi et al. [17], but our data are in conflict with the results of Fialkow and coworkers [8], who showed that 4 parathyroid adenomas were polyclonal, based on the detection of G6PD isoenzymes. In this study, 1 adenoma also showed a polyclonal pattern. This pattern is also consistent with admixture of monoclonal tumour cells and normal stromal tissue. We could not exclude this reason for polyclonality, because this adenoma was found to be

Table 1 Summary of the pathological features and clonalities (*PHP* primary hyperplasia, *SHP* secondary hyperplasia)

Gland no.	Pathological diagnosis	Glands involved	Single or multiple nodules	Rim of normal parathyroid tissue	Fat cells within or among nodules	Clonality
1	Adenoma	Single	Single	Absent	Absent	Polyclonal
2	Adenoma	Single	Single	Present	Absent	Monoclonal
3	Adenoma	Single	Single	Present	Absent	Monoclonal
4	Adenoma	Single	Single	Present	Absent	Monoclonal
5	Adenoma	Single	Single	Present	Absent	Monoclonal
6	Adenoma	Single	Single	Absent	Absent	Monoclonal
7	PHP	Multiple	Multiple	Present	Present	Monoclonal
8	PHP	Multiple	Multiple	Absent	Absent	Monoclonal
9	SHP	Multiple	Multiple	Present	Absent	Monoclonal
10	SHP	Multiple	Multiple	Absent	Present	Monoclonal
11	SHP	Multiple	Multiple	Absent	Absent	Monoclonal
12-1	SHP	Multiple	Multiple	Absent	Absent	Polyclonal
12-2	SHP	Multiple	Multiple	Absent	Present	Polyclonal
12-3	SHP	Multiple	Multiple	Absent	Absent	Monoclonal

rich in blood vessels when the haematoxylin-eosin-stained sections were examined.

In secondary hyperparathyroidism with multiple nodules, several questions have to be considered in the interpretation of clonal results. Do clonal patterns represent the true nature of the hypercellular glands? Monoclonality is so defined that somatic mutation of certain genes occurring in single cells leads to a selective growth advantage for them and their progeny. The only definitive finding in monoclonality is revealed by X-inactivation analyses. For secondary hyperparathyroidism, the possibility that two or more nodules in one gland share the same allelic inactivation cannot be excluded. In this situation, clonal analysis using X-linked genes will not help in differentiating these nodules, whether developing from a single cell or developing separately. In any case, it is certain that a monoclonal pattern indicates the existence of monoclonal components in the hypercellular gland. A polyclonal pattern, however, could be misinterpreted for a variety of reasons [3]. It should be emphasized that it is possible that different nodules have different monoclonal patterns or monoclonal and polyclonal nodules co-existing in one gland. Our work indicates that this may be true, according to the clonal analysis of different nodules in one case (data not shown). To answer these questions, separate analysis of different nodules may be very helpful. For these reasons, the proportion of cases bearing monoclonal tumours could be underestimated.

Our previous studies [14, 18, 20] have shown that there are different growth properties and immunohistochemical reactivity against PTH, PTHrP (parathyroid hormone-related protein) and chromogranin A antibodies in adenomas and in hyperplasia. In this study, we have compared the clonality with pathological features and found that inconsistency was prominent in secondary hyperparathyroidism. Macroscopically, all the 6 glands with secondary hyperparathyroidism were enlarged, with a mean diameter of 1.7 cm, and 4 of the 6 had an asymmetric appearance. Histological examination demonstrated a multinodular arrangement of chief cells; some nodules were composed of oxyphilic cells and a focal "adenomatoid" pattern of growth was seen in some nodules. There were no histological differences between glands with monoclonal and with polyclonal patterns. A finding of monoclonal tumours may indicate a progression from secondary to tertiary hyperparathyroidism in some cases. This may explain why autografted parathyroid tissues in some cases exhibit persistent hyperfunction although they are thought to represent hyperplasia pathologically, and why some parathyroid nodules secrete PTH autonomously without appropriate response to physiological influences. If classical morphological criteria are not reliable in the diagnosis of monoclonal tumours, clonality will provide an important tool assisting in the interpretation and diagnosis of morphologically controversial cases.

In 1994, PCR-based *PGK-1* gene inactivation analysis was reported to make it possible to detect clonality in FFPE tissues [19]. For FFPE tissues, DNA was difficult to amplify in some cases because of the DNA degeneration or the presence of some PCR-inhibiting materials in fixatives. In addition, the lack of normal control tissues in some cases also limits us to the use of archival tissues [1]. The disadvantage of using *PGK-1* gene is the lower rate of heterozygosity (30–40% females). Several reports have shown that the human androgen receptor gene and M27β are highly polymorphic, reaching 90% of females [7, 15]. Analysis using M27β was carried out only by Southern blot hybridization, which requires moderate amounts of relatively intact DNA. It is not possible to analyse small lesions using this procedure. The androgen receptor gene has been reported to be suitable for FFPE tissue, but in our laboratory we could not obtain either specific or adequate PCR products of androgen receptor gene from FFPE tissue.

The preferential methylation in our study may reflect an accidental phenomenon attributable to the relatively small sample size. A similar phenomenon has been reported by Shroyer and Gudlaugsson [19] in five cases of uterine endometrioid adenocarcinoma. Arnold et al. [4] have demonstrated a tumour-specific loss at M27β and other X-chromosome loci in three parathyroid adenomas. They thought that somatic inactivation of an unknown X-linked tumour suppressor gene might contribute to clonal outgrowths in at least some cases of parathyroid hyperplasia.

In summary, we have analysed the clonality of hyperparathyroidism and compared it with pathological features in this report. There is inconsistency between the clonal origins and the pathology in hyperparathyroidism with multigland involvement, which indicates that some of the current views in parathyroid pathology may not be reasonable and may need to be revised on the basis of molecular analysis.

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