Clonal Emergence in Uremic Parathyroid Hyperplasia Is Not Related to MEN1 Gene Abnormality

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It is difficult to differentiate between parathyroid neoplasia and hyperplasia. In an attempt to elucidate the clonality of uremic parathyroid hyperplasia and the molecular genetic abnormalities accounting for clonal emergence, we analyzed 20 cases of uremic parathyroid hyperplasia. Clonality was determined using the X-chromosome-linked human androgen receptor (HUMARA) gene and the phosphoglycerate kinase (PGK) gene, and multiple endocrine neoplasia type 1 (MEN1) gene abnormality was analyzed by studying loss of heterozygosity (LOH) in 11q13 and somatic mutations in the MEN1 gene. As a positive control, a case of MEN1 with Zollinger-Ellison syndrome was analyzed simultaneously. Our analysis revealed that a majority (75%) of the uremic parathyroid hyperplasia tissues, including an autograft with recurrent hyperparathyroidism, was of monoclonal origin. Clonality did not correlate with serum carboxyl-terminal parathyroid hormone (C-PTH) level, calcium level, hemodialytic duration, gland weight or pathological features. Neither LOH in 11q13 nor somatic mutation in the MEN1 gene was detected. For the MEN1 case, a germline mutation (W198X) was detected in exon 3. We concluded that a majority of the uremic parathyroid hyperplasia cases was in fact monoclonal neoplasia. MEN1 gene abnormality played a minor role, if any, in the clonal emergence in uremic parathyroid hyperplasia.

Key words: Parathyroid — Uremic parathyroid hyperplasia — Clonality — MEN1 gene

Uremic parathyroid hyperplasia is characterized by hypersecretion of parathyroid hormone (PTH) and enlargement of more than one gland. Because of these features, uremic parathyroid hyperplasia is traditionally interpreted as reactive hyperplasia of the parenchymal cells.1, 2 Parathyroid tumors may occur in uremic parathyroid hyperplasia but are considered rare. Recently this concept has been challenged by molecular analysis and clinical long-term follow-up. Molecular analysis has demonstrated that some uremic parathyroid hyperplasia specimens are of monoclonal origin and should therefore be interpreted as neoplastic.3, 4 Clinical studies showed that about 3% and 7% of patients receiving a subtotal parathyroidectomy developed recurrent hyperparathyroidism 5 and 7 years later, respectively. A much higher recurrence rate was observed in autografts, with a frequency of 10, 20 and 30% occurring 3, 5 and 7 years, respectively, after total parathyroidectomy and autotransplantation.5 In these cases, pathological observation of the autografts often revealed a distinct nodular proliferation of the parenchymal cells with larger and more irregular nuclei. Occasionally, apparent mitotic activity and nuclear pleomorphism were noted. A striking finding by Klempa et al. was the presence of small nests of parathyroid tissue next to or at some distance from the autografts.6 These observations and evidence are suggestive of a neoplastic nature of uremic parathyroid hyperplasia. However, little is known about the molecular abnormalities accounting for this neoplastic proliferation. In the last year, the multiple endocrine neoplasia type 1 (MEN1) gene was cloned, and abnormalities in the gene were identified in MEN1 and related sporadic endocrine tumors, including parathyroid adenomas.7–9 Previous studies have revealed that some of the gland tissues from uremic parathyroid hyperplasia patients harbor loss of heterozygosity (LOH) in 11q13, the locus of the MEN1 gene. Therefore, a MEN1 gene abnormality may play a role in clonal emergence in uremic parathyroid hyperplasia.10, 11 In an attempt to elucidate whether uremic parathyroid hyperplasia is of monoclonal origin and how often monoclonal proliferation is associated with MEN1 gene abnormality, we examined this issue in uremic parathyroid hyperplasia.

MATERIALS AND METHODS

Patients Twenty unselected and unrelated patients with uremic parathyroid hyperplasia were included in this study. The male to female ratio was 9:11. All patients were on hemodialytic treatment for chronic renal failure. They were operated upon in Nagoya Kyoritsu Hospital because of resistance to medical treatment. Eighteen of the patients underwent total parathyroidectomy and forearm autotransplantation, 1 patient underwent resection of an autograft,

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and 1 patient underwent resection of residual parathyroid tissue. The latter 2 resections were necessary due to recurrent hyperparathyroidism. One had undergone a total parathyroidectomy and forearm autotransplantation 40 months previously and the other a subtotal parathyroidectomy 19 months previously. Serum carboxyl-terminal PTH and calcium levels were markedly elevated in all patients, with average values of 20.78 ng/ml (range 6–28, less than 1.3 normal) and 4.66 mEq/liter (range 2.6–5.63, 4.1–5.0 normal) respectively. The average duration of hemodialysis before operation was 155.63 months (range 24–297) for the former 18 cases, and the mean interval between the 2 operations was 29.5 months (40 and 19, individually) for the 2 recurrent cases. In addition, a case of MEN1 was also included as a positive control for MEN1 gene analysis. This case was a 28-year-old female, showing symptoms of Zollinger-Ellison syndrome clinically, with a family history.

Samples and DNA extraction After surgical removal, each parathyroid gland was weighed and then divided into 2 parts; one was fixed in 10% neutral formalin and embedded in paraffin for pathological examination and one was stored at −80°C for molecular analysis. In this study, only the largest gland in each patient was analyzed. In the event that an abnormal pattern in polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) or LOH analysis had been observed, all glands from this patient would have been subjected to analysis. The average weight of the glands was 1148.13 mg (range 695–2114) for the former 18 cases and 2189.75 mg (2850 and 1529.5, individually) for the 2 recurrent cases. Pathological examination of all glands showed a multinodular growth of chief cells, oxyphilic cells or both. There was no evidence of malignancy in any case, clinically or pathologically. The peripheral blood leukocytes (PBL) from the same patients were used as controls in molecular analysis. Genomic DNA was extracted from tumor tissues and PBL using the QIAamp kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s protocol.

Clonal analysis Clonality of the uremic parathyroid hyperplasia tissues was analyzed using the X-chromosome-linked human androgen receptor (HUMARA) gene and the phosphoglycerate kinase (PGK) gene as previously reported by us.1, 12, 13 Briefly, a portion of the HUMARA gene was amplified using matched pairs of genomic DNA from parathyroid tissue and PBL with and without pretreatment by HpaII methylation-sensitive enzyme in pairs. The PCR products were resolved on a 12% polyacrylamide gel and stained with ethidium bromide. For the analysis using PGK gene, DNA with and without HpaII treatment was first amplified using outer primers and then re-amplified using inner primers. The PCR products were digested with BstXI enzyme and resolved on a 2% agarose gel. All cases were first analyzed at the HUMARA gene to determine heterozygosity and clonality. If the patients were homozygous at the HUMARA gene, the PGK gene was studied to analyze heterozygosity and clonality.

LOH analysis In 16 cases the DNA of the PBL was available for LOH analysis. Three highly polymorphic markers at the MEN1 gene locus were used, including the intragenic marker D11S4946 and the flanking markers D11S4941 and D11S4945, as reported by Manickam et al.14 PCR amplification was performed in a 50 μl reaction mixture for 35 cycles consisting of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C. Six microliters of the PCR products was mixed with the same volume of loading buffer (99% deionized formamide, 20 mM EDTA, 0.05% xylene cyanol FF and 0.05% bromophenol blue), denatured for 5 min at 95°C, and loaded onto a 6% polyacrylamide gel containing 7 M urea and 32% formamide. After electrophoresis, the gel was stained by silver staining using a PlusOne DNA silver staining kit in the GeneStain Automated Gel Stainer according to the manufacturer’s instruc-

![Fig. 1. Clonal analysis. A: The hyperplastic glands obtained from cases 1, 2 and 4 show a monoclonal pattern, and case 3 shows a polyclonal pattern using the HUMARA gene. The results for the peripheral blood leukocytes (PBL) in each case are not shown. B: One case analyzed using the PGK gene demonstrated a monoclonal pattern of the parathyroid gland after treatment with the BstXI polymorphic restriction enzyme versus a polyclonal pattern of the control PBL. Marker: PhiX174 RF DNA HaeIII digest. (−) and (+) indicate the DNA without and with HpaII treatment before PCR amplification.](image-url)
tions (Pharmacia Biotech AB, Uppsala, Sweden). LOH was determined visually based on complete or near complete loss of an allele. All samples were analyzed a minimum of two times.

**MEN1 gene mutation analysis** The MEN1 gene from exons 2 to 10 was amplified for 35–40 cycles as previously described by us. The PCR products were assessed by agarose gel electrophoresis (2% Nusieve GTG and 1% Seakem, FMC, Rockland, ME). Mutations were detected using nonradioactive SSCP analysis. Two microliters of the PCR products was mixed with 4 µl of loading buffer, denatured for 5 min at 95°C, and then plunged immediately into ice. The electrophoretic analysis was carried out at 7°C on a GenePhor Electrophoresis Unit using GeneGel Excel 12.5/24 kit (Pharmacia Biotech AB) under conditions of 200 V and 3 W for 4 h. The gels were stained by silver staining as described above. If an abnormal product migration had been identified, DNA sequencing was performed.

**Statistical analysis** Statistical analysis was carried out on clonality and clinicopathological data by using the t test with Microsoft Excel 5.0 (Microsoft, Tokyo).

**RESULTS**

**Clonality** Fifteen of the 20 cases (75%) were heterozygous at the HUMARA gene, including 14 of the former 18 cases and 1 of the recurrent cases. Eleven hyperplastic glands, including the resected autograft, showed a monoclonal pattern exhibiting only one band in the gel, as the tumor DNA was treated with HpaII enzyme before PCR amplification (Fig. 1A). Four hyperplastic glands showed a polyclonal pattern, exhibiting two bands in the gel. The remaining 5 homozygous cases were further analyzed at the PGK gene locus, and 1 case was heterozygous. This gland was demonstrated to be monoclonal (Fig. 1B). One of the cases with recurrent hyperparathyroidism was homozygous at both the HUMARA gene and the PGK gene. In total, 75% (12/16) of the uremic hyperplastic glands showed a monoclonal pattern.

**Relationship between clonality and clinicopathological information** The determined clonality (polyclonality and monoclonality) was compared with the serum carboxyl-terminal parathyroid hormone (C-PTH) level, calcium level, hemodialytic duration and the gland weight (Table I). No relationship was observed between clonality and the clinical data; also, no relationship was found between clonality and pathological features such as nodularity, presence or absence of fat cells within the glands, and presence or absence of a rim of normal parathyroid tissue. In this study only one of the 2 cases of recurrent hyperparathyroidism was informative, and the autograft in this case was demonstrated to be monoclonal. The interval between the 2 operations was only 3 years, yet the resected autograft (2850 mg) was much larger than the initially resected glands, even the largest one (288 mg).

### Table I. Relationship between Clonality and Clinical Data

| Clonality | No. of cases | Male:female | C-PTH level (ng/ml) | Calcium level (mEq/liter) | Dialysis duration (months) | Gland weight (mg) |
|-----------|--------------|-------------|---------------------|--------------------------|---------------------------|------------------|
| Monoclonal| 11           | 6:5         | 21.94±8.8          | 4.64±0.9                 | 155.63±58.8              | 1291.25±491.5    |
| Polyclonal| 4            | 2:2         | 19.63±8.1          | 4.68±0.2                 | 155.75±118.5             | 1005±246.9       |

The normal value for serum carboxyl-terminal PTH (C-PTH) level is less than 1.3 ng/ml and that for serum calcium level is 4.10–5.00 mEq/liter. None of the differences between monoclonal and polyclonal groups reached statistical significance.

![image](PBL_Tumor_PBL_Tumor.png)

**ig. 2.** Representative LOH analysis using D11S4945 marker. The case shown in lanes 1 and 2 is homozygous and the case shown in lanes 3 and 4 is heterozygous. Both alleles are retained in the tumor of the latter case.

![image](Seq-MEN1.png)

**ig. 3.** Sequencing of MEN1 gene exon 3, showing the heterozygous germline mutation (W198X) detected in the MEN1 gene. Heterozygous base change (G to A) is indicated by an arrow.
Allelic loss and MEN1 gene mutation Twelve of the 16 cases (75%) displayed heterozygosity in the PBL DNA with at least one of the 3 markers (D11S4946, D11S4941 and D11S4945). The lower heterozygosity rate might be related to the ethnic factor. All glands retained heterozygosity (Fig. 2). No glands, including the recurrent parathyroid tissues, displayed LOH at 11q13. Independent of the clonality of the parathyroid tissues, we did not detect any somatic mutation in any exon of the MEN1 gene. For the MEN1 case, a germline mutation (W198X) was detected in exon 3 of the MEN1 gene (Fig. 3), in agreement with another report.17)

DISCUSSION
Clinico-pathological interpretation of uremic parathyroid hyperplasia is still controversial, particularly the differentiation between parathyroid hyperplasia and neoplasia.15, 16) We have applied two X-chromosome-linked genes, HUMARA and PGK, to analyze the clonality of uremic parathyroid hyperplasia, and these allowed us to evaluate most of the isolated glands. Previous studies have revealed that large glands are more prone to be neoplastic.17) Therefore, only the largest gland in each case was analyzed in this study. One of our purposes was to determine the clonality of uremic parathyroid hyperplasia and its correlation with the clinico-pathological characteristics. Our study revealed that a majority of the uremic parathyroid hyperplasia tissues and the resected autograft were monoclonal, consistent with the term “tertiary hyperparathyroidism.” Emergence of clonal proliferation did not correlate with serum C-PTH level, calcium level, dialytic duration or gland weight. Pathological features were not adequate to differentiate monoclonal from polyclonal glands. These results indicate that a progression from generalized hyperplasia to neoplastic proliferation is present in uremic parathyroid hyperplasia. Within a gland undergoing hyperplasia, one or more cells may undergo a somatic mutation that confers a selective growth advantage on the progeny of these cells and forms a monoclonal nodule. The progression did not correlate with the clinico-pathological information.

LITTLE is known about the molecular mechanisms involved in clonal emergence in uremic parathyroid hyperplasia. Some reports have revealed that a small number of uremic hyperplastic glands display LOH at several loci. 3q, the calcium-sensing receptor gene locus, has been demonstrated to possess a relatively high frequency of LOH in uremic parathyroid hyperplasia, but mutational analysis has excluded a role of calcium-sensing receptor gene mutation in tumorigenesis.18–20) 11q13, the MEN1 gene locus, is another spot where LOH has been demonstrated in uremic parathyroid hyperplasia.20, 10) Whether or not MEN1 gene abnormality plays a role in the tumorigenesis of uremic parathyroid hyperplasia has not been studied previously, so unraveling this issue was another purpose of our study. Neither LOH nor somatic mutation in the MEN1 gene was detected. It is possible that abnormalities were present in some tumors but failed to be detected by our method or lay in the regulatory or untranslated regions of the MEN1 gene. For the PCR-SSCP analysis, we employed a GenePhor Electrophoresis Unit with a GeneGel Excel 12.5/24 Kit and Genestain Automated Gel Stainer in this study. It is estimated that about 90% of mutations can be detected by this Unit. We successfully detected the germline mutation in the MEN1 case using this method. Thus, we consider that absence of mutation in uremic parathyroid hyperplasia is not due to methodologic limitations. Moreover, the fact that neither LOH nor mutation was found indicates that MEN1 gene abnormalities do not play an important role in tumorigenesis in uremic parathyroid hyperplasia. The molecular mechanisms involved in the clonal emergence of uremic parathyroid hyperplasia remain to be identified.

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