CLINICAL STUDY

Homozygous inactivation of the *MEN1* gene as a specific somatic event in a case of secondary hyperparathyroidism

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Abstract

Background: Most patients who have been surgically treated for secondary hyperparathyroidism (HPT) harbor at least one pathological parathyroid gland with a tumor of monoclonal origin.

Objective: To elucidate the underlying genetic mechanisms behind secondary HPT, by studying a panel of such tumors for numerical alterations.

Methods: Sixteen parathyroid glands from eight patients (median age 58 years, range 31–74 years), were screened for numerical chromosomal imbalances, using comparative genomic hybridization (CGH). Mutation analysis of the multiple endocrine neoplasia type 1 gene (*MEN1*) was also performed by sequencing of the coding region.

Results: The results show that gross chromosomal alterations occur rarely in secondary HPT. In one of the three glands analyzed from one patient, a complete loss of chromosome 11 was detected. This gland also had an inactivating nonsense mutation, E469X, of the *MEN1* gene. The mutation was present neither in the other two glands, nor in the constitutional tissue of the same patient, thus confirming its somatic origin.

Conclusions: The relative lack of numerical chromosomal alterations would suggest that more discrete genetic alterations are responsible for the monoclonal growth in the majority of cases of secondary HPT. Furthermore, somatic inactivation of the *MEN1* tumor suppressor gene contributes to the tumorigenesis in a small proportion of the cases.

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Introduction

Secondary hyperparathyroidism (HPT) arises in patients with renal failure and chronic phosphate retention in combination with hypocalcemia. A subgroup of patients with secondary HPT develop hypercalcemia and are usually classified as having tertiary HPT or secondary hypercalcemic HPT. The pathogenesis of parathyroid tumor development in secondary HPT involves an early polyclonal hyperplasia of all four glands, which is followed by the outgrowth of individual tumors of monoclonal origin. Consequently, the majority of patients surgically treated for secondary HPT have also been shown, by X-chromosome inactivation analysis, to harbor at least one monoclonal gland. However, histopathology cannot be used to predict whether a certain gland is monoclonal or not. The monoclonal tumor growth in secondary HPT can be anticipated to be caused by a hitherto unidentified genetic mechanism. Altered genes leading to primary HPT also constitute good candidates for the development of monoclonal tumors in secondary HPT.

In primary HPT, comparative genomic hybridization (CGH) and loss of heterozygosity (LOH) studies have identified chromosomal regions putatively involved in the tumorigenesis. Loss of the 11q13 region, which harbors the multiple endocrine neoplasia type 1 gene (*MEN1*) (1–3), is the most frequent aberration found (4–6), and in half of the reported cases a somatic *MEN1* gene mutation could be demonstrated (7–9). Losses of chromosomes 1p, 6q, 9p, 11p, 13q and 15q, and gains of chromosomes 7, 16p and 19p, have frequently been demonstrated in sporadic parathyroid adenomas (5, 6, 10). Furthermore, overexpression of the *PRAD1* oncogene has also been demonstrated in a subset of the tumors. Familial forms of hyperparathyroidism are associated with syndromes such as MEN 1, familial hypocalciuric hypercalcemia (FHH), the HPT–jaw-tumor syndrome (HPT–JT) and familial isolated hyperparathyroidism (FIHP). The genes responsible for MEN 1 (11q13), and FHH (3q13.3–q21) have been identified, whereas FIHP can occur as a genetic variant of MEN 1 or be linked to the HPT–JT region in 1q (2, 3, 11–13).

To date, none of the genetic aberrations characteristic of primary HPT have been linked to the development of secondary HPT. However, inactivation of the *MEN1* tumor suppressor gene has been suggested as one mechanism, and has also been supported by findings of 11q13 LOH in a limited number of cases (14–16), and of a *MEN1* mutation in one case (16). In order to examine the genetic background of tumorigenesis in secondary HPT, we have screened a panel of such tumors for numerical alterations by CGH. Mutation analyses of the *MEN1* gene were also performed.

Patients and methods

Patient population

The study material included 16 parathyroid glands from eight patients (I–VIII, six men and two women, median age 58 years, range 31–74 years), who underwent exploration of the neck for hypercalcemic secondary HPT (Table 1). The median preoperative serum calcium concentration for the patients was 2.79 mmol/l (range 2.22–3.22 mmol/l). Histopathologically, six of the glands were diagnosed as diffuse hyperplasia and seven as nodular hyperplasia; three glands showed a mixed pattern of nodular and diffuse hyperplasia. Results from LOH analyses of chromosome 11 have been published previously for all cases (15, 17). This study was approved by the local ethics committee of the Karolinska Hospital.

DNA analyses

Tumor DNA was isolated from cryopreserved or paraffin-embedded parathyroid tissue samples. The cryopreserved tissue was homogenised (17) and high-molecular-weight DNA was isolated according to the QIAamp DNA extraction kit procedure provided by the manufacturer (Qiagen, Hilden, Germany). For the paraffin-embedded tissues, DNA was extracted from 5- μ m sections as described previously (15). To ensure that the samples used contained a sufficient proportion of tumor cells for CGH analyses (i.e. >70%), histopathological examination of representative sections from all 16 glands were performed. For the frozen tumors, a tissue slice was cut from the middle of the sample used for DNA extraction, embedded in paraffin, sectioned and stained with hematoxylin and eosin. For the paraffin-embedded samples, consecutive cuts were made and the first and last sections stained with hematoxylin and eosin. For patient V, high-molecular-weight constitutional DNA was extracted from peripheral leukocytes and used as a normal matched control.

Comparative genomic hybridization

CGH was carried out as described previously (5, 18, 19). Briefly, tumor DNA samples were labeled with fluorescein isothiocyanate-conjugated (FITC) dUTP (DuPont, Boston, MA, USA) by nick translation, mixed together with Texas Red-labeled normal DNA (Vysis, Downers Grove, IL, USA) and unlabeled Cot-1 DNA (Gibco-BRL, Bethesda, MD, USA) and denatured. The denatured mixture was hybridized onto normal denatured lymphocyte metaphase slides (Vysis) for 72 h at 37 °C. After hybridization, the slides were washed in $0.4 \times$ SSC/0.3% NP-40 at 74 °C for 2 min, and in $2 \times$ SSC/0.1% NP-40 at room temperature for 1 min. Following air drying, the slides were counterstained with DAPI (Vysis). In control experiments, normal DNA from a reference woman was hybridized against normal human metaphase spreads.

Digital image analyses

At least 10 three-color digital images (DAPI, FITC, and Texas Red fluorescence) were captured from each hybridization using a Zeiss Axioplan 2 (Carl Zeiss Jena GmbH, Jena, Germany) epifluorescence microscope and a Sensys (Photometrics, Tucson, AZ, USA) charged-coupled-device camera interfaced to an IPLab Spectrum 10 workstation (Signal Analytics Corporation, Vienna, VA, USA). DNA sequence copy number changes were detected by analyzing the hybridization intensities of tumor and normal DNAs along the length of all chromosomes in each metaphase spread as described previously (19). The absolute fluorescence intensities were normalized and the green-to-red fluorescence ratios <0.8 were considered as losses and ratios >1.2 were scored as gains of chromosomal material. For high-level amplifications, a cut-off value of 1.5 was used. The Y chromosome and heterochromatic regions in the centromeric and paracentromeric parts of some chromosomes and on the short arm of the acrocentric chromosomes were not included in the evaluation. The final results were plotted as a series of green-to-red ratio profiles and corresponding standard deviations.

Sequencing of the *MEN1* gene

The nine coding exons (2–10) of *MEN1* were amplified by PCR using primers derived from the intronic sequences as described elsewhere (2, 12). The DNA (50 ng) was amplified in 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 9.0) 2 mmol/l $MgCl_2$ (PE Applied Biosystems, Stockholm, Sweden), 0.1 mmol/l dTTP, dATP, dGTP and dCTP, 0.1 mmol/l of each M13 primer and 5 U/ μ l Ampli Taq Gold (PE Applied Biosystems) in a final volume of 15 μ l. The thermocycling conditions were: denaturing at 96 °C for 15 min, followed by 30 step cycles at 96 °C for 30 s, 62 °C for 20 s and 72 °C

Table 1 Clinical data and CGH results of the eight patients with secondary HPT in the study.

Patient No.	Age at surgery (years)	Sex (M/F)	Diagnosis	Duration of dialysis (years)	Serum calcium (mmol/l)	Serum PTH (ng/l)	Serum creatinine (μmol/l)	Parathyroid gland		Genetic findings			
								No.	Histopathological diagnosis	Weight (mg)	CGH results	LOH of 11	MEN1 mutation
I	54	M	Renal tuberculosis	12	2.78	70	132	1	Mixed pattern	203	Normal	Normal	—
II	31	M	Nephrotic syndrome	11	2.88	121	120	2	Nodular hyperplasia	432	Normal	Normal	—
								1	Diffuse hyperplasia	428	Normal	Normal	—
III	34	M	Chronic glomerulonephritis	19	2.61	125	200	2	Diffuse hyperplasia	477	Normal	Normal	—
								1	Diffuse hyperplasia	306	Normal	Normal	—
IV	52	F	Chronic glomerulonephritis	8	2.8	99	101	2	Diffuse hyperplasia	218	Normal	Normal	—
								3	Diffuse hyperplasia	193	Normal	Normal	—
V	71	M	Acute renal failure	0	3.22	277	345	1	Mixed pattern	938	Normal	Normal	—
								2	Mixed pattern	173	Normal	Normal	—
VI	74	F	Chronic pyelonephritis	5	3.00	600	462	1	Nodular hyperplasia	703	Normal	Normal	No
								2	Nodular hyperplasia	146	Normal	Normal	No
VII	61	M	Chronic pyelonephritis	<1	2.60	500	994	3	Nodular hyperplasia	647	Loss of 11	LOH of 11	E469X
								1	Nodular hyperplasia	2630	Normal	Normal	—
VIII	74	M	Interstitial nephritis	<1	2.22	1600	400	2	Nodular hyperplasia	184	Normal	Normal	—
								1	Diffuse hyperplasia	2250	Normal	Normal	—
								1	Nodular hyperplasia	1530	Normal	Normal	—

Reference values: Serum calcium: 2.20–2.60 mmol/l; serum PTH: 12–55 ng/l; serum creatinine <110 μ mol/l. Results from the LOH analyses of chromosome 11 have been published previously (15, 17).
—, Not determined.

for 30 s, and a final extension for 2 min at 72 °C. The PCR products were run in a cycle sequencing reaction using the ABI PRISM 377 BigDye Primer cycle sequencing kit (PE Applied Biosystems), with thermocycling conditions as follows: denaturing at 95 °C for 2 min, 15 cycles of 95 °C for 30 s and 70 °C for 1 min and 15 cycles of 95 °C for 30 s and 70 °C for 1 min. Finally, the products were run on an automated sequencer ABI 377 (Applied Biosystems, The Perkin Elmer Corp., Foster City, CA, USA).

Results

The study included 16 tumor samples from eight patients (I–VIII) with secondary HPT who had required surgical treatment. All patients demonstrated secondary HPT due to renal failure as indicated by the requirement for long-term dialysis or by increased serum creatinine concentrations, and which could not be controlled by medical therapy. The clinical information for each case and gland analyzed is given in Table 1. Histopathological examination showed multiglandular hyperplasia in all cases, of nodular type in seven of the glands, of diffuse type in six glands, and with a mixed pattern in three of the 16 glands analyzed.

The results from the CGH analyses are detailed in Table 1 and illustrated in Fig. 1. CGH abnormalities were detected only in gland 3 from patient V (Fig. 1). This gland (weight 647 mg) showed loss of the entire chromosome 11, whereas no abnormalities were detected in either of the other two glands (703 mg and 146 mg respectively) from the same patient. The results from the CGH analyses were confirmed by comparison with previous analyses of LOH for chromosome 11 (Table 1). The findings by the two methods were completely in agreement, with loss of 11 demonstrated only in gland 3 of patient V.

The three glands and the constitutional tissue of patient V were further analyzed with respect to the *MEN1* gene. Sequencing of all coding exons of *MEN1* in gland 3 revealed a base substitution at codon 469 (GAG $\rightarrow$ TAG), leading to the introduction of a premature stop codon (E469X) in exon 10 (Fig. 1). Sequencing of exon 10 in glands 1 and 2 and in the constitutional tissue showed that this nonsense mutation was present only in gland 3, and had thus occurred as a somatic event.

Discussion

The present work demonstrates that the majority of secondary HPT tumors do not harbor gross DNA sequence copy number alterations. The lack of numerical alterations could be due to other smaller 'genetic hits' such as balanced structural aberrations, not affecting the DNA sequence copy number, or aberrations beyond the resolution capacity of CGH.

Figure 1 (A) Results from CGH analyses of chromosome 11 and sequencing of the *MEN1* gene in glands 1–3 from patient V. The CGH profiles of chromosome 11 are shown to the right of the ideograms. In gland no 3, the entire loss of chromosome 11 is demonstrated with a red bar to the left of the ideogram. A nonsense mutation (GAG→TAG) in exon 10 of *MEN1* was detected in gland 3, but not in glands 1 or 2. (B) Demonstration of the wild-type (wt) *MEN1* sequence in the constitutional DNA obtained from peripheral leukocytes of the same patient.

Histopathologically, parathyroid hyperplasia induced by chronic renal failure is usually divided into two types: diffuse hyperplasia and nodular hyperplasia. It is assumed that there is a progression from diffuse to nodular hyperplasia in which each nodule is monoclonal and shows proliferative potential. It is unlikely that the different clones in nodular hyperplasia, which display significant growth advantages, have no

acquired genetic changes. An attractive speculation is that diffuse hyperplasia arises from extrinsic and/or intrinsic stimulation (e.g. phosphate retention, vitamin D deficiency and/or increased setpoint for parathyroid hormone (PTH) secretion) that induces a high cellular proliferation rate, triggering 'genetic hits' that might give clones a growth advantage, resulting in clonal expansion. This would correspond to the model proposed by Parfitt (20) for tumor growth in primary HPT, in which two types of pathological growth of parathyroid cells have been postulated. Calculations based on estimations of the frequency of mitoses and the date of tumor initiation indicate that most of the parathyroid adenomas have ceased to proliferate at the time of surgery. This could be explained by a change in set-point for PTH secretion, which would have given the cell a growth advantage because of continuous stimulation by the relative hypocalcemia. Such a clone of cells will have continued to grow until the secretion of PTH is so great that it increases serum calcium to a concentration that matches the new set-point, and then the growth will be slowed down. The second type of parathyroid growth is postulated to be the result of a mutation in, for instance, a cell-cycle gene. The tumors are then expected to grow exponentially in a similar way as tumors in other tissues. Possible genetic events contributing to this type of growth would include somatic deletions and amplifications of specific chromosomal regions harboring putative tumor suppressor genes and oncogenes.

Alternative genetic mechanisms could relate to the reduced expression of the calcium sensing receptor (CASR), the vitamin D receptor (VDR) and the suggested calcium sensor (CAS) belonging to the low-density lipoprotein receptor superfamily that is observed in the majority of cases of HPT (21–25). The genes CAS (maps to chromosome 2q21–q22) and CASR (maps to chromosome 3q13.1–q21) have previously been demonstrated to show a reduced pattern of expression in sporadic parathyroid adenomas (22, 25). In a similar fashion, CASR and CAS showed reduced expression and LOH in secondary HPT (22–24). A similar situation has been suggested to occur in a newly diagnosed family with a constitutional FHH mutation. In this family, the hypercalcemic disorder was caused by an inactivating point mutation in the cytoplasmic tail of the CASR (26). In addition, a recent study showed reduced VDR mRNA levels in secondary HPT (27), and the VDR gene located in 12cen–12q12 (28) has been suggested as candidate tumor suppressor gene in secondary HPT. The VDR gene has been screened for mutations or allelic losses, but no positive findings were reported (29). Therefore, the identification of the genes involved in the neoplastic transformation of pre-existing polyclonal parathyroid hyperplasia remains an open issue.

However, Tominaga *et al.* (30) indicated that, in parathyroid hyperplasia induced by chronic renal

failure, the parathyroid glands are initially diffuse and polyclonally growing. Later, the clonally transformed nodules proliferate during the development of nodular hyperplasia and may be followed by an adenoma in one or more glands. One clonal lesion of chromosome 11 has been reported to occur in a small minority of secondary HPT (14–16). In 1995, Arnold *et al.* (31) reported that a majority of patients with chronic renal failure and severe refractory HPT harbor at least one monoclonal parathyroid tumor, which also indicates that monoclonal parathyroid neoplasms are common in patients with uremic refractory HPT. These findings led to our LOH study on chromosome 11 in tumors from patients with chronic renal failure (15), because tumor development in secondary HPT has been suggested to involve loss of the *MEN1* locus (14). However, this alteration seems to occur only in a small subset of these tumors (14–16). Therefore, in the present study we have furthered the investigation of our patient material by screening the genome for chromosomal copy number changes, using CGH. Here we were able to detect aberrations in only one tumor, which showed loss of the entire chromosome 11, and a novel somatic nonsense mutation within the *MEN1* gene (not yet identified as a *MEN1* germline mutation) on the other allele (32). Sequencing of the *MEN1* gene, using DNA from blood and the other two hyperplastic glands from the same patient, failed to demonstrate a mutation. The finding of this sporadic nonsense mutation supports the developmental mechanism suggested above.

In conclusion, this study suggests that secondary HPT tumors are chromosomally stable, in the sense that the pathological glands do not demonstrate frequent gross numerical alterations. One disadvantage with the conventional CGH is that the mapping resolution is limited to about 20 Mb (33). CGH microarray, which has recently been introduced, could therefore be a useful tool to overcome this problem and increase the resolution to obtain a more stringent screening. An array-based CGH provides a locus-by-locus measure of DNA copy number variations in order to achieve better mapping resolution. This method would improve the detection of the putative genetic alterations contributing to the development of secondary HPT.

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