EXPERIMENTAL STUDY

Distinction in gene expression profiles demonstrated in parathyroid adenomas by high-density oligoarray technology

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Abstract

Objectives: Somatic deletion of chromosome 11q13 is the most frequent genetic aberration in parathyroid adenoma. To gain further insight into the genetic etiology of parathyroid tumor development, we examined a comprehensive gene expression profile of parathyroid adenomas and normal parathyroid tissues. The results were then evaluated with respect to differences between adenomas and normal parathyroid tissue, and to the presence of loss of heterozygosity (LOH) in chromosomal region 11q13. **Design and methods:** Sporadic parathyroid adenomas and normal parathyroids were hybridized against HG-U95Av2 oligonucleotide arrays (Affymetrix) containing a total of 12 625 probe sets. Quantitative real-time PCR (QRT-PCR) was performed in a larger series of parathyroid adenomas, in order to confirm the microarray results.

Results: *Cyclin D1* and *c-Jun* showed increased expression in adenomas vs normal parathyroids by microarray analysis and QRT-PCR, suggesting an oncogenic role of these genes in parathyroid tumor development. At unsupervised hierarchical clustering, the adenomas fell into two groups: Group I adenomas were characterized by 11q13 LOH, while Group II adenomas lacked this abnormality. In addition, a *t*-test analysis identified largely non-overlapping genes with differential expression in the tumors subgroups; e.g. in Group I tumors the putative oncogene *ENC 1* was found highly over-expressed vs Group II adenomas.

Conclusions: The microarray analyses revealed partly distinctive and partly common expression profiles in parathyroid adenomas with and without 11q13 LOH. In addition, approximately half of the under-expressed genes were mapped to chromosome 11, in agreement with a dose effect following loss of this chromosome.

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Introduction

Primary hyperparathyroidism (PHPT) is the second most common endocrinopathy following diabetes. In the typical form, a benign adenoma overproduces parathyroid hormone (PTH) leading to hypercalcemia and symptoms following therefrom. Parathyroid tumors are also recognized in a variety of less common conditions, in which one or more glands can be involved and the tumors may be benign or malignant. In addition to the more common sporadic cases, various familial forms have also been recognized, such as multiple endocrine neoplasia type 1 (MEN 1), the hyperparathyroidism-jaw tumor syndrome (HPT-JT) and familial isolated hyperparathyroidism (FIHP). To date, a number of parathyroid tumor-related genes have been identified. For the hereditary disorders, these include

germline mutations of the *MEN1* gene in MEN 1 and FIHP, of *CASR* in familial hypocalciuric hypercalcemia variants and of *HRPT2* in HPT-JT and FIHP (1–5).

In sporadic forms of the disease, inactivation of *HRPT2* is preferentially found in the malignant cases while *MEN1* inactivation is mainly found in the benign form (3, 6–8). Furthermore, the *Cyclin D1* gene as well as several genes related to calcium homeostasis have been suggested to be involved in the development of PHPT (9–11). Somatic deletion of chromosome 11 is the most frequent genetic aberration in parathyroid adenoma, and has been demonstrated by both comparative genomic hybridization (CGH) and loss of heterozygosity (LOH) techniques (12–14). This alteration is seen in a third of the tumors, and generally represents a quite specific chromosomal alteration accompanied by inactivating mutation

of the *MEN1* gene in the other allele (6, 15). In addition, losses of chromosomes 1p, 6q, 9p, 11p, 13q and 15q as well as gains of chromosomes 7, 16p and 19p are also demonstrated in sporadic hyperparathyroidism (12, 13, 16).

In the present study, we have characterized the global gene expression profiles in a series of sporadic parathyroid adenomas, in an attempt to obtain an improved picture of the genetic etiology behind parathyroid tumor development.

Materials and methods

Clinical cases

Seventeen parathyroid tumors (T1–T17) were collected from patients treated surgically for PHPT at the Karolinska University Hospital (Table 1). Informed consent was obtained from each patient and the local ethics committee approved the study of the clinical material. All patients had a sporadic form of the disease without a personal or family history of MEN 1, HPT-JT or other familial forms of PHPT. The tumors were classified histopathologically as uniglandular parathyroid adenomas according to WHO criteria (17). Representative sections were analysed to ensure that the samples used for DNA and RNA extractions contained a sufficient proportion of tumor cells for the analysis (i.e. >70%). Tumors T1–T8 were analysed by microarray, LOH and CGH, while tumors T1–T17 were all analysed by real-time PCR. The two biopsies from normal parathyroid glands (N1 and N2) were obtained from patients treated for nodular thyroid lesions when

parathyroid glands had to be excised and reimplanted into the sterno-mastoid muscle for anatomical reasons. Both these patients had normal levels of PTH, serum calcium and thyroid hormones preoperatively. They were both verified as histopathologically normal, and were used as reference samples in the microarray and real-time PCR analysis.

Isolation of DNA and RNA

All tissue samples were collected immediately after surgery and stored at -70°C until use. Total RNA was extracted from all 17 tumors (T1–T17) and the two normal parathyroid biopsies (N1 and N2) using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), followed by purification with an Rneasy mini kit (Qiagen, Hilden, Germany). The RNA purity and quality was confirmed by analysis in an Agilent 2100 Bioanalyser (Agilent Technologies, Inc, Palo Alto, CA, USA). For eight of the adenomas (T1–T8), DNA was isolated in parallel with the RNA during the TRIzol extraction. Peripheral blood leukocytes were used as a source of constitutional DNA in the microsatellite analysis (T1–T8).

LOH analyses

The eight matched blood and tumor DNA samples from T1–T8 were genotyped for three microsatellite markers located at the *MEN1* locus in 11q13: cen-(*D11S4946/MEN1*)-*D11S4938-D11S4937*-tel. For T2 and T3, LOH analyses of markers from the *MEN1* region have been previously published (15). The markers were analysed using fluorescent detection in an ABI 377 laser fluorescent sequencer and the GeneScan 3.1 software (Applied Biosystems, Perkin Elmer Corp., Foster City, CA, USA).

Microarray experiments and data analyses

For eight of the tumors (T1–T8) and the two normal biopsies (N1 and N2), cDNA was synthesized from total RNA using a T-7 linked oligo-dT primer, and then used for cRNA synthesis with biotinylated UTP and CTP. After fragmenting, the labeled RNA was hybridized to HG-U95Av2 oligonucleotide arrays (Affymetrix Inc., Santa Clara, CA, USA) according to the protocol recommended by the supplier. After hybridization, the arrays were washed and stained with streptavidin-phycoerythrin (Molecular Probes, Inc., Eugene, OR, USA) in an Affymetrix fluidics station. The arrays were then scanned in an Affymetrix scanner and the expression values for each probe set (represented by 16–20 selected oligomers) were estimated using the Affymetrix Microarray Suite Software (MAS v5.0). To minimize discrepancies resulting from individual variations regarding sample preparations, hybridization conditions, staining or different oligoarray lots, all probe sets were scaled from each array to a target

Table 1 Clinical data for the 17 cases with sporadic uniglandular parathyroid adenoma in this study.

Case		Age ^a	Sex	S-Ca ^b	S-PTH ^c	Tumor weight (g)
No.	Id.					
T1	3161	55	M	2.77	108	0.85
T2	1879	53	F	2.94	111	1.62
T3	2076	40	F	2.86	79	2.38
T4	3648	59	M	2.65	74	0.30
T5	3046	63	M	2.85	79	4.44
T6	2877	52	F	1.41*	91	1.70
T7	2613	53	F	3.00	105	2.77
T8	3480	25	F	2.66	284	0.65
T9	3575	75	F	1.47*	>100	1.44
T10	3223	53	F	2.75	88	0.46
T11	3355	58	M	2.60	60	1.15
T12	3371	46	F	3.16	176	4.00
T13	3261	47	M	2.84	80	1.33
T14	2896	34	M	2.70	66	0.39
T15	2766	65	F	2.50	140	1.13
T16	2884	82	M	1.50*	172	2.08
T17	3647	83	F	2.75	174	2.30

^aAge at operation. ^bTotal serum calcium (S-Ca; reference range, 2.20–2.60 mmol/l) preoperatively. *S-Ca level originally reported as ionized Ca (reference range, 1.15–1.35 mmol/l). ^cIntact serum PTH (S-PTH; reference range, 17–55 ng/l) preoperatively.

intensity value of 100 (using Affymetrix MAS v5.0), thus allowing interarray comparisons. Scaling factors (range 2.28–5.26) varied within the acceptable range i.e. <threefold. 3'/5' ratios for *GAPDH* and *β -actin* were confirmed to be within acceptable limits (< threefold), and BioB spike controls were found to be present on all chips. In addition, the internal controls BioC, BioD and CreX were present in increasing intensity.

By using the statistical detection algorithm in Affymetrix MAS v5.0, we were able to separate the transcripts of particular probe sets that are reliably detected (present call), from the transcripts below the threshold of detection and therefore considered as absent. From the 12 626 probe sets on the array, control probes and genes scored as marginal or absent (not detected) were excluded, leaving 4436 present probe sets for the subsequent analyses.

Comparison files were created using the batch analysis tool in the MAS v5.0. These included files for each of the parathyroid adenomas (T1–T8), which were then individually compared with each of the normal parathyroids (N1 and N2), thus yielding 16 comparison files. To identify differentially expressed genes between parathyroid adenomas and normal parathyroids, two different algorithms from the MAS v5.0 tool were applied. The change algorithm reported the probe sets to be unchanged, increased, decreased or marginal. The signal log ratio algorithm instead calculated an estimated log value for each probe set in each sample. The algorithms are described at http://www.affymetrix.com/support/technical/technotes/statistical_reference_guide.pdf. For both algorithms the signal values were imported into the GeneSpring 5.0 software tool (Silicon Genetics, Redwood City, CA, USA) to sort out genes with increased or decreased expression. Unsupervised hierarchical clustering analysis of the 4436 present probe sets was performed using GeneSpring 5.0 software with the minimum distance set to 0.001 and the separation ratio set to 0.95. The comparisons of mean expression levels were compared in the two groups of tumors identified by clustering, i.e. T1–T4 with 11q13 LOH (Group I) and T5–T8 without 11q13 LOH (Group II). The comparisons were carried out using the expression values estimated by dChip analysis (www.dchip.org) (18). This was done to obtain more sensitive estimates of the low-abundance genes than were available through MAS v5.0. To avoid effects related to the wide variation at the upper end of the distribution of values, the logarithmic values were used. *t*-statistics were computed for each gene between the two groups of tumors identified by clustering. The false positive rate was estimated at several different thresholds for the *t*-score, by permutating the sample labels and re-computing all gene-wise *t*-scores. The *t*-score threshold was set so that the estimated number of false positives was half the number of genes with *t*-scores exceeding that threshold (i.e. the 'false discovery rate' was one half).

CGH

CGH was carried out on tumors T1–T8 using methods previously described (13). For T2 and T3 the results have been previously published (13). A minimum of ten three-color digital images (DAPI, FITC and Spectrum Red fluorescence) were captured from each hybridization using an Axioplan 2 epifluorescence microscope (Carl Zeiss Jena GmbH, Jena, Germany) equipped with a Sensys charged-coupled-device camera (Photometrics, Tucson, AZ, USA) and analysed with the isis/CGH software (Metasystem, Altlußheim, Germany). Relative DNA sequence copy number changes were detected along the length of all chromosomes in each metaphase spread. Green to red ratios <0.8 were scored as losses, ratios >1.2 as gains and ratios >2.0 as high-level amplifications. Heterochromatic regions in the centromeric and paracentromeric parts of some chromosomes, the Y chromosome, the short arm of the acrocentric chromosomes as well as the region next to the telomeres were not included in the evaluation. All tumor samples were hybridized and analysed on two different occasions, with concordant results.

Quantitative real-time PCR (QRT-PCR)

Total RNA was isolated from T1–T17 and N1 and N2 as described above. To avoid the presence of genomic DNA all samples were treated with DNAase after RNA extraction. Ten micrograms of RNA were reverse transcribed into cDNA using the TaqMan reverse transcription Kit (Applied Biosystems). Reverse transcription was performed in a thermal cycler at 25 °C for 10 min and 37 °C for 120 min. For five of the six genes analysed by TaqMan real-time PCR, Assays-on-Demand kits (Applied Biosystems) were available including *Cyclin D1* (assay identification (ID) is Hs00233498_m1), *c-Jun* (Hs00277190_s1), *β -actin* (Hs99999903_m1), *TBP* (Hs99999910_m1) and *18S rRNA* (Hs99999901_s1). For each single sample, the master-mix included: 12.5 μ l TaqMan 2 $\times$ Universal Master-Mix (Applied Biosystems), 8.25 μ l RNase-free water and 1.25 μ l 20 $\times$ Assays-on-Demand gene expression assay mix. For the *36B4* gene (public ID M17885) no Assays-on-Demand are available, while the following previously published primers and probe were used (19): forward primer: 5'-AGATGCAGCAGATCCGCAT-3'; reverse primer: 5'-ATATGAGG AGCAGTTTCTCCAG-3'. The corresponding TaqMan probe 5'-AGGCTGTGGTGC-TGATGGGCAAGAAC-3' was purchased from MedProbe (catalogue number OL-0361-FAMDQ02). For *36B4*, the TaqMan PCR Master-Mix was constituted of 12.5 μ l TaqMan 2 $\times$ Universal Master-Mix, 7 μ l RNase free water, 1.5 μ l primers (10 μ M) and 1 μ l *36B4* probemix (5 μ M); per sample to be analysed. For each sample and gene, 22 μ l aliquots of the MasterMix and 3 μ l (~15 ng) cDNA were transferred in duplicates to

adjacent wells of a MicroAmp Optical 96-well reaction plate (Applied Biosystems) and covered with Optical Caps (Applied Biosystems). The plate was then placed in the ABI Prism 7700 Sequence Detector (Applied Biosystems) and the run commenced. The conditions for every TaqMan PCR reaction were: 2 min at 50°C (stage 1), 10 min at 95°C (stage 2) and 40 cycles of 15 s at 95°C followed by 60 s at 60°C (stage 3). When the run was completed, output data were analysed using the SDS software and exported into a Microsoft Excel file for analysis. The 36B4 housekeeping gene was used as an endogenous control to normalize the expression level of target genes. Standard curves for 36B4 and target genes were constructed using 1:10 serial dilutions of control cDNA prepared from the human neuroblastoma cell-line SK-NAS.

In the fold-change analysis, the RNA level was standardized by correcting for the 36B4 level in the same sample. For all experimental samples (T1–T17), the quantity was determined from the standard curve and divided by the quantity of the mean normal quantity (N1/N2). Thus, the normal quantity becomes the 1 × sample, and all other quantities are expressed as a relative *n*-fold difference.

Results

Expression profiling was performed on eight parathyroid adenomas (T1–T8) and two normal parathyroid tissues (N1 and N2) by hybridization to high-density oligonucleotide arrays. Some of the array results were then verified by QRT-PCR in an extended series of 17 sporadic parathyroid adenomas (T1–T17). The clinical details of the 17 cases with PHPT are listed in Table 1. The findings and expression analyses are illustrated in Fig. 1 and Tables 2–5.

Comparison of parathyroid adenomas and normal parathyroid

A total of 4436 of the 12 625 probe sets represented on the array gave a present call in at least eight of the ten arrays. This set of present genes was considered as informative and used in all comparisons in this study. However, for the analysis of the genes with decreased expression in the adenomas, the 4533 genes detected as present in both normal parathyroids were included in the analysis.

Differentially expressed genes between parathyroid adenomas (T1–T8) and normal parathyroids (N1 and N2) were identified by pair-wise comparison using change and signal log ratio algorithms in the Affymetrix MAS v5.0. The computing results from both analyses were then imported into the GeneSpring software tool, in order to identify probe sets with increased or decreased expression. A total of 224 probe sets showed increased expression in the

adenomas vs normal parathyroids using at least one of the algorithms. The 35 probe sets (representing 31 genes) that were independently identified with increased expression in the adenomas using both algorithms are listed in Table 2. Several of the over-expressed genes are putative oncogenes involved in cell cycle control or transcriptional regulation, e.g. the *Cyclin D1* gene and the *c-Jun* proto-oncogene. Similarly, 361 probe sets were detected with decreased expression in the adenomas by at least one algorithm. Eighty of these (representing 75 genes) were identified as decreased by both algorithms (Table 4). Noteworthy, *CACNB2* and *CXCL12* involved in calcium transport and homeostasis were present among these genes with decreased expression.

Expression of parathyroid-related genes

In addition, we investigated the microarray expression of genes selected because of their demonstrated or implicated relation to parathyroid functions (Table 3). The calcium-sensing receptor gene (*CASR*) showed a distinct pattern of decreased expression in all eight adenomas. The mean expression level was 0.61 (range 0.43–0.80) as compared with the mean expression of the two normal parathyroids (1.00). Similarly, a reduced expression was noted for the vitamin D receptor gene (*VDR*), with a mean reduction from 1.0 in the normal parathyroids to 0.47 (range 0.20–0.64) in the eight adenomas.

Different expression profiles in parathyroid adenomas with and without 11q13 LOH

Similarities in the global gene expression patterns of normal and pathological parathyroids were studied by creating a hierarchical tree. The unsupervised hierarchical clustering analysis of the present probe sets (4436) revealed that the eight tumor samples fell into two distinct cluster groups (Fig. 1A). Group I was found to consist of the four tumors with 11q13 LOH (T1–T4) and Group II of the four tumors without 11q13 LOH (T5–T8). The expression profiles between the tumors in the two groups were then compared by direct *t*-test comparisons of the means between the two groups. The *t*-tests revealed 85 genes that showed significantly ($P < 0.0032$) differential mean expression between the two groups (Table 5). The results obtained from the two independent approaches (MAS v5.0: Fig. 1 and dChip: Table 5) support the distinction in expression between Group I and Group II adenomas.

As is evident from Table 5, approximately 50% of the genes with decreased expression in Group I vs Group II were located on chromosome 11 within and outside the q13 region (Fig. 1B). This indicated that the lower expression of some genes in Group I could be a result of low DNA dose, i.e. a reduction in DNA copies. In

Figure 1 (A) Unsupervised hierarchical clustering of the eight parathyroid adenomas (T1–T8) and two normal parathyroids (N1 and N2) analyzed by microarray. All probe sets that were present in at least eight of the ten arrays, i.e. 4436, were used to create the hierarchical tree. The dendrogram was based on MAS v5.0 estimates. The hierarchical tree revealed two distinct cluster subgroups, which were found to consist of the four tumors with 11q13 LOH (Group I) and the four with no 11q13 LOH (Group II). All eight adenomas were distinctly separated from the two normal parathyroids (N1 and N2). The highlighted genes showed significantly altered expression in Group I vs Group II adenomas based on dChip estimates (Table 5). Red indicates relatively higher and green indicates relatively lower gene expression. (B) Chromosomal location of the 85 genes showing significantly ($P < 0.0032$) differential mean expression between adenoma Groups I and II. The t -tests were performed by comparing the mean expression levels between Group I and Group II using the dChip software. Genes detected with increased expression in Group I compared with Group II are shown by green circles to the right of the chromosomes, whereas those with decreased expression are shown by red circles to the left. (C and D) QRT-PCR analysis of the RNA expression levels of (C) *Cyclin D1* and (D) *c-Jun* in normal parathyroid (Norm) and parathyroid adenomas (T1–T17). The results presented represent the mean of duplicate PCR assays and after normalization against the housekeeping gene *36B4*. Norm shows the average expression level from the two normal parathyroids analysed (N1 and N2). The numbers above the bars are relative quantities.

addition, no gene located on chromosome 11 was detected with increased expression when comparing the adenomas in Group I vs Group II. In order to detect the chromosome 11 status in the adenomas used for array analyses we complemented the LOH analysis with CGH. Of the eight adenomas hybridized onto microrarrays, tumors T1–T4 were by LOH performance identified with a deletion in 11q13 while T5–T8 had retention of the same chromosomal region. CGH results confirmed that the entire chromosome 11 was lost in three (T1–T3) of the four tumors in Group I. In T4, no loss of 11 was detected by CGH, suggesting that tumor T4 carries a balanced alteration or a small deletion of chromosome 11q13 beyond the resolution of CGH. The four adenomas in Group II (T5–T8) all showed retention of chromosome 11 by CGH. In addition, losses were detected in 1p32-ter (T4), 6cen-q22 (T4), 15q (T3, T8), 21q (T3, T8) and 22q (T8),

while trisomies were observed for chromosomes 6, 8, 14 and 16 (T7).

QRT-PCR analyses of selected genes

Two genes (*Cyclin D1* and *c-Jun*), detected with increased expression in tumors vs normal parathyroid by the array analysis, were selected for QRT-PCR on a larger series of sporadic parathyroid tumors, including 17 adenomas T1–T17 (Table 1) and two normal parathyroid biopsies (N1 and N2). In these analyses, the quantity of the tumor cDNA was determined from the standard curve and divided by the quantity of the normal parathyroid. The *36B4* housekeeping gene was used as an endogenous control to normalize the expression level of target genes. *Cyclin D1* thus showed more than twofold increased expression in 4/17 (24%) of the adenomas compared with the average expression of the two

Table 2 Genes with increased expression in parathyroid adenomas (T1–T8) vs normal parathyroids (N1 and N2).

Accession number	Gene symbol	Chromosomal location	GO biological process description ^a	Probe set ID
J04111	<i>c-JUN</i>	1p32–p31	Regulation of transcription	32583_at
J04111	<i>c-JUN</i>	1p32–p31	Regulation of transcription	1895_at
AF065388	<i>TSPAN-1</i>	1p34.1	Cell proliferation and adhesion	34775_at
M97815	<i>CRABP-II</i>	1q21.3	Regulation of transcription	1057_at
X57985	<i>HIST2H2BE</i>	1q21–q23	Regulation of transcription	33352_at
M82809	<i>ANXA4</i>	2p13		37374_at
U17077	<i>BENE</i>	2q13		33331_at
X05323	<i>MOX2</i>	3q12–q13		37716_at
D16815	<i>NRID2</i>	3p24.1	Regulation of transcription	35705_at
D16815	<i>NRID2</i>	3p24.1	Regulation of transcription	35706_at
AF049884	<i>ARGBP2</i>	4q35.1		39295_s_at
M31661	<i>PRLR</i>	5p14–p13	Embryo implantation	1078_at
X68742	<i>ITGA1</i>	5q11.1	Cell–matrix adhesion	120_at
X70683	<i>SOX4</i>	6p22.3	Regulation of transcription	33131_at
X78947	<i>CTGF</i>	6q23.1	Regulation of cell growth	36638_at
AL033377		6q23.1–q24.3	Neuropeptide signalling	36014_at
M15518	<i>PLAT</i>	8p12	Protein modification	33452_at
D84111	<i>RBPMS</i>	8p12–p11	RNA processing	34162_at
D84111	<i>RBPMS</i>	8p12–p11	RNA processing	34163_g_at
U90878	<i>PDLIM1</i>	10q22–q26.3	Intracellular signalling	36937_a_at
X76488	<i>LIPA</i>	10q23.2–q23.3	Lipid catabolism	38745_at
M64349	<i>CCND1</i>	11q13	Regulation of cell cycle	2017_s_at
W28869	<i>TEGT</i>	12q12–q13	Apoptosis	33989_f_at
U82256	<i>ARG2</i>	14q24.1–q24.3	Urea cycle catabolism	32722_at
D31885	<i>ARL6IP</i>	16p11.2–p12		36572_r_at
Y00503	<i>KRT19</i>	17q21		40899_at
AF002020	<i>NPC1</i>	18q11–q12	Protein transport	37047_at
AF002020	<i>NPC1</i>	18q11–q12	Protein transport	980_at
D00096	<i>TTR</i>	18q12.1	Thyroid transport	32252_at
A1263885	<i>WSX1</i>	19p13.11	Immune response	37844_at
U87947	<i>EMP3</i>	19q13.3	Regulation of cell growth	39182_at
M68840	<i>MAOA</i>	Xp11.4–p11.3	Neurotransmitter catabolism	41772_at
M68840	<i>MAOA</i>	Xp11.4–p11.3	Neurotransmitter catabolism	41771_g_at
AL049250		16p12.2		40951_at
AF052107	LOC129642	2p25.1		38262_at

All genes listed were, in pairwise comparison, identified with increased expression using two algorithms in MAS v5.0. ^aGene ontology description according to the NetAffxTM analysis center: <http://www.affymetrix.com/analysis/index.affx>

Table 3 Relative expression levels of selected genes with parathyroid-related functions in adenomas as compared with normal parathyroid.

Case number	CASR (3q21–q24)	PTH (11p15)	VDR (12q12–q14)
Group I: With 11q13 LOH			
T1	0.48	0.51	0.64
T2	0.46	0.35	0.51
T3	0.60	0.70	0.47
T4	0.72	0.70	0.49
Group II: no 11q13 LOH			
T5	0.80	1.76	0.54
T6	0.43	1.10	0.50
T7	0.76	0.94	0.38
T8	0.66	0.84	0.20
Normal parathyroids			
Average of N1 and N2	1.00	1.00	1.00
Range of N1/N2	1.00/1.00	0.95/1.05	1.21/0.79

CASR = calcium sensing receptor. Probe set ID 38958_at/accession number U20760.
PTH = parathyroid hormone: 708_at/J00301. VDR = vitamin D receptor: 1388_g_at/J03258.

normal parathyroid samples (Fig. 1C). Similarly, *c-Jun* showed more than a twofold increased expression in 6/17 (35%) of the adenomas (Fig. 1D).

Discussion

In approaching the molecular genetic pathways of parathyroid tumor development we have characterized global expression profiles in parathyroid adenomas and normal parathyroid tissues, using the oligonucleotide microarray technology. Although the number of samples studied was limited, several significant results were obtained. We could identify genes that were differentially expressed in tumors vs normal tissue. We also investigated the expression profiles of selected genes, previously implicated in parathyroid-related functions, and observed altered expression profiles for some of them. In addition, we detected different expression patterns in parathyroid adenoma subgroups.

Table 4 Genes with decreased expression in parathyroid adenomas (T1–T8) vs normal parathyroid (N1 and N2).

Accession number	Gene symbol	Chromosomal location	GO biological process description ^a	Probe set ID
X08020	<i>GSTM1</i>	1p13.3		39054_at
U20938	<i>DPYD</i>	1p22		38220_at
AL049798	<i>DPT</i>	1q12–q23	Cell adhesion	38057_at
Z97171	<i>MYOC</i>	1q23–q24	Vision morphogenesis	40403_at
M25280	<i>SELL</i>	1q23–q25	Cell motility	245_at
AB007972	<i>PP1R12B</i>	1q32.1	Signal transduction	41137_at
M69199	<i>G0S2</i>	1q32.2–q41	Regulation of cell cycle	38326_at
M81883	<i>GAD1</i>	2q31	Glutamate decarboxylation	37183_at
X51408	<i>CHN1</i>	2q31–q32.1	Intracellular signalling	40512_at
AJ001014	<i>RAMP1</i>	2q36–q37.1	Intracellular signalling	35668_at
S95936	<i>TF</i>	3q21	Ion transport	32538_at
D64142	<i>H1FX</i>	3q21.3	Regulation of transcription	318_at
AF035296	<i>DC12</i>	3q21.3		38989_at
J02611	<i>APOD</i>	3q26.2–qter	Lipid metabolism	36681_at
D45371	<i>APM1</i>	3q27	Energy pathways	40658_r_at
U51712	<i>HOP</i>	4q11–q12		39698_at
M12963	<i>ADH1A</i>	4q21–q23	Alcohol metabolism	34637_f_at
X03350	<i>ADH1B</i>	4q21–q23	Alcohol metabolism	35730_at
AL049963	<i>SLC39A8</i>	4q22–q24	Ion transport	40456_at
D45299	<i>BASP1</i>	5p15.1–p14	Regulation of transcription	32607_at
AB005060	<i>NRG2</i>	5q23–q33	Regulation of transcription	35089_at
X68277	<i>DUSP1</i>	5q34	Amino acid dephosphorylation	1005_at
AW015732	<i>AIM1</i>	6q21	Cell adhesion	32112_s_at
X56777	<i>ZP3</i>	7q11.23	Cell adhesion	39719_at
AA128249	<i>FABP4</i>	8q21	Transport	38430_at
N74607	<i>QP3</i>	9p13	Transport	39248_at
X92493	<i>PIP5K1B</i>	9q13		37253_at
Y10812	<i>FBP2</i>	9q22.3	Carbohydrate metabolism	40293_at
S60415	<i>CACNB2</i>	10p12	Calcium ion transport	39646_at
L36033	<i>CXCL12</i>	10q11.1	Calcium ion homeostasis	32666_at
L36033	<i>CXCL12</i>	10q11.1	Calcium ion homeostasis	33834_at
AI381790	<i>APM2</i>	10q23.31		32527_at
X00129	<i>RBP4</i>	10q23–q24		32552_at
D42073	<i>RCN1</i>	11p13		40556_at
X76732	<i>NUCB2</i>	11p15.1–p14		35643_at
X57351	<i>IFITM2</i>	11p15.5	Immune response	411_i_at
M55409	<i>EEF1G</i>	11q12.3		1676_s_at
X92814	<i>HRASLS3</i>	11q13.1		35704_at
AA158243	<i>FKBP2</i>	11q13.1–q13.3	Protein folding	33409_at
S72370	<i>PC</i>	11q13.4–q13.5	Gluconeogenesis	38000_at
AF060568	<i>ZNF145</i>	11q23.1		39681_at
AB017563	<i>IGSF4</i>	11q23.2		35829_at
AB017563	<i>IGSF4</i>	11q23.2		37929_at
U60060	<i>FEZ1</i>	11q24.2	Cell adhesion	37743_at
J03802	<i>PTHLH</i>	12p12.1–p11.2	Cell–cell signalling	37989_at
J03802	<i>PTHLH</i>	12p12.1–p11.2	Cell–cell signalling	615_s_at
J04080	<i>C1S</i>	12p13	Proteolysis	40496_at
M12807	<i>CD4</i>	12pter–p12	Immune response	35517_at
X05409	<i>ALDH2</i>	12q24.2	Alcohol metabolism	32747_at
AB018273	<i>SACS</i>	13q12		32102_at
L26336	<i>HSPA2</i>	14q24.1	Spermatid development	36925_at
L26336	<i>HSPA2</i>	14q24.1	Spermatid development	645_at
V01512	<i>FOS</i>	14q24.3		1915_s_at
V1512	<i>FOS</i>	14q24.3		1916_s_at
AF0933118	<i>FBLN5</i>	14q32.1	Cell–matrix adhesion	39038_at
AF047348	<i>APBA2</i>	15q11–q12	Neurogenesis	39590_at
U90942	<i>MYO5A</i>	15q21		40571_at
AB005293	<i>PLIN</i>	15q26	Lipid metabolism	37122_at
AL109695	<i>SLC03A1</i>	15q26	Ion transport	37451_at
U07919	<i>ALDH1A3</i>	15q26.3	Alcohol metabolism	36686_at
AB002351	<i>DMN</i>	15q26.3		39544_at
AC004381	<i>LOC81691</i>	16p13.11		41149_at
K01383	<i>MT1G</i>	1p35.1		31623_f_at
AA224832	<i>MT1X</i>	16q13	Metal ion response	39120_at
D21254	<i>CDH11</i>	16q22.1		2087_s_at

Table 4 continued on following page

Table 4 Continued.

Accession number	Gene symbol	Chromosomal location	GO biological process description ^a	Probe set ID
U39447	<i>AOC3</i>	17q21	Immune response	33756_at
M34064	<i>CDH2</i>	18q11.2	Cell adhesion	2053_at
U56102	<i>CD226</i>	18q22.3	Cell adhesion	33575_at
M84526	<i>DF</i>	19p13.3	Proteolysis	40282_s_at
M92843	<i>ZFP36</i>	19q13.1	mRNA catabolism	40448_at
X73113	<i>MYBPC2</i>	19q13.33	Muscle contraction regulation	37941_at
J02854	<i>MYL9</i>	20q11.23	Muscle contraction regulation	39145_at
AA181053	<i>PARVB</i>	22q13.2–q13.33		37965_at
M69177	<i>MAOB</i>	Xp11.4–p11.3	Electron transport	37628_at
L27560	<i>IGFBP5</i>	2q35		1396_at
L27560	<i>IGFBP5</i>	2q35		38650_at
AF007153	<i>FLJ44318</i>	2q34		37273_at
HG3477-HT3670				1146_at
U05861		10p15.1		32805_at
H15814				40657_r_at

All genes listed were, in pairwise comparison, identified with decreased expression using two algorithms in MAS v5.0. ^aGene ontology description according to the NetAffxTM analysis center: <http://www.affymetrix.com/analysis/index.affx>

Comparison of parathyroid adenomas and normal parathyroid

The result of over-expressed genes in adenomas vs normal parathyroid identifies several putative oncogenes implicated in cell growth and transcriptional regulation, such as the *Cyclin D1* gene and the *c-Jun* proto-oncogene (Table 2). *Cyclin D1* over-expression is in agreement with the protein over-expression previously detected by immunohistochemistry in parathyroid adenomas (20, 21). A pericentric inversion with breakpoints at 11q13 and 11p15 is suggested to bring the *Cyclin D1* oncogene under the influence of the 5'-regulatory sequences of the *PTH* gene (9), which results in dramatic over-expression of *Cyclin D1*. While this rearrangement has been described in a few tumors only, Cyclin D1 protein over-expression has been reported in 18–40% of adenomas by immunohistochemistry studies. Cyclin D1 promotes the G1–S-phase transition, probably by activating a cyclin-dependent kinase, but may also interact with other proteins and influence other pathways related to cell proliferation (22). In our study, over-expression of *Cyclin D1* and *c-Jun* in the adenomas vs normal parathyroid tissue was confirmed by real-time PCR, which represents an accurate method for mRNA expression quantification. In the extended series of 17 adenomas, *Cyclin D1* was over-expressed (> twofold) in 24% of the cases, which is in good agreement with the reported immunohistochemistry results (20, 21). Notably, in the four adenomas with *Cyclin D1* over-expression, the level was largely increased compared with the normal parathyroid (4- to 36-fold), suggesting its relevance for tumor development.

The *c-Jun* gene, located in chromosomal region 1p32, has previously been shown to induce oncogenic transformation, probably due to several mechanisms: *c-Jun* is upregulated in many carcinomas and one suggested function (e.g. in primary hepatocytes and in liver tumors) is to antagonize the proapoptotic activity of

p53 (23). Furthermore, *c-Jun* is a member of the same family of Jun transcription factors as JunD (24). Menin, encoded by the *MEN1* gene in 11q13, interacts with the AP-1 transcription factor leading to a repression of the JunD-mediated transcription (25). Interestingly, menin was recently shown to uncouple JunD and *c-Jun* phosphorylation from MAP kinase activation (26). Given the role of *MEN1* in parathyroid tumor development, it is tempting to speculate that *c-Jun* could also play a role in this tumor type. In our array analysis, over-expression of *c-Jun* was confirmed by two independent probe sets (Table 2). Furthermore, real-time PCR showed a > twofold increased expression in 6/17 of the adenomas (35%) as compared with normal parathyroid.

QRT-PCR analyses was performed on two of the most interesting over-expressed genes and correlated well with the array results, thus supporting the accuracy of the array methodology applied. In the real-time PCR, the normalization to a housekeeping gene is currently the most acceptable method to correct for minor variations due to differences in RNA amounts or in efficiencies of reverse transcription. An ideal housekeeping gene should be expressed at an approximately constant level among different tissues and between, for example, tumor and normal samples. We found that the *36B4* housekeeping gene (coding for the acidic ribosomal phosphoprotein) is suitable for studies of normal and adenomatous parathyroid tissues.

Expression of parathyroid-related genes

In the array analyses, Group I adenomas showed lower levels of *PTH* expression as compared with Group II adenomas. Since the *PTH* gene is located in 11p15, the lower expression might be explained by a reduced dose effect following loss of a chromosome 11 homologue in Group I adenomas. Two receptors of main

Table 5 Genes showing significantly differential mean expression levels in parathyroid adenomas with 11q13 LOH (Group I) vs adenomas without 11q13 LOH (Group II).

Accession number	Gene symbol	Chromosomal location	GO biological process description ^a	Relative expression (Group I/Group II)	P
High expression in adenoma: Group I					
AL049654	<i>PRKCABP</i>	22q13.1	Amino acid phosphorylation	1.2/1.0	0.0001
U77948	<i>GTF2I</i>	7q11.23	Regulation of transcription	1.4/1.0	0.0004
U07231	<i>GRSF1</i>	4q13		1.3/1.0	0.0005
AF059611	<i>ENC1</i>	5q12–q13.3	Neurogenesis	19.6/1.0	0.0005
AL050166	<i>CYLD</i>	16q12.1		1.9/1.0	0.0006
D86981	<i>APPBP2</i>	17q21–q23	Intracellular protein transport	1.4/1.0	0.0006
AL031714	<i>UBE21</i>	16p13.3	Protein modification	2.3/1.0	0.0007
U23803	<i>HNRPA0</i>	5q31	mRNA processing	1.7/1.0	0.0007
AB015344	<i>UBQLN2</i>	Xp11.23		1.7/1.0	0.0007
AL021786	<i>ITM2A</i>	Xp13.3–Xq21.2		2.2/1.0	0.0008
X01630	<i>ASS</i>	9q34.1		1.4/1.0	0.0009
Z283840	<i>NHP2L1</i>	22q13.2–q13.31	Regulation of cell cycle	1.5/1.0	0.0010
D87075	<i>SLC23A1</i>	20p13	Sodium ion transport	1.5/1.0	0.0011
AL080097	<i>C3orf4</i>	3p11–q11		1.3/1.0	0.0012
X96924	<i>SLC25A1</i>	22q11.21	Mitochondrial transport	1.3/1.0	0.0012
S67247	<i>MYH10</i>	17p13.1	Cytokinesis	1.6/1.0	0.0012
AL049981	<i>WBP2</i>	17q25	Signal transduction	1.4/1.0	0.0013
M69013	<i>GNA11</i>	19p13.3		1.7/1.0	0.0014
AF072810	<i>BAZ1B</i>	7q11.23	Regulation of transcription	1.6/1.0	0.0015
D16481	<i>HADHB</i>	2p23	Fatty acid metabolism	2.0/1.0	0.0017
AL041663	<i>MDC1</i>	6pter–p21.31		1.9/1.0	0.0017
AB002347	<i>C6orf133</i>	6p21.1	Ubiquitin cycle transport	1.3/1.0	0.0017
AL080113	<i>KDELR3</i>	22q13.1	Intracellular prot transport	1.6/1.0	0.0018
D21851	<i>LARS2</i>	3p21.3	Leucyl-tRNA aminoacylation	1.4/1.0	0.0018
AB018264	<i>TSPYL4</i>	6q22.31	Nucleosome assembly	1.6/1.0	0.0018
AB017019	<i>HNRPDL</i>	4q13–q21	RNA processing	1.3/1.0	0.0020
U91641	<i>SIAT8E</i>	18q12.3–q21.1	Amino acid glycosylation	16.0/1.0	0.0020
D67029	<i>SEC14L1</i>	17q25.1	Transport	1.7/1.0	0.0020
AF002697	<i>BNIP3</i>	14q11.2–q12	Anti-apoptosis	2.0/1.0	0.0021
AJ243310	<i>AHSA1</i>	14q23.3–31		1.3/1.0	0.0022
U66309	<i>RXRA</i>	9q34.3	Signal transduction	1.6/1.0	0.0023
D50929	<i>EIF3S10</i>	10q26	Regulation of translation	1.4/1.0	0.0024
M26683	<i>CCL2</i>	17q11.2–q21.1	Amino acid phosphorylation etc.	1.9/1.0	0.0025
U28389	<i>EPB49</i>	8p21.1	Cytoskeleton biosyntes	1.4/1.0	0.0025
D87466	<i>KIAA0276</i>	4p12		1.9/1.0	0.0025
D87119	<i>TRB2</i>	2p25.1	Amino acid phosphorylation	1.9/1.0	0.0026
AB007855	<i>TIX1</i>	20q12	Regulation of transcription	2.1/1.0	0.0027
X81625	<i>ETF1</i>	5q31.1	Regulation of translation	1.3/1.0	0.0029
L17131	<i>HMGA1</i>	6p21	Regulation of transcription	1.6/1.0	0.0029
AL022326	<i>SYNGR1</i>	22q13.1		2.8/1.0	0.0029
AB014512	<i>BZRAP1</i>	17q22–q23		2.5/1.0	0.0031
Lower expression in adenoma: Group 1					
AF042386	<i>PPIE</i>	1p32	Regulation of transcription	0.58/1.0	0.0001
X92814	<i>HRASLS3</i>	11q13.1		0.14/1.0	0.0001
AF052149	<i>BSCL2</i>	11q12–q13.5		0.57/1.0	0.0002
M55409	<i>EEFIG</i>	11q13.2		0.58/1.0	0.0002
U39400	<i>MRPL49</i>	11q13	Cell growth	0.67/1.0	0.0002
AI033692	<i>BANF1</i>	11q13.1	Virus response	0.59/1.0	0.0002
U60644	<i>PLD3</i>	19q13.2	Metabolism	0.50/1.0	0.0002
X65923	<i>FAU</i>	11q13	Protein biosynthesis	0.49/1.0	0.0003
X05323	<i>MOX2</i>	3q12–q13		0.18/1.0	0.0003
X02344		9		0.75/1.0	0.0004
HG1614-HT1614				0.50/1.0	0.0005
AF022813	<i>TM4SF7</i>	11p15.5	Protein assembly	0.78/1.0	0.0005
AF018631	<i>BTD</i>	3p25	CNS development etc.	0.59/1.0	0.0005
X81372	<i>BPHL</i>	6p25	Amino acid metabolism	0.67/1.0	0.0006
AL050204		11q22.3		0.47/1.0	0.0007
AW044624	<i>RER1</i>	1pter–q24	ER transport	0.56/1.0	0.0007
D29643	<i>DDOST</i>	1p36.1	N-linked glycosylation	0.62/1.0	0.0007
U43368	<i>VEGFB</i>	11q13	Regulation of cell growth	0.48/1.0	0.0007
W26480	<i>FADS1</i>	11q12.2–q13.1		0.47/1.0	0.0011
L13687	<i>SNX15</i>	11q13	Protein transport and signalling	0.80/1.0	0.0012

Table 5 continued on following page

Table 5 Continued.

Accession number	Gene symbol	Chromosomal location	GO biological process description ^a	Relative expression (Group I/Group II)	P
AF043325	<i>NMT2</i>	10p12.33	Protein myristoylation	0.40/1.0	0.0012
U62961	<i>OXCT</i>	5p13	Succinyl-CoA metabolism	0.43/1.0	0.0013
AL080190		8p23.1		0.86/1.0	0.0017
AA845349	<i>HMGN3</i>	6q15	Regulation of transcription	0.83/1.0	0.0020
M15036	<i>PROS1</i>	3p11–q11.2	Blood coagulation	0.31/1.0	0.0020
U52427	<i>POLR2G</i>	11q13.1	Nucleotide-excision repair	0.49/1.0	0.0020
AA158243	<i>FKBP2</i>	11q13.1–q13.3	Protein folding	0.45/1.0	0.0022
M62895	<i>ANXA2P3</i>	10q21–q22	Skeletal development	0.34/1.0	0.0023
AF010187	<i>FIBP</i>	11q13.1	FGF receptor signaling	0.55/1.0	0.0023
U66879	<i>BAD</i>	11q13.1	Apoptosis	0.37/1.0	0.0024
U90916	<i>SORL1</i>	11q23.3	Lipid transport	0.39/1.0	0.0025
AB020637	<i>KIAA0830</i>	11q21		0.48/1.0	0.0025
AL050089	<i>BAZ1A</i>	14q12–q13	Regulation of transcription	0.54/1.0	0.0025
AA917672	<i>ATP5L</i>	3q27	ATP biosynthesis	0.64/1.0	0.0025
Y14155	<i>MOB</i>	10q		0.41/1.0	0.0026
D00017	<i>ANXA2</i>	15q21–q22	Skeletal development	0.31/1.0	0.0027
L37043	<i>CSNK1E</i>	22q13.1	DNA repair and amino acid phosphorylation	0.68/1.0	0.0029
AF038406	<i>NDUFS8</i>	11q13	Mitochondrial transport	0.47/1.0	0.0030
L40397	<i>TMP21</i>	14q24.3	ER transport	0.52/1.0	0.0030
AL050008	<i>BRMS1</i>	11q13–q13.2		0.52/1.0	0.0031
D12676	<i>SCARB2</i>	4q21.1	Cell adhesion	0.57/1.0	0.0031
U93237	<i>MEN1</i>	11q13	Regulation of transcription	0.25/1.0	0.0031
S57501	<i>PPP1CA</i>	11q13	Amino acid dephosphorylation	0.56/1.0	0.0031
M68864	<i>LOC51035</i>	11q13.1		0.70/1.0	0.0031

t-tests were performed by comparing the mean expression levels between the groups of adenomas with 11q13 LOH and without 11q13 LOH using the dChip software. ^aGene ontology description according to the NetAffxTM analysis center: <http://www.affymetrix.com/analysis/index.affx>. The genes are listed by ascending *P* values. ER, estrogen receptor.

interest for parathyroid tumorigenesis are *CASR* and *VDR*. Both these receptors showed a distinct pattern of under-expression in all adenomas as compared with the normal parathyroids (Table 3). Both *CASR* and *VDR* participate in the calcium homeostasis controlled by the parathyroid gland, and it is suggested that the reduced expression of *CASR* and *VDR* has a major impact on the altered calcium homeostasis in PHPT. Our array data regarding *CASR* expression (median 63%, range 43–80%) correlate well with the mRNA *in situ* data previously published which also showed reduced expression in adenomas vs normal parathyroid (reduced to median 64%, range 41–98%) (10). Furthermore, in our study the *VDR* expression was reduced in the adenomas (to median 47%, range 20–64%) compared with the expression in normal parathyroid tissue. A similar pattern of *VDR* under-expression in parathyroid adenomas was also reported by Carling *et al.* (27). The consistent reduced levels of both *VDR* and *CASR* expression are of interest given the recent demonstration that the *CASR* gene promoters have vitamin D response elements (VDREs) that confer transcriptional responsiveness to 1,25-dihydroxyvitamin D levels (28).

Expression profiles in parathyroid adenomas with and without 11q13 LOH

In order to display potential parathyroid adenoma subgroups, we performed an 'unsupervised' clustering ana-

lysis, in which no external input was used to guide the analysis process. We used the most common unsupervised analysis method, i.e. hierarchical cluster analysis (29). This method is used to search for patterns without any expectation concerning the number or type of groups that creates the dendrogram, as illustrated in Fig. 1A. The resulting hierarchical tree demonstrated that the adenomas clustered together in two individual groups. Group I consisted of the four adenomas with 11q13 LOH and Group II of the four adenomas without 11q13 LOH (Fig. 1A). In addition, several genes were detected with a significantly ($P < 0.0032$) distinct expression profile in Group I and II (Table 5). Among the genes over-expressed in Group I can be noted *ENC1* (5q12–q13.3), which showed 20-fold increase compared with Group II. This may reflect an important downstream effect. *ENC1* is over-expressed in primary colon cancer, neuroblastoma and haircell leukemia (30). Functional *in vitro* assays suggest that the p53 inducible *ENC1* is regulated by the β -catenin/Tcf pathway, and that its over-expression stimulates proliferation and suppresses differentiation. The present series of tumors is too limited in number to draw conclusions about clinical phenotypes and expression profiles. However, it has been suggested that sporadic and MEN 1-associated parathyroid tumors with LOH of the *MEN1* locus in 11q13 are larger than tumors without this abnormality (14).

Approximately 50% of the under-expressed genes in Group I are located on chromosome 11 suggesting an

underlying gene dose effect (Table 5 and Fig. 1B). This hypothesis was substantiated by the demonstration of loss of the entire chromosome 11 by CGH. The results suggest that adenomas in Group I have partly similar and partly distinguishing expression profiles as compared with the Group II adenomas. The distinct expression profiles would suggest that adenomas with 11q13 LOH partly affect different pathways as compared with the adenomas without LOH. Nevertheless, another set of genes showed similarly differential expression in the entire adenoma group as compared with normal parathyroid. This may result from late downstream effects in common for tumor development of most adenomas, e.g. over-expression of *Cyclin D1* and *c-Jun*. The common alterations could also represent secondary effects of the tumor status or altered calcium homeostasis. The reduced expression observed for, for example *CASR* and *VDR*, would preferentially be placed in this last category. PHPT is clearly a heterogeneous disease both regarding clinical development and molecular genetic alterations. Although the biological effect of the altered expression pattern is difficult to survey, it can be speculated that partly different molecular pathways could lead to benign tumor development in the parathyroid gland.

In summary, we have shown that genetic subsets in parathyroid adenomas distinguished by the 11q13 LOH status are reflected in the gene expression profiles, but that commonly altered gene expression patterns are also evident. In the future, detailed analysis at the protein level will help us to understand the impact on tumorigenesis of the genes identified in this study.

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