Molecular pathways associated with transcriptional alterations in hyperparathyroidism

FANG LEE[1], JIE-JEN LEE[1-3], WOAN-CHING JAN[2], CHIH-JEN WU[3,4], HAN-HSIANG CHEN[3,4] and SHIH-PING CHENG[1,3]

[1]Department of Surgery, MacKay Memorial Hospital and MacKay Medical College, Taipei 10449; [2]Department of Nursing, MacKay Junior College of Medicine, Nursing and Management, Tapei 11260; [3]Department of Pharmacology and Graduate Institute of Medical Sciences, Taipei Medical University, Taipei 11031; [4]Department of Nephrology, MacKay Memorial Hospital and Mackay Medical College, Taipei 10449, Taiwan, R.O.C.

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Abstract. Hyperparathyroidism is characterized by the oversecretion of parathyroid hormone biochemically and increased cell proliferation histologically. Primary and secondary hyperparathyroidism exhibit distinct pathophysiology but share certain common microscopic features. The present study performed the first genome-wide expression analysis directly comparing the expression profile of primary and secondary hyperparathyroidism. Microarray gene expression analyses were performed in parathyroid tissues from 2 primary hyperparathyroidism patients and 3 secondary hyperparathyroidism patients. Unsupervised hierarchical clustering analysis identified two natural subgroups containing different types of hyperparathyroidism. Combined with additional data extracted from a publicly available database, a meta-signature was constructed to represent an intersection of two sets of differential expression profile. Multiple pathways were identified that are aberrantly regulated in hyperparathyroidism. In primary hyperparathyroidism, dysregulated pathways included cell adhesion molecules, peroxisome proliferator-activated receptor signaling pathway, and neuroactive ligand-receptor interaction. Pathways implicated in secondary hyperparathyroidism included tryptophan metabolism, tight junctions, renin-angiotensin system, steroid hormone biosynthesis, and O-glycan biosynthesis. The present study demonstrates that different pathophysiology is associated with differential gene profiling in hyperparathyroidism. Several pathways are involved in parathyroid dysregulation and may be future targets for therapeutic intervention.

Correspondence to: Dr Shih-Ping Cheng, Department of Surgery, MacKay Memorial Hospital and MacKay Medical College, 92 Chung-Shan North Road, Taipei 10449, Taiwan, R.O.C.
E-mail: surg.mmh@gmail.com

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Introduction

Parathyroid hormone is the principal physiologic regulator of calcium homeostasis. Hyperparathyroidism is a result of increased activity of the parathyroid glands, either from an intrinsic change altering excretion of parathyroid hormone (primary hyperparathyroidism, pHPT) or from an extrinsic change affecting calcium homeostasis stimulating production of parathyroid hormone (secondary hyperparathyroidism, sHPT) (1). Clinically, hyperparathyroidism leads to skeletal and renal complications in addition to an impairment in quality of life (2-4). Prolonged oversecretion of parathyroid hormone is accompanied by histologically abnormal parathyroid glands that are typically enlarged and hypercellular with decreased stromal fat (5,6).

Parathyroid glands are derived from the third and fourth pharyngeal pouches and are endodermal in origin (7). Generally, pHPT is caused by a single adenoma (80-85%) or four-gland hyperplasia (10-15%). Parathyroid carcinomas are rare and account for <1% of pHPT cases. By contrast, four-gland hyperplasia is the rule in sHPT, ranging from diffuse chief cell hyperplasia to nodular formations. Significantly increased proliferation and apoptosis were both observed in pHPT and sHPT in comparison with normal parathyroids (8). Nonetheless, different genetic alterations are implicated in the development of different types of hyperparathyroidism (9,10).

Although pHPT mainly occurs as a sporadic disease, it may be part of a hereditary syndrome (e.g., multiple endocrine neoplasia types 1 and 2A). On the other hand, chronic renal failure is the primary cause of sHPT. The phenomenon that different pathophysiology and genetic alterations of hyperparathyroidism lead to partially overlapping histological phenotype is notable. However, to the best of our knowledge, there have been no reports comparing transcriptional alterations in different types of hyperparathyroidism. In the present study, the gene expression differences between pHPT and sHPT were analyzed and molecular pathways that are dysregulated in different contexts of hyperparathyroidism were identified.

Materials and methods

Patients and tissue samples. The present study was approved by the Institutional Review Board of MacKay Memorial Hospital (Taipei, Taiwan; approval no. 11MMHIS194), and all patients gave written informed consent. Parathyroid samples were obtained from patients undergoing surgical treatment of hyperparathyroidism at MacKay Memorial Hospital, Taipei, Taiwan (11). All samples were snap frozen in liquid nitrogen within 10 min of resection and stored at -80˚C. Diagnosis was histologically confirmed by a senior endocrine pathologist using hematoxylin-eosin staining.

RNA extraction. Total RNA was extracted from homogenized frozen tissue samples using TRIzol reagent (Life Technologies, ThermoFisher Scientific, Inc., Waltham, MA, USA) and purified using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations (12). Sample purity was confirmed by measuring ratios of sample absorbance at 260 and 280 nm (ranging from 1.8 to 2.2). The quality of RNA was determined before labeling using the 2,100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Microarray hybridizations. A total of 200 μg of total RNA was amplified by a Low Input Quick Amp Labeling Kit (Agilent Technologies) and labeled with Cy3 during the *in vitro* transcription process. Cy3-labled cRNA (600 μg) was fragmented to an average size of ~50-100 nucleotides by incubation with fragmentation buffer at 60˚C for 30 min. Fragmented labeled cRNA was then pooled and hybridized to Agilent SurePrint G3 Human Gene Expression v2 8x60K Microarray at 65˚C for 17 h. After washing and drying, microarrays were scanned with an Agilent microarray scanner at 535 nm for Cy3. Scanned images were analyzed by Feature Extraction software version 10.5.1.1 (Agilent Technologies) to quantify signal and background intensity.

Data analysis and comparison with public microarray data. The microarray data were subjected to linear normalization to allow comparison between arrays. Hierarchical cluster analysis was performed with Cluster 3.0 (bonsai.hgc.jp/~mdehoon/software/cluster/software.htm), and heat maps were constructed with Java Treeview software (www.princeton.edu/~abarysh/treeview/). A public microarray dataset (GSE10317) was retrieved from the National Center for Biotechnology Information Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/). GSE10317 comprises gene expression data of a case of pHPT (13). Gene expression levels of the parathyroid tumor and normal parathyroid tissue were analyzed using Affymetrix Human Genome U133 Plus 2.0 Arrays. (Affymetrix, Inc., Santa Clara, CA, USA)

t statistics were used to estimate the significance of expression difference between pHPT and sHPT. R software version 3.0.2 (www.r-project.org) was used for Bayes-regularized *t* tests. Associated P-values were adjusted for multiple testing by controlling for a false discovery rate <5% using the Benjamini-Hochberg procedure (14), and adjusted P<0.05 was considered to indicate a statistically significant difference. For the GSE10317 data, probes with a differential expression of at least 2-fold were considered to be significant. A meta-signature that characterized the intersection of differentially expressed genes from both datasets were constructed. Genes that demonstrated significantly altered expression changes in the same direction for both dataset were considered to be pHPT-associated. The intersection of differentially expressed genes of the two dataset in the opposite direction was considered to be sHPT-associated.

Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg/) pathway analyses were performed to annotate the biological functions and pathways in which the aberrantly expressed genes of pHPT and sHPT were involved.

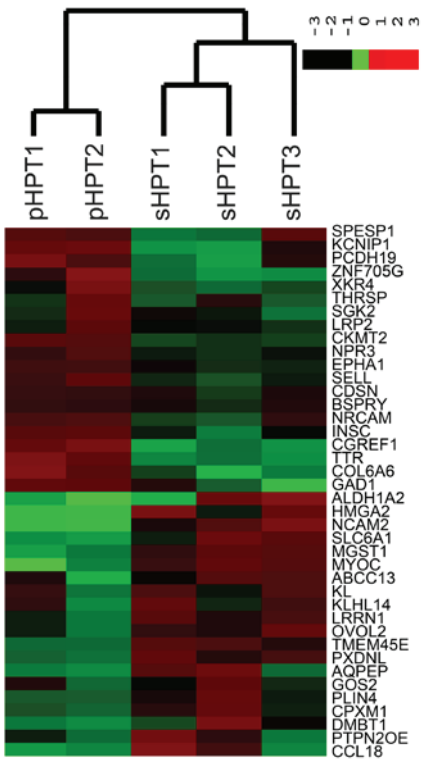

Figure 1. Hierarchical clustering of microarray data in patients with hyperparathyroidism.

Results

Microarray gene expression analyses were performed in parathyroid tissues from 2 pHPT and 3 sHPT patients. The 2 pHPT patients were female and had single parathyroid adenoma. All sHPT patients including 2 women and 1 man had four-gland nodular hyperplasia. Unsupervised hierarchical clustering analysis for the expression of all genes revealed two natural subgroups containing pHPT and sHPT, respectively.

A meta-signature was constructed to represent an intersection of two sets of differential expression profile. Based on predefined criteria, 339 genes were upregulated and 261 genes were downregulated in pHPT. The ten most common leading-edge genes are summarized in Tables I and II. A total of 218 genes were upregulated and 367 genes were downregulated in sHPT. The top upregulated and downregulated genes are shown in Tables III and IV, respectively. A heat map generated from the most differently expressed genes is presented in Fig. 1.

Table I. Upregulated genes in primary hyperparathyroidism.

Probe	Gene	Accession #	Description
A_23_P130333	TTR	NM_000371	*Homo sapiens* transthyretin
A_33_P3814721	INSC	NM_001031853	*Homo sapiens* inscuteable homolog (Drosophila), transcript variant 1
A_32_P224525	COL6A6	NM_001102608	*Homo sapiens* collagen, type VI, alpha 6
A_33_P3400273	SELL	NM_000655	*Homo sapiens* selectin L, transcript variant 1
A_24_P252364	NRCAM	NM_001037132	*Homo sapiens* neuronal cell adhesion molecule, transcript variant 1
A_23_P157333	EPHA1	NM_005232	*Homo sapiens* EPH receptor A1
A_23_P350396	CDSN	NM_001264	*Homo sapiens* corneodesmosin
A_33_P3244728	LRP2	NM_004525	*Homo sapiens* low density lipoprotein receptor-related protein 2
A_23_P374689	GAD1	NM_000817	*Homo sapiens* glutamate decarboxylase 1 (brain, 67kDa), transcript variant GAD67
A_23_P71946	BSPRY	NM_017688	*Homo sapiens* B-box and SPRY domain containing

Table II. Downregulated genes in primary hyperparathyroidism.

Probe	Gene	Accession #	Description
A_23_P36658	MGST1	NM_145791	*Homo sapiens* microsomal glutathione S-transferase 1, transcript variant 3
A_33_P3300253	PTPN20B	NM_001042357	*Homo sapiens* protein tyrosine phosphatase, non-receptor type 20B, transcript variant 1
A_23_P74609	G0S2	NM_015714	*Homo sapiens* G0/G1 switch 2
A_33_P3251522	AQPEP	NM_173800	*Homo sapiens* laeverin
A_33_P3400763	PLIN4	NM_001080400	*Homo sapiens* perilipin 4
A_23_P23783	MYOC	NM_000261	*Homo sapiens* myocilin, trabecular meshwork inducible glucocorticoid response
A_21_P0000096	CPXM1	NM_019609	*Homo sapiens* carboxypeptidase X (M14 family), member 1, transcript variant 1
A_23_P258310	PXDNL	NM_144651	*Homo sapiens* peroxidasin homolog (Drosophila)-like
A_23_P55270	CCL18	NM_002988	*Homo sapiens* chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)
A_23_P86599	DMBT1	NM_007329	*Homo sapiens* deleted in malignant brain tumors 1, transcript variant 2

Table III. Upregulated genes in secondary hyperparathyroidism.

Probe	Gene	Accession #	Description
A_24_P268685	SLC6A1	NM_003042	*Homo sapiens* solute carrier family 6 (neurotransmitter transporter), member 1
A_23_P503064	KL	NM_004795	*Homo sapiens* klotho
A_24_P73577	ALDH1A2	NM_170697	*Homo sapiens* aldehyde dehydrogenase 1 family, member A2, transcript variant 3
A_23_P1682	TMEM45B	NM_138788	*Homo sapiens* transmembrane protein 45B
A_23_P95930	HMGA2	NM_003483	*Homo sapiens* high mobility group AT-hook 2, transcript variant 1
A_24_P240187	LRRN1	NM_020873	*Homo sapiens* leucine rich repeat neuronal 1
A_23_P143348	OVOL2	NM_021220	*Homo sapiens* ovo-like zinc finger 2
A_32_P199429	NCAM2	NM_004540	*Homo sapiens* neural cell adhesion molecule 2
A_23_P370830	KLHL14	NM_020805	*Homo sapiens* kelch-like family member 14
A_23_P99253	LIN7A	NM_004664	*Homo sapiens* lin-7 homolog A (*C. elegans*)

Table IV. Downregulated genes in secondary hyperparathyroidism.

Probe	Gene	Accession #	Description
A_23_P144778	CKMT2	NM_001825	*Homo sapiens* creatine kinase, mitochondrial 2 (sarcomeric), transcript variant 1
A_33_P3319248	ZNF705G	NM_001164457	*Homo sapiens* zinc finger protein 705G
A_23_P131801	SGK2	NM_170693	*Homo sapiens* serum/glucocorticoid regulated kinase 2, transcript variant 1
A_23_P30554	KCNIP1	NM_001034837	*Homo sapiens* Kv channel interacting protein 1, transcript variant 1
A_23_P129085	SPESP1	NM_145658	*Homo sapiens* sperm equatorial segment protein 1
A_23_P363954	THRSP	NM_003251	*Homo sapiens* thyroid hormone responsive
A_32_P134007	XKR4	NM_052898	*Homo sapiens* XK, Kell blood group complex subunit-related family, member 4
A_33_P3423230	PCDH19	NM_001184880	*Homo sapiens* protocadherin 19, transcript variant 3
A_33_P3227793	CGREF1	NM_006569	*Homo sapiens* cell growth regulator with EF-hand domain 1, transcript variant 1
A_23_P58676	NPR3	NM_001204375	*Homo sapiens* natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C), transcript variant 1

Table V. Pathway analysis of genes with differential expression in primary hyperparathyroidism.

Pathway	Count	P-value	Genes
Cell adhesion molecules	9	0.022	HLA-DQB1, NRCAM, ALCAM, SDC1, NRXN3, CD40LG, SELL, ITGA8, ITGA4
PPAR signaling pathway	6	0.034	HMGCS2, APOA5, APOC3, SLC27A6, ACSL3, SLC27A2
Neuroactive ligand-receptor interaction	13	0.035	CSH1, S1PR3, GABRG2, PTGER3, PRLR, RXFP1, HTR7, GRIN2A, TAAR1, ADRA1A, GABBR2, PTGFR, GCGR

Table VI. Pathway analysis of genes with differential expression in secondary hyperparathyroidism.

Pathway	Count	P-value	Genes
Tryptophan metabolism	6	0.003	TDO2, CYP1B1, MAOA, AOX1, ALDH2, INMT
Tight junction	10	0.007	PRKCQ, INADL, MYH11, ACTN1, CLDN10, MYH7, CLDN11, CTNNA3, CTNNA2, MYL9
Renin-angiotensin system	4	0.009	ACE2, MAS1, ANPEP, CTSG
Steroid hormone biosynthesis	5	0.030	AKR1C3, CYP3A5, CYP1B1, AKR1C4, CYP19A1
O-glycan biosynthesis	4	0.041	GALNT3, GCNT1, GALNT13, GALNT14

The gene function annotations were evaluated according to the GO and KEGG pathway databases. For genes with differential expression in pHPT, involved molecular functions in order were: Fatty-acid ligase activity, calcium ion binding, alkali metal ion binding, ligase activity forming carbon-sulfur bonds, and symporter activity. Involved biological processes were: Ion transport, cell adhesion, biological adhesion, cation transport, and metal ion transport. Dysregulated pathways in pHPT are presented in Table V.

Molecular interaction and networks contributing to sHPT were identified. Molecular functions involved in sHPT were: Retinal dehydrogenase activity, steroid dehydrogenase activity acting on the CH-OH group of donors NAD or NADP as acceptor, carbohydrate binding, sulfotransferase activity, and acetylgalactosaminyltransferase activity. Involved biological processes were: Cell adhesion, biological adhesion, secondary metabolic process, skeletal system development, and regulation of nucleotide metabolic process. Dysregulated pathways in sHPT are listed in Table VI.

Discussion

The exact mechanism underlying the development of pHPT remains poorly understood (15). In patients with multiglandular pHPT, independent genetic events may be present in separate glands within the same individual (16).

Previous studies have indicated that parathyroid adenomas typically harbor few somatic variants (17,18). Mutations in the *MEN1* tumor suppressor gene and alterations in the *CCND1* (cyclin D1/PRAD1) oncogene represent the major driver in sporadic parathyroid tumorigenesis. Cell cycle regulators (including *CDC73* aka HRPT2), growth factors, apoptosis-inducing ligands, death receptors, and other transmitter substances have also been implicated in the pathogenesis (19,20). Although there is an abundance of data examining the gene expression profiles of parathyroid adenoma, few studies have been performed in comparing gene expressions of pHPT with sHPT tissues.

In a previous study examining clonality in pHPT and sHPT, 7/8 pHPT glands (6 adenomas and 2 hyperplasias) exhibited monoclonal proliferation (21). One parathyroid adenoma demonstrated a polyclonal pattern. In an sHPT patient, one of the 3 hyperplastic lesions was monoclonal and the other 2 lesions were polyclonal. This finding underscores the complexity of pHPT pathogenesis. The results from later studies also attest that pHPT can arise by clonal and polyclonal mechanisms (22). This is different from sHPT in which polyclonal growth transforms to monoclonal proliferation during disease progression (23). Nonetheless, there remains the possibility that similar mechanisms regulate parathyroid cell growth in both entities. The current study represents the first effort to compare the transcriptional profiles between pHPT and sHPT using modern microarray technology.

One of the limitations of the study design is the lack of transcriptional data from normal parathyroid controls. To overcome this ethical constraint, additional data were extracted from a publicly available database and combined to form a meta-signature. Regardless, the aberrantly regulated pathways that the present study identified in different types of hyperparathyroidism provide critical insights into the differences in pathophysiology. For instance, cell adhesion molecules were upregulated in pHPT but downregulated in sHPT. It is well known that nodular hyperplasia in patients with chronic kidney disease is associated with progressive downregulation of calcium-sensing receptor and vitamin D receptor (6). Activation of calcium-sensing receptor may potentiate cell adhesion by promoting integrin binding to a fibronectin-rich matrix (24). It is therefore reasonable that downregulated tight junction and cell adhesion were among the dysregulated pathways in sHPT. Conversely, expression of some adhesion molecules including selectin L and neuronal cell adhesion molecule were significantly increased in pHPT (Table I).

It was also noted that renin-angiotensin system was among the dysregulated pathways in sHPT. Increasing evidence links the renin-angiotensin-aldosterone system to calcium regulatory systems (25). A high calcium diet was demonstrated to downregulate angiotensin-converting enzyme of the kidney in experimental renal failure (26). In the present study, it was demonstrated that the expression of angiotensin I converting enzyme 2 (*ACE2*) was downregulated in sHPT. Recently, it has been shown that angiotensin II infusion acutely stimulated the secretion of parathyroid hormone in a dose-dependent manner (27). It is possible that downregulation of angiotensin-converting enzymes in sHPT results from a negative feedback mechanism to reduce further stimulation from angiotensin II. Renin-angiotensin system may be a potential target for therapeutic intervention in hyperparathyroidism.

The result of the present study point to alterations in peroxisome proliferator-activated receptor pathway in addition to fatty acid and amino acid metabolism in pHPT and sHPT. Metabolic aberrations have already been established as serving essential roles in imaging studies of hyperparathyroidism. Uptake and accumulation of technetium-99m sestamibi in mitochondria-rich oxyphil cells are employed as the basis of scintigraphic detection of hyperfunctional parathyroids (28). In addition, various tracers for positron emission tomographic scan have been exploited in localization of abnormal parathyroid glands (29,30). Metabolic reprogramming in neoplasms has recently been indicated as another general hallmark of cancer. Nonetheless, metabolic rearrangements in hyperparathyroidism remain a virtually untapped area of investigation. Elucidating the complex interplay between calcium homeostasis and parathyroid metabolic activity is an exciting challenge for future research.

In conclusion, the present study demonstrates that different pathophysiology led to differential gene profiling in hyperparathyroidism. Systemic analysis and annotated pathway resources were used to identify several pathways that are dysregulated in hyperparathyroidism which may be targets of interest.

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