Published as: *Genes Chromosomes Cancer*. 2011 September ; 50(9): 735–745.

The DNA Methylome of Benign and Malignant Parathyroid Tumors

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Abstract

The role of DNA methylation of CpG islands in parathyroid tumorigenesis has not been analyzed in an unbiased, systematic fashion. DNA was isolated from normal and pathologic parathyroid tissues, bisulphite modified and analyzed using the Infinium HumanMethylation27 BeadChip. Distinct hierarchical clustering of genes with altered DNA methylation profiles in normal and pathologic parathyroid tissue was evident. Comparing normal parathyroid tissue with parathyroid adenomas, 367 genes were significantly altered, while 175 genes significantly differed when comparing parathyroid carcinomas and normal parathyroid tissues. A comparison between parathyroid adenomas and parathyroid carcinomas identified 263 genes with significantly distinct methylation levels. Results were confirmed for certain genes in a validation cohort of 40 parathyroid adenomas by methylation-specific PCR. Genes of known or putative importance in the development of parathyroid tumors showed significant and frequent hypermethylation. DNA hypermethylation of *CDKN2B*, *CDKN2A*, *WT1*, *SFRP1*, *SFRP2* and *SFRP4* was associated with reduced gene expression in both benign and malignant parathyroid tumors. Treatment with 5-aza-2'-deoxycytidine of primary cell cultures restores expression of hypermethylated genes in benign and malignant parathyroid tumors. In conclusion, the unbiased, genome-wide study of the parathyroid tumor DNA methylome identified a number of genes with altered DNA methylation patterns of putative importance to benign and malignant parathyroid tumorigenesis.

INTRODUCTION

Primary hyperparathyroidism (pHPT) is common, especially in postmenopausal females, and population-based screening studies suggests a prevalence of 0.7 to 2.3% (Lundgren et al., 1997; Siilin et al., 2011). The disease is due to a single parathyroid adenoma in about 85% of cases whereas parathyroid carcinoma is rare (0.5–1% of cases). Apart from surgical therapy, there exist no effective treatment for pHPT and parathyroid carcinoma is associated with significant morbidity and mortality (Fraker, 2008). The molecular pathogenesis of

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sporadic pHPT has been partly elucidated; inactivating somatic mutations of the tumor suppressor genes *MEN1* and *HRPT2/CDC73* have been identified in a subset of parathyroid tumors (Heppner et al., 1997; Carling et al., 1998). The *CCND1* oncogene, now recognized to have a central role in many forms of human neoplasia, was initially identified at the breakpoint of a parathyroid adenoma DNA rearrangement (Arnold and Kim 1989; Motokura et al., 1991), and cyclin D1 overexpression has been detected in 18–40% of sporadic parathyroid adenomas (Hsi et al., 1996; Yi et al., 2008). Aberrations in the Wnt/ β -catenin signaling pathway have been identified in parathyroid tumors and an aberrantly spliced, internally truncated variant of *LRP5*, a co-receptor for Wnt ligands, results in stabilization and accumulation of β -catenin in a majority of parathyroid tumors of pHPT (Bjorklund et al., 2007). Parafibromin, the protein product of *HRPT2/CDC73*, is a part of the human polymerase associated factor complex (PAF), required for facilitating transcriptional elongation and histone modification (Rozenblatt-Rosen et al., 2005). Additionally, parafibromin down-regulates MYC, suggesting its role as a tumor suppressor through inhibition of Wnt signaling (Mosimann et al., 2006). Moreover, *CCND1* is a target of the Wnt/ β -catenin signaling pathway (Shtutman et al., 1999).

Growing evidence shows that acquired epigenetic abnormalities, including DNA methylation, along with genetic alterations lead to altered patterns of gene expression/function in tumorigenesis. Much is now known about the importance of promoter cytosine methylation in cytosine phosphate guanine (CpG) islands and gene silencing (Jones and Baylin, 2007). It has been established that such methylation is intimately involved in cancer development (Jones and Baylin, 2007). Characterization of DNA methylation information, collectively denoted the DNA methylome, has recently been successfully employed to characterize the molecular pathogenesis and epigenetically classify both solid and hematological malignancies (Kulis and Esteller 2010).

Hypermethylation of promoter regions has also been analyzed in parathyroid tumors and frequent hypermethylation of *RIZ1/PRDM2*, *CDKN2A*, *RASSF1A*, and *APC* has been reported (Carling et al., 2003; Juhlin et al., 2010; Svedlund et al., 2010). However, such studies have focused on individual genes or a small cohort of genes. In contrast to most other neoplastic processes, parathyroid adenomas are less aggressive, homogeneous, and diagnosed at an earlier stage because of related hormone excess. Thus, they represent an interesting model system to study epigenetic aberrations involved in tumor development.

The current study represents the first comprehensive, unbiased analysis of quantitative DNA methylation alterations in benign and malignant parathyroid tumors.

MATERIALS AND METHODS

Subjects and Tissues

Parathyroid adenomas (n=51) and carcinomas (n=7) were acquired from pHPT patients diagnosed and surgically treated in the clinical routine at Yale-New Haven Hospital, Uppsala University Hospital or Martin-Luther University Hospital, and clinical characteristics are presented in Supplementary Table 1. Inclusion criteria were inappropriate elevation of PTH in relation to serum calcium, normal creatinine levels, no history of familial hyperparathyroidism or exposure to calcimimetic therapy. All tumors were carefully evaluated and dissected by an experienced endocrine pathologist prior to use in the study. The diagnosis of parathyroid carcinoma was unequivocal with all patients demonstrating widely invasive and/or distant metastatic disease. Normal parathyroid tissue (n=3) was obtained from glands inadvertently removed in conjunction with thyroid surgery where auto-transplantation was not required, or as normal parathyroid gland biopsies in patients subjected to parathyroidectomy. Therefore, a small number of non-pathologic tissues were

available for analysis; however, previous parathyroid studies have utilized similar numbers (Juhlin et al., 2010). Tissues were snap-frozen in liquid nitrogen and stored in -80°C or were prepared for primary cultures. Informed consent and approval by institutional review boards at participating institutions were obtained. All cases were analyzed by Sanger sequencing for germline mutations of the *MEN1* gene prior to further analysis, and found to be wild-type.

Comprehensive DNA Methylation Profiling

High molecular weight genomic DNA was isolated from normal and pathological parathyroid tissue, as previously described (Carling et al., 1995). Genomic DNA (500 ng) from normal parathyroid tissue (n=3), parathyroid adenoma (n=14), and cancer (n=7) were simultaneously bisulphite modified using the EZ DNA Methylation kit (Zymo Research, Orange, CA) according to the instructions from the manufacturer and analyzed using Infinium HumanMethylation27 BeadChip (Illumina, San Diego, CA). The Infinium HumanMethylation27 BeadChip protocol comprises six steps (whole-genome amplification, fragmentation, hybridization, washing, counterstaining and scanning) (Thirlwell et al., 2010), which were carried out at the Yale Center for Genome Analysis at Yale University according to the manufacturer's recommendation. The HumanMethylation27 panel targets CpG sites located within the proximal promoter regions of transcription start sites of 14,475 consensus coding sequencing (CCDS) in the NCBI Database (Genome Build 36). In addition, 254 assays cover 110 miRNA promoters. On average, two assays were selected per CCDS gene and from 3–20 CpG sites for >200 cancer-related and imprinted genes. The platform also allows interrogation of 27,578 highly informative CpG sites per sample at single-nucleotide resolution (Bibikova et al., 2006; Killian et al., 2009). The CpG sites are located in promoter regions, up to 1 kb upstream or 0.5 kb downstream of transcription start sites. The BeadChip was scanned on the Illumina iScan and the resulting files were analyzed with the Beadstudio software (Version 3.2; Illumina). The output of the Beadstudio analysis is a β -value for each CpG site interrogated. This is a continuous value between 0 and 1, where 0 indicates 0% methylation and 1 indicates 100% methylation at a given CpG site. Therefore, this assay provides quantitative methylation measurement at the single CpG site level. The calculation of the β -value is performed as described (Thirlwell et al., 2010).

Methylation-Specific PCR Analysis Using SYBR Green

To verify the findings from the methylation arrays, 6 highly hypermethylated genes were analyzed using methylation-specific PCR (MSP). Methylated and unmethylated specific primers were designed using the Methyl Primer Express software (Applied Biosystems, Foster City, CA, USA) and the primer sequences are presented in supplementary Table 2. Both unmethylated and methylated specific primers displayed an identical target amplicon. Semi-quantitative PCR was performed using SYBR-Green PCR Master Mix (#4309155) and results were analyzed using StepOne Software v2.1 (Applied Biosystems). Human methylated DNA (Epitect Control DNA; Qiagen, Valencia, CA, USA) was utilized as the reference DNA to quantitatively assess the methylation status of the target CpG island, when using methylated specific primers. Similarly, human demethylated DNA (Epitect Control DNA; Qiagen) was used as reference for the unmethylated specific primers. The relative percentage of the values from the methylated and unmethylated measurements was calculated.

Quantitative RT-PCR Analysis

cDNA was synthesized using 1 μg of total RNA and iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc. Hercules, CA). Quantitative real-time PCR was performed on StepOnePlus™ Real-Time PCR systems (Applied Biosystems) using assays for *CDKN2B* (Hs 00793225_m1), *CDKN2A* (Hs 00233365_m1), *WT1* (Hs 01103754_m1), *SFRP4* (Hs

00180066_m1), *SFRP1* (Hs 00610060_m1), *SFRP2* (Hs 293258_m1) and *GAPDH* (Hs99999905_m1; all from Applied Biosystems). Each cDNA sample was analyzed in triplicate. Standard curves for each experiment were established by amplifying a purified PCR fragment covering the sites for probes and primers.

Primary Cell Cultures, and Treatment With 5-Aza-2'-Deoxycytidine

Parathyroid carcinoma (n=1) and adenoma cells (n=18) were prepared fresh, directly after operation according to published procedures with minor modifications (Carling et al., 2000). Briefly collagenase digestion was performed for 1 h. Cells were then cultured in 35-mm dishes in DMEM, containing 10% fetal bovine serum and penicillin/fungizone/L-glutamine (Sigma-Aldrich St.Louis, MO) and were treated in triplicates with 5 μ M 5-aza-2'-deoxycytidine (Sigma-Aldrich). Cell viability was measured on PC1 cells distributed in triplicates (2×10^4) onto 96 well plates using the cell proliferation reagent WST-1 (Roche Diagnostics GmbH, Mannheim, Germany) (Svedlund et al., 2010). Fresh 5-aza-2'-deoxycytidine was added every 24 h. Cells were harvested after 24 h and 48 h for RNA extraction based upon previously published literature (Svedlund et al., 2010). Primary cell cultures were utilized due to the lack of either parathyroid adenoma or carcinoma cell lines.

Statistical Analysis

Wilcoxon rank test, Student's unpaired t-test, and Fisher exact test were used for statistical evaluation, with $p < 0.05$ considered to be significant. All results are expressed as mean $\pm$ SEM (standard error of the mean).

RESULTS

Comprehensive DNA Methylation Profiling of Parathyroid Tumors

Using a discovery cohort of normal parathyroid tissue, parathyroid adenomas and parathyroid carcinomas we characterized the DNA methylome of these tumors using the Illumina Infinium HumanMethylation27 BeadChip. Comparing normal parathyroid tissue with parathyroid adenomas, 367 genes were significantly altered, while 175 genes differed comparing parathyroid carcinomas and normal parathyroid tissues ($P < 0.005$ for both). A comparison between parathyroid adenomas and parathyroid carcinomas identified 263 genes with distinct methylation levels ($P < 0.005$). Distinct hierarchical clustering of genes with altered DNA methylation profiles in normal parathyroid tissue, parathyroid adenomas, and parathyroid carcinomas was evident by unsupervised clustering (Fig. 1A). Furthermore, when performing unsupervised examination of the top 100 differentially methylated CpG islands, the normal parathyroid tissue displayed low levels of hypermethylation, parathyroid adenomas intermediate levels of hypermethylation, whereas parathyroid carcinomas displayed hypermethylation of all examined CpG islands (Fig. 1B). When examining the known function of annotated genes showing significantly altered DNA methylation levels, genes involved in regulation of apoptosis and cell cycle control as well as ion channels were frequently altered (Fig. 1C). There was no association between DNA methylation levels and patient characteristics such as age, gender and biochemical indices of the disease in the discovery cohort when analyzing the most frequently hypermethylated genes.

Genes or members of pathways of known or putative importance in the development of parathyroid tumors showed significant and frequent hypermethylation. Such genes included those involved in regulation of the cell cycle and transcription (*CDKN2B*, *CDKN2A*, *RBI*, *WT1*, *RASSF1A* and *RIZ1/PRDM2*) and members in the Wnt/ β -catenin signaling pathway (*APC*, *SFRP1*, *SFRP2* and *SFRP4*). Interestingly, significant hypermethylation was seen frequently in parathyroid tumors at multiple examined CpG islands of individual genes, such as *CDKN2B*, *CDKN2A*, *APC*, *GATA4*, and *PYCARD*. The top 50 hypermethylated genes in

parathyroid adenomas versus normal parathyroid tissue (Table 1), parathyroid carcinomas versus normal parathyroid tissue (Table 2), and parathyroid carcinomas versus parathyroid adenomas (Supplementary Table 3) are presented. For each comparison, the top 25 hypomethylated genes are presented in supplementary Tables 4–6.

Verification of Hypermethylation of Selected Genes in Benign and Malignant Parathyroid Tumors

Based on data from the comprehensive DNA methylation profiling of parathyroid tumors, six genes (*CDKN2B*, *CDKN2A*, *WT1*, *SFRP1*, *SFRP2* and *SFRP4*) showing frequent and significant hypermethylation were selected for further analysis. Validation of promoter hypermethylation by semi-quantitative MSP (SYBR green) was analyzed in the selected genes. *CDKN2B*, *CDKN2A*, *WT1*, *SFRP1*, *SFRP2* and *SFRP4* were found to be significantly hypermethylated in both parathyroid adenomas and carcinomas compared to normal parathyroid tissue (Supplementary Fig. 1). Validation of the array results corroborated with highly reproducible results from bisulphite converted DNA allowed us to negate potential batch effect.

Hypermethylated Genes Show Reduced/Lack of Expression in Primary Benign and Malignant Parathyroid Tumors

Next we evaluated whether the hypermethylation of *CDKN2B*, *CDKN2A*, *WT1*, *SFRP1*, *SFRP2* and *SFRP4* was related to gene expression in parathyroid tumors. Total RNA was extracted from primary parathyroid tissues and gene expression was analyzed using quantitative RT-PCR. Gene expression of *CDKN2B*, *CDKN2A*, *WT1*, *SFRP1*, *SFRP2* and *SFRP4* were significantly reduced in both parathyroid adenomas and carcinomas compared to normal parathyroid tissue (Fig. 2B).

Treatment with 5-Aza-2'-Deoxycytidine of Primary Cell Cultures Restores Expression of Hypermethylated Genes in Benign and Malignant Parathyroid Tumors

In order to examine whether transcriptional repression to the *CDKN2B*, *CDKN2A*, *WT1*, *SFRP1*, *SFRP2* and *SFRP4* genes was due to reversible epigenetic alterations, primary cell cultures from parathyroid adenomas (n=18) and a parathyroid carcinoma (n=1) were utilized. Cell cultures were treated with 5-aza-2'-deoxycytidine, an inhibitor of methyltransferase activity causing demethylation, and cells were harvested at 24h and 48h after treatment. Total RNA was prepared and gene expression of the previously demonstrated highly methylated genes *CDKN2B*, *CDKN2A*, *WT1*, *SFRP1*, *SFRP2* and *SFRP4* were examined using quantitative RT-PCR. Treatment with 5-aza-2'-deoxycytidine restored measurable gene expression of the hypermethylated genes in cell cultures from parathyroid adenomas (Fig. 3) and the carcinoma (Supplementary Fig. 2). Thereby, indicating that for these 6 genes analyzed, all specimens (18 adenomas and 1 carcinoma) demonstrated evidence of promoter hypermethylation at the CpG island investigated.

DISCUSSION

Among epigenetic alterations, promoter hypermethylation of CpG islands, short regions 0.5–4.0 kilobases rich in CpG contents, has emerged as the best described epigenetic changes in tumors. Two key hypotheses have been formulated in understanding epigenetic changes in cancer development. Promoter hypermethylation can silence tumor suppressor genes and aberrant events occur across the genome resulting in specific patterns of gene inactivation contributing to the cancer state. Recently, the appreciation that DNA methylation alterations in tumors are genome-wide events has led to the development of technologies to study the global patterns of methylation (Noushmehr et al., 2010).

Using the Illumina HumanMethylation27 platform, we characterized the DNA methylome of benign and malignant parathyroid tumors. We found it to be a sensitive, robust and reproducible technique for mapping DNA methylation patterns in parathyroid tumor genomes. Normal parathyroid tissue, adenomas and carcinomas suggested a distinct DNA methylation profile. Genes involved in regulation of apoptosis and cell cycle control as well as ion channels were frequently altered in benign and malignant parathyroid tumors. Overall, there were a higher number of genes that showed a significantly different β -value in parathyroid adenomas vs. normal parathyroid tissue (n=367) than parathyroid carcinomas vs. normal parathyroid tissue (n=175). However, when analyzing the unsupervised top 100 differentially methylated CpG islands it was evident that parathyroid carcinomas displayed a greater degree of hypermethylation as compared to parathyroid adenomas. This discrepancy is most likely due to the fact that a fewer number of the very rare parathyroid carcinomas (n=7) than adenomas (n=14) were available for analysis. It is conceivable that more genes would have reached statistically significant differences in parathyroid carcinomas than adenomas had the number of available cases been equal. Nonetheless, our goal was to identify genes that were highly and frequently altered and the used approach was able to identify such genes. Among the top 50 most hypermethylated genes in both benign and malignant parathyroid tumors were those previously reported to be frequently hypermethylated in parathyroid tumors such as *RIZ1/PRDM2*, *CDKN2A*, *RASSF1A*, and *APC*. Furthermore, we selected six genes (*CDKN2B*, *CDKN2A*, *WT1*, *SFRP1*, *SFRP2* and *SFRP4*) based on the array data, their known protein function and previous implication in oncogenesis. Frequent hypermethylation of these genes were validated by semi-quantitative MSP.

CDKN2B and *CDKN2A* are tumor suppressor genes located on 9p21, and encode a cyclin-dependent kinase inhibitors. They regulate two critical cell cycle regulatory pathways, the TP53 pathway and the retinoblastoma (RB1) pathway. They are frequently deleted in cancer and hypermethylation of the genes occurs in a number of both solid and hematological malignancies (Yu et al., 2008). Studies have failed to identify mutational aberrations of *CDKN2B* and *CDKN2A* in parathyroid tumors, although 9p21 is among the 5 most frequently deleted loci in parathyroid tumors (Tahara et al., 1996). The *WT1* (Wilm's tumor 1) gene encodes a transcription factor and plays an important role in cell growth and differentiation and is a well characterized tumor suppressor gene in leukemia and various types of solid tumors. Hypermethylation of *WT1* has been demonstrated in cancer, including in breast, ovarian, hepatocellular carcinoma as well as myelodysplastic syndrome (Huang et al., 1997; Kaneuchi et al., 2005; Yu et al., 2003; Hopfer et al., 2009). The secreted frizzled-related proteins (*SFRPs*) comprise a family of five secreted glycoproteins that antagonize Wnt signaling and are putative tumor suppressors. Epigenetic inactivation of *SFRP* genes causing constitutive Wnt signaling was initially demonstrated in colorectal cancer (Suzuki et al., 2004), and has now been substantiated in a number of solid tumors (Dahl et al., 2007). Hypermethylation of *CDKN2B*, *CDKN2A*, *WT1*, *SFRP1*, *SFRP2* and *SFRP4* was related to lack/or reduced expression of these genes in both benign and malignant parathyroid tumors. Furthermore, treatment with a 5 μ M solution of the demethylating agent 5-aza-2'-deoxycytidine restored gene expression of the hypermethylated genes in primary cell cultures from parathyroid adenomas and a parathyroid carcinoma (only one fresh specimen could be obtained due to the rarity of this specific pathology) suggesting that the epigenetic alterations are reversible.

Our studies substantiate the importance of alterations of cell cycle regulation and transcriptional control in parathyroid tumors via epigenetic inactivation of pathways controlled by *CDKN2B* and *CDKN2A* and *RB1* and *RIZ/PRDM2*. The *APC* tumor suppressor gene has been shown previously to be hypermethylated in parathyroid adenomas and carcinomas (Svedlund et al., 2010), which is in agreement with the results presented here. In

the examined parathyroid carcinomas versus normal parathyroid tissue, hypermethylation at 5 different CpG sites of the *APC* gene was noted with a differential β -value of 0.44 at the most differentially methylated sites. Furthermore, the frequent hypermethylation of *SFRP* genes likely resulting in constitutive Wnt signaling further implicates an altered Wnt/ β -catenin signaling pathway in parathyroid tumorigenesis. This study provides the framework for understanding epigenetic mechanisms contributing to parathyroid tumor development. In addition, it may aid in the notoriously difficult histopathological distinction between benign and malignant parathyroid tumors. Finally, it identifies genes and pathways showing frequent epigenetic alterations, which may be pharmaceutically targeted in metastatic parathyroid carcinoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Sheila Umlauf and Shrikant Mane and the Yale Center for Genome Analysis for assistance in the development of critical methodologies.

Supported by: Ohse Research Award, the Yale Clinical and Translational Science Award UL1 RR024139 from NIH, and Ake Wiberg Foundation. R.P.L. is an Investigator of the Howard Hughes Medical Institute. P.B. is a Swedish Society for Medical Research fellow. T.C. is a Doris Duke-Damon Runyon Clinical Investigator supported in part by the Damon Runyon Cancer Research Foundation and the Doris Duke Charitable Foundation.

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Figure 1A

Figure 1.

(A) Unsupervised hierarchical clustering of parathyroid specimens analyzed using the genome-wide DNA methylation platform (Illumina Infinium HumanMethylation27 BeadChip). (B) Heat map of unsupervised top 100 differentially methylated CpG islands. Green indicated unmethylated genes (β -value ≤ 0.2), whereas red indicates hypermethylated genes (β -value ≥ 0.6). (C) Functional annotation of genes differentially methylated (diff. β -value ≥ 0.25 and $p < 0.05$) in parathyroid adenomas (top) and carcinomas (bottom) compared with normal parathyroid tissue.

Figure 2.

Quantitative RT-PCR of *CDKN2A*, *CDKN2B*, *WT1*, *SFRP1*, *SFRP2* and *SFRP4* mRNA in parathyroid tumors of the validation cohort, showing reduced gene expression of all six selected genes in parathyroid adenomas (n=37) and carcinomas (n=7). Reduction of gene expression was significant versus normal parathyroid ($p < 0.01$). Values represent normalized target gene/GAPDH mRNA ratio.

Figure 3.

In vitro effect of 5-aza-2'-deoxycytidine treatment of primary cultures of parathyroid adenoma cells (n=18) showing restoration of gene expression of *CDKN2A*, *CDKN2B*, *WT1*, *SFRP1*, *SFRP2* and *SFRP4* after treatment at 24 and 48 hours compared to untreated cells (control). Target gene/GAPDH mRNA ratio was determined by quantitative RT-PCR analysis, normalized values are presented. Gene expression restoration was significant in all experiments ($P \leq 0.05$)

TABLE 1

The Top-50 Most Differentially Hypermethylated Genes in Parathyroid Adenomas Versus Normal Parathyroid Glands.

Gene symbol	P Value	β -value difference	Function
CDKN2B *	0.001	0.728	Tumor suppressor
RAMP1	0.034	0.445	Protein transport
KCTD1	0.002	0.431	Ion channel
SEMA3F	0.002	0.430	Unknown
SOCS3	0.006	0.417	Anti- apoptosis
ESPN	0.002	0.408	Cell adhesion
SFRP1	0.003	0.401	Cell differentiation
GJA5	0.012	0.399	Connexon channel
TNFRSF10C*	0.011	0.381	Signal transduction, apoptosis
NBL1	0.003	0.0378	Cell cycle regulator
SCARA3	0.040	0.378	response to oxidative stress
APC*	0.007	0.372	Tumor suppressor
PRDM2	0.003	0.372	Transcription factor
HCG9*	0.011	0.370	Unknown
CDKN2A	0.009	0.365	Tumor suppressor
MCAM	0.038	0.363	Cell adhesion
SLC2A3	0.049	0.357	Glucose transport
BHMT2	0.001	0.355	Homocysteine S-methyltransferase
P8	0.007	0.354	Apoptosis
PDGFRB	0.012	0.353	Signal transduction
RASSF2	0.020	0.350	Signal transduction
TMPRSS2	0.009	0.349	Proteolysis
PIK3R2	0.013	0.347	Apoptosis
GNB4	0.019	0.347	Signal trasduction
WT1	0.006	0.344	Transcription factor, cell cycle regulator
PSKH2	0.022	0.342	Kinase, tranferase
SLC16A3	0.003	0.329	Anion transport
CFTR	0.032	0.329	Ion channel
PSTPIP1	0.012	0.328	Signal transduction
SFRP4	0.006	0.325	Cell differentiation
GIMAP1	0.001	0.324	Immunity associated protein
GIMAP5	0.015	0.320	Immunity associated protein
KCNE3	0.005	0.320	Ion transport
RASSF1	0.004	0.319	Cell cycle regulator
ADCY9	0.009	0.318	Signal transduction

Gene symbol	P Value	β -value difference	Function
C20orf100	0.026	0.318	Transcription
ANXA6	0.021	0.316	Calcium ion binding
SFRP2	0.001	0.315	Cell differentiation
C12orf34	0.003	0.315	Unknown
FZD2	0.017	0.315	Signal transduction
CA3	0.020	0.314	Metal ion binding
KIF17	0.021	0.311	Protein transport
TGIF	0.001	0.310	Transcription
WDR72	0.023	0.309	Unknown
RB1	0.006	0.309	Cell cycle regulator, transcription
GPR132	0.010	0.308	Signal transduction
HIST1H4I	0.016	0.306	Maintenance of chromatin architecture
ANGPTL2	0.008	0.303	Development
DNAJB8	0.029	0.303	Protein folding
RRP22	0.016	0.301	Transduction

* indicates genes where multiple CpG islands were differentially hypermethylated. Genes in bold were analyzed in the validation cohort of parathyroid tumors. β - Value Difference is the difference between the raw β - Values parathyroid adenomas and normal parathyroid tissue.

TABLE 2

The Top-50 Most Differentially Hypermethylated Genes in Parathyroid Carcinomas Versus Normal Parathyroid Glands.

Gene symbol	P Value	β - Value difference	Function
CDKN2B *	0.001	0.728	Tumor suppressor
KIAA0323	0.023	0.679	Catalytic activity
CLDN6	0.004	0.679	Cell adhesion
CDKN2A *	0.008	0.675	Tumor suppressor
CFTR	0.001	0.664	Ion transport
CMTM2	0.002	0.659	Cytokine
PYCARD*	0.018	0.653	Cell cycle regulation, apoptosis
UBTD1*	0.002	0.649	Protein modification
BNC1	0.027	0.643	Transcription regulation
GPC2	0.024	0.625	Galactosylceramide sulfotransferase
AJAP1	0.031	0.615	Unknown
LYPD3	0.028	0.606	Unknown
HIST1HEJ	0.001	0.602	Maintenance of chromatin structure
HOXC11	0.005	0.598	Transcription factor
SFRP1	0.001	0.596	Cell differentiation
DGKI	0.016	0.595	Intracellular signaling
SFRP4	0.002	0.595	Cell differentiation
ABCA3	0.001	0.595	Transporter
CTPS	0.007	0.585	Nucleotide metabolism
C10orf116	0.035	0.584	Unknown
WT1 *	0.047	0.574	Transcription factor
TCL1A	0.016	0.570	Development
ALS2CR11	0.002	0.570	Calcium ion binding
DUSP8	0.026	0.569	Phosphatase
GATA4	0.044	0.567	Transcription regulation
RRP22	0.006	0.567	Signal transduction
SPIB*	0.007	0.562	Transcription factor
BTG3	0.010	0.558	Cell cycle regulation
VILL	0.048	0.552	Calcium ion binding
TAL1	0.003	0.545	Transcription regulation
WNK4	0.027	0.542	Ion transport
NHMT	0.034	0.541	S- methyltransferase
SFRP2	0.007	0.537	Cell differentiation
PSKH2	0.018	0.536	Kinase
HIST1H4I	0.006	0.536	Maintenance of chromatin structure

Gene symbol	P Value	β - Value difference	Function
hCAP-D3	0.028	0.534	Unknown
PTPN20B*	0.003	0.530	Phosphatase
C12orf34	0.031	0.527	Unknown
OTOP3	0.025	0.524	Unknown
TCF21	0.002	0.518	Transcription factor
KIAA1822	0.049	0.517	Scavenger receptor
SAMD4A	0.036	0.516	Unknown
COG4	0.017	0.516	Protein transport
SCARA3	0.011	0.514	Scavenger receptor
SLC22A16	0.016	0.511	Ion transport
SOCS3	0.010	0.511	Apoptosis
DLEC1	0.060	0.511	Cell proliferation
C20orf100	0.001	0.510	Transcription regulation
GEFT	0.032	0.509	Guanyl- nucleotide exchange factor
KCNG3	0.001	0.507	Ion transport

* indicates genes where multiple CpG islands were differentially hypermethylated. Genes in bold were analyzed in the validation cohort of parathyroid tumors. β - Value Difference is the difference between the raw β - Values parathyroid carcinomas and normal parathyroid tissue