DNA Methylation Signatures Identify Biologically Distinct Thyroid Cancer Subtypes

Sandra Rodríguez-Rodero,* Agustín F. Fernández,* Juan Luís Fernández-Morera, Patricia Castro-Santos, Gustavo F. Bayon, Cecilia Ferrero, Rocio G. Urdinguio, Rocío Gonzalez-Marquez, Carlos Suarez, Iván Fernández-Vega, Manuel Florentino Fresno Forcelledo, Pablo Martínez-Camblor, Veronika Mancikova, Esmeralda Castelblanco, Marco Perez, Pablo Isidro Marrón, Marta Mendiola, David Hardisson, Pilar Santisteban, Garcilaso Riesco-Eizaguirre, Xavier Matías-Guiu, Amancio Carnero, Mercedes Robledo, Elías Delgado-Álvarez, Edelmiro Menéndez-Torre, and Mario F. Fraga†

Objective: The purpose of this study was to determine the global patterns of aberrant DNA methylation in thyroid cancer.

Research Design and Methods: We have used DNA methylation arrays to determine, for the first time, the genome-wide promoter methylation status of papillary, follicular, medullary, and anaplastic thyroid tumors.

Results: We identified 262 and 352 hypermethylated and 13 and 21 hypomethylated genes in differentiated papillary and follicular tumors, respectively. Interestingly, the other tumor types analyzed displayed more hypomethylated genes (280 in anaplastic and 393 in medullary tumors) than aberrantly hypermethylated genes (86 in anaplastic and 131 in medullary tumors). Among the genes indentified, we show that 4 potential tumor suppressor genes (*ADAMTS8*, *HOXB4*, *ZIC1*, and *KISS1R*) and 4 potential oncogenes (*INSL4*, *DPPA2*, *TCL1B*, and *NOTCH4*) are frequently regulated by aberrant methylation in primary thyroid tumors. In addition, we show that aberrant promoter hypomethylation-associated overexpression of *MAP17* might promote tumor growth in thyroid cancer.

Conclusions: Thyroid cancer subtypes present differential promoter methylation signatures, and nondifferentiated subtypes are characterized by aberrant promoter hypomethylation rather than hypermethylation. Additional studies are needed to determine the potential clinical interest of the tumor subtype-specific DNA methylation signatures described herein and the role of aberrant promoter hypomethylation in nondifferentiated thyroid tumors. **(*J Clin Endocrinol Metab* 98: 2811–2821, 2013)**

Thyroid cancer is the most frequent malignant neoplasm in the endocrine system. According to their histopathological characteristics, thyroid tumors are classified as differentiated or nondifferentiated cancers. Differentiated tumors are subdivided into papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC). Nondifferentiated tumors include 2 main subtypes: anaplastic thyroid cancer (ATC) and medullary thyroid carcinoma (MTC). PTCs and FTCs often have a good prognosis when identified in the early stages of the disease. In contrast, ATC represents the most aggressive and undifferentiated subtype of thyroid cancer, responds poorly to treatment, and is associated with the worst prognosis due to the generally invasive local growth and distant metastasis (lungs) (1). Thyroid malignancies are mainly derived from follicular cells; PTC, FTC, and ATC together constitute 95% of thyroid cancers with the papillary variant representing 80% to 85% of the total. In contrast, parafollicular C cells develop MTCs, whose incidence accounts for 3% to 12% of thyroid cancers. Factors involved in the etiology of thy-

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.
Copyright © 2013 by The Endocrine Society
Received October 9, 2012. Accepted April 17, 2013.
First Published Online May 10, 2013

* S.R.-R. and A.F.F. contributed equally to this work.
† Author affiliations are shown at the bottom of the next page
Abbreviations: ATC, anaplastic thyroid cancer; FTC, follicular thyroid cancer; GO, gene ontology; MTC, medullary thyroid cancer; PTC, papillary thyroid cancer.

Downloaded from https://academic.oup.com/jcem/article/98/7/2811/2537028 by guest on 18 April 2024

roid cancer include radiation exposure or iodine-deficient diets (2), but the underlying molecular mechanisms are still largely unknown.

Thyroid tumors have been shown to present many genetic alterations. Examples include germline mutations at the protein kinase, cAMP-dependent, regulatory, type I, α (*PRKAR1A*) and somatic mutations involved in the activation of oncogenes such as the v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) and the RAS family (3, 4) or in the inactivation of tumor suppressor genes such as the phosphatase and tensin homolog (*PTEN*) and the tumor protein p53 (*TP53*) (5). Furthermore, mutations in the rearranged during transfection proto-oncogene (*RET*) are associated with development of thyroid tumors (6, 7). Besides genetic mistakes, recent research has demonstrated that epigenetic alterations might also play an essential role in thyroid cancer. For example, the thyroid-specific genes solute carrier family 5 (sodium iodide symporter), member 5 (*SLC5A5*) and TSH receptor (*THSR*) have been shown to be frequently repressed by aberrant DNA promoter hypermethylation in thyroid cancer (8–10). It is worth mentioning that, because both genes play an important role in iodine uptake, it has been proposed that tumors displaying aberrant hypermethylation at their gene promoters could be good candidates for receiving demethylating agents in conjunction with TSH-promoted radioiodine therapy (10). Other examples of genes frequently hypermethylated in thyroid cancer include the apoptosis-related cysteine protease (*CASP8*) (11), the tissue inhibitor of metalloprotease 3 (*TIMP3*) (12, 13), the ataxia telangiectasia mutated gene (*ATM*) (8), and RAS association domain family protein 1 (*RASSF1*) (14).

Although the aberrant methylation status of a number of candidate genes in specific thyroid cancer subtypes are well characterized, the genome-wide promoter DNA methylation patterns of thyroid cancer are still unknown. To address this issue, we used 27 K Infinium Methylation Arrays to analyze the methylation status of healthy thyroid tissues, thyroid cancer cell lines, and primary PTCs, FTCs, ATCs, and MTCs. Results identified tumor subtype-specific promoter DNA methylation signatures with potential as clinical predictors.

Materials and Methods

The experimental details, including human samples, genetic and epigenetic analyses, and functional assays, are presented as Supplemental Materials and Methods (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

Human tissue samples

We analyzed a cohort of 114 snap-frozen thyroid samples, part of which were obtained from the Tumor Bank at the Institute of Oncology of Asturias (Asturias, Spain), part from the Pathology and Otolaryngology Departments at the Hospital Universitario Central de Asturias (Asturias, Spain) and the remainder from the Hereditary Endocrine Cancer Group (Spanish National Cancer Research Centre, CNIO), the Hospital Universitario La Paz (Madrid, Spain), and the Department of Pathology and Molecular Genetics (Hospital Arnau de Vilanova, Lerida, Spain). The samples consisted of 24 PTCs, 7 FTCs, 11 ATCs, 26 MTCs, and 4 goiter and 6 normal thyroid samples. Additionally, 18 paired normal-tumor samples (12 PTCs and 6 ATCs) were analyzed.

Statistical analysis

All data imports, processing, and downstream analyses were conducted in R (version 2.15.0) (http://www.r-project.org/) and Bioconductor (development version 2.11) (15). In general, all statistical tests were performed using a significance level of .05.

Results

Genome-wide promoter DNA methylation profiling and differentially methylated genes in thyroid cancer

We compared the DNA methylation status of 27 578 sequences in 2 PTCs, 2 FTCs, 2 MTCs, 2 ATCs, 2 nontumorigenic thyroid samples, and 4 independently isolated human thyroid cancer cell lines (K1, FTC-133, 8305C, and TT) using Illumina Infinium Methylation Arrays. As expected, the methylation status of 1063 CpG sites in 591 genes located in the X and Y chromosomes were perfectly correlated with gender (Supplemental Figure 1). Unsupervised clustering of samples exclusively using the methylation signals of the autosomal probes (26 239; 13 789 genes) revealed 2 main clusters (Figure 1): one cluster comprising the healthy tissues and the primary tumors and the other containing 3 of the 4 cancer cell lines

Departments of Endocrinology and Nutrition (S.R.-R., J.L.F.-M., E.D.-A., E.M.-T.) and Pathology (I.F.-V., M.F.F.F.) and Cancer Epigenetics Laboratory (A.F.F., P.C.-S., G.F.B., C.F., R.G.U., M.F.F.) and Servicio de Otorrinolaringología (R.G.-M., C.S.), Instituto Universitario de Oncología del Principado de Asturias, Hospital Universitario Central de Asturias; Biobanco Hospital Universitario Central de Asturias (P.M.) 33006 Oviedo, Spain; and Oficina de Investigación Biosanitaria de Asturias (P.M.-C.) 33005 Oviedo, Spain, and Department of Medicine (E.D.-A.), Universidad de Oviedo, 33006 Oviedo, Spain; Facultad de Ciencias de la Salud (P.C.-S.), Universidad Autónoma de Chile, Alemania 1090, Chile; Hereditary Endocrine Cancer Group (V.M., M.R.), Spanish National Cancer Research Center, Madrid, Spain, and Centro de Investigación Biomédica en Red de Enfermedades 28029 Raras, Spain; Departamento de Patologia y Genetica Molecular (E.C., X.M.-G.), Hospital Universitario Arnau de Vilanova, Universidad de Lerida, 25198 Lerida, Spain; Instituto de Biomedicina de Sevilla (M.P., A.C.), Hospital Universitario Virgen del Rocío/Consejo Superior de Investigaciones Científicas (CSIC)/Universidad de Sevilla, Departamento de Oncohematologia y Genetica, 41013 Sevilla, Spain; Laboratory of Pathology and Oncology (M.M.), Research Unit, and Departments of Pathology (D.H.) and Endocrinology (G.R.-E.), Hospital Universitario La Paz, Instituto de Investigación Hospital Universitario La Paz 28046; and Instituto de Investigaciones Biomédicas "Alberto Sols" (P.S.) 28029 Madrid, Spain, CSIC-Universidad Autónoma de Madrid; and Department of Immunology and Oncology (M.F.F.), Centro Nacional de Biotecnología/CSIC, Cantoblanco, Madrid 28049, Spain

Downloaded from https://academic.oup.com/jcem/article/98/7/2811/2537028 by guest on 18 April 2024

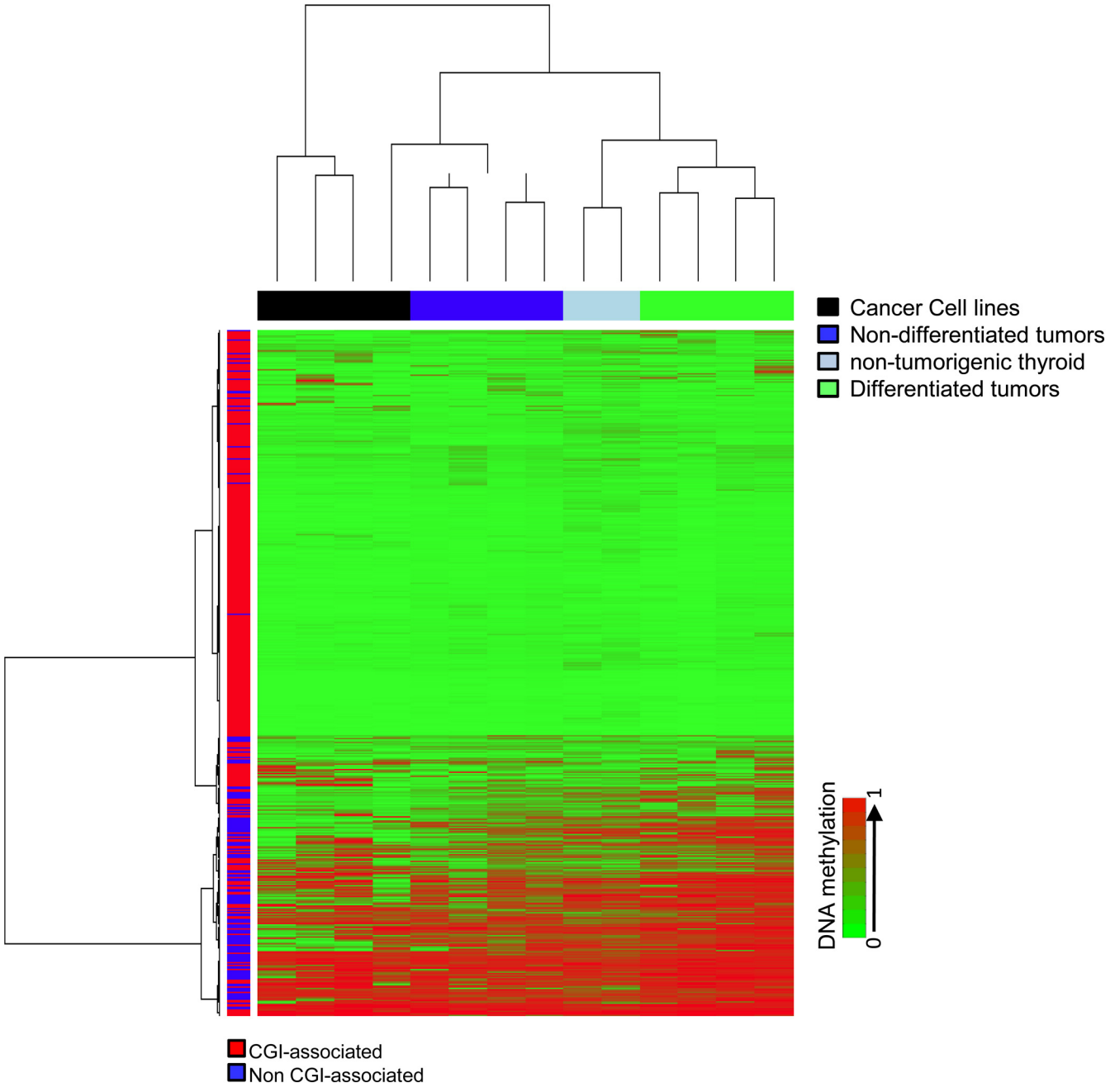

Figure 1. Hierarchical clustering heatmap showing the DNA methylation patterns of autosomal probes in 2 normal thyroid samples, 8 thyroid primary tumors, and 4 thyroid cancer cell lines. Whether the CpG site analyzed is associated to a CpG island (CGI) or not can clearly be distinguished.

(K1, FTC-133, and 8305C). Interestingly, papillary and follicular tumors subclustered together with healthy tissues, and nondifferentiated tumors were more similar to cancer cell lines than differentiated tumors (Figure 1).

To identify differentially methylated genes in thyroid cancer, we initially classified CpG sites according to their methylation status in healthy samples, and following the criteria described in the methodology, we found that 2946 CpGs were unmethylated and 16 901 were methylated. Initial data analysis revealed 8613 CpG sites (6904 genes) in primary tumors that showed consistent DNA methylation changes compared with methylated and unmethylated genes in normal thyroid tissues (Supplemental Table 2). To study these differentially methylated genes in more detail, in each thyroid cancer subtype, we classified CpG sites depending on their methylation status (see Materials and Methods). We then identified genes that were differentially methylated in each thyroid cancer subtype compared with normal tissues: 309 CpGs (corresponding to 262 genes) that were hypermethylated in PTCs, 408 (352 genes) in FTCs, 148 (131 genes) in MTCs, and 114 (86 genes) in ATCs (Supplemental Table 3 and Figure 2, A and

Downloaded from https://academic.oup.com/jcem/article/98/7/2811/2537028 by guest on 18 April 2024

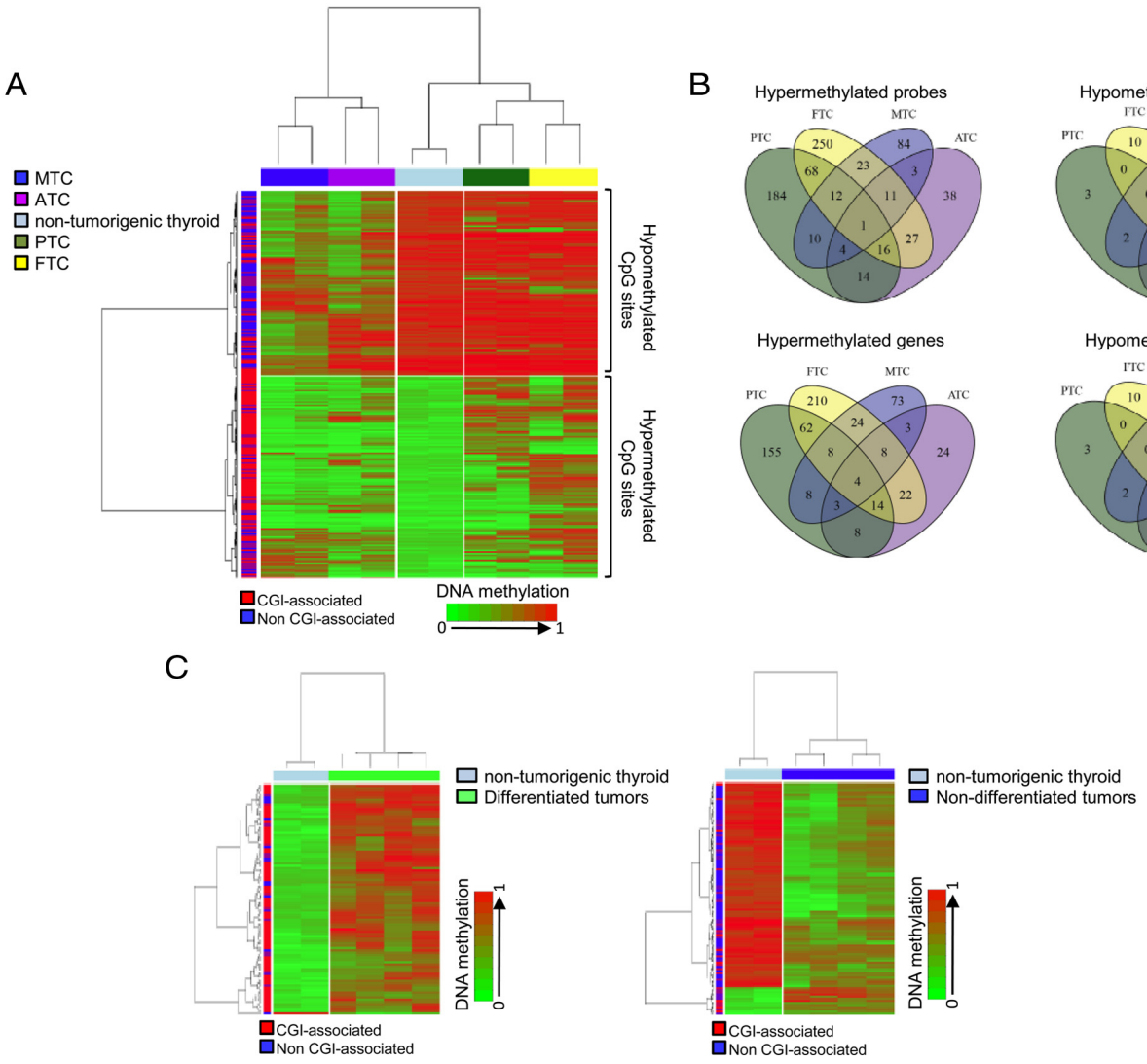

Figure 2. A, Hierarchical clustering heatmap including all CpG dinucleotides with differential DNA methylation values (hypermethylated and hypomethylated) from each thyroid cancer subtype. B, Venn diagrams showing the number of differentially methylated CpG sites and genes in each thyroid cancer subtype and differentially methylated CpG sites and genes shared by the different subtypes. C, Hierarchical clustering heatmap including common hypermethylated and hypomethylated CpG sites in differentiated (left) and nondifferentiated primary tumors (right).

B). Of these, 184 CpG sites (corresponding to 155 genes) were hypermethylated only in PTCs, 250 (210 genes) only in FTCs, 84 (73 genes) only in MTCs, and 38 (24 genes) only in ATCs (Supplemental Table 4 and Figure 2B). Similarly, these criteria allowed the identification of 14 (corresponding to 13 genes) hypomethylated probes in PTCs, 24 (21 genes) in FTCs, 490 (393 genes) in MTCs, and 328 (280 genes) in ATCs (Supplemental Table 5 and Figure 2, A and B). Of these, 3 CpG sites (corresponding to 3 genes) were hypomethylated specifically in PTCs (and in no other tissue), 10 (10 genes) in FTCs, 338 (261 genes) in MTCs, and 174 (147 genes) in ATCs (Supplemental Table 6 and Figure 2B). It is worth noting that the detailed examination of the CpG content of the sequences targeted by alterations of DNA methylation showed that hypermethylation occurred more frequently at CpG islands (77.2%), wherease hypomethylation was more frequent at CpG-poor regions (22.8%) (Fisher's exact test, $P < .001$) (Figure 2A). However, we did not find any statistically significant differences between hypermethylated and hypomethylated CpG sites with respect to their average distance to the transcription start site (Supplemental Figure 2).

To study the DNA methylation changes in thyroid cancer cell lines, we first identified CpG sites that were differentially methylated in each thyroid cancer cell line compared with normal tissues. We observed that 548 (74%) hypermethylated and 637 (93%) hypomethylated CpG sites in primary tissues were also hypermethylated and hypomethylated in cancer cell lines. A more detailed analysis showed that 334 (53.9%) hypermethylated and 25 (81.5%) hypomethylated CpG sites identified in differentiated primary tumors (PTCs and FTCs) were also present in differentiated thyroid carcinoma-derived cancer cell

Downloaded from https://academic.oup.com/jcem/article/98/7/2811/2537028 by guest on 18 April 2024

lines (K1 and FTC133). In addition, we found that 198 (81.5%) hypermethylated and 598 (89%) hypomethylated CpG sites identified in nondifferentiated primary tumors (MTCs and ATCs) were also present in nondifferentiated thyroid carcinoma-derived cancer cell lines (TT and 8305C).

Collectively, differentiated tumors (PTCs and FTCs) showed more hypermethylated than hypomethylated CpG sites (620 vs 37; Fisher's exact test, $P < .001$), whereas in nondifferentiated tumors (MTCs and ATCs), the opposite was found: 243 CpG sites that were hypermethylated vs 672 hypomethylated (Fisher's exact test, $P < .001$) (Figure 2, A and B). Specific analysis of common hypermethylated and hypomethylated CpG sites in differentiated and nondifferentiated primary tumors again showed that the location of these DNA methylation events differed; CpG dinucleotide hypermethylation mostly occurred at CpG islands, whereas CpG hypomethylation was present mainly in non–CpG-island genes (Fisher's exact test, $P < .001$) (Figure 2C).

Relationship between promoter hypermethylation and hypomethylation and genome-wide expression

To determine the role of aberrant promoter methylation on gene expression, we compared our methylation data with the available expression data of normal thyroid tissue and that of primary tumors of each subtype (GEO database GSE27155) (16). We first identified differentially expressed genes for each thyroid cancer subtype (*t* test, false discovery rate <0.05), and compared the associations between hypermethylated and hypomethylated genes with gene expression (Figure 3). We found that 7.5% of the genes hypermethylated in PTC, 1.5% in FTC, 7.3% in MTC, and 7.7% in ATC were downregulated. Moreover, we found that 12.5% of the genes hypomethylated in PTC, 5.2% in MTC, and 3.2% in ATC were upregulated (Figure 3). We did not, however, find any association between hypomethylated genes in FTC and changes in gene expression. Furthermore, no preferential association between promoter hypermethylation or hypomethylation and changes in gene expression was found.

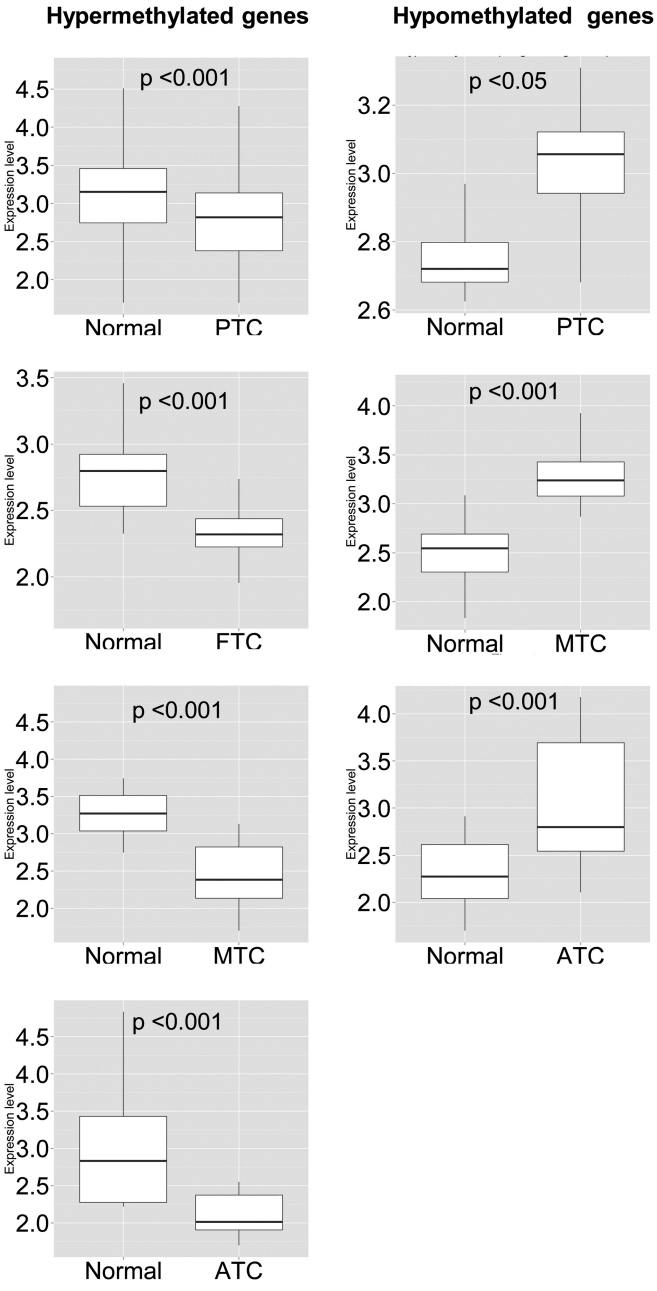

Figure 3. Box plots of microarray-based gene expression data including genes differentially expressed by thyroid cancer subtypes. In each thyroid cancer subtype, differentially methylated genes (hypermethylated and hypomethylated) exhibited lower and higher expression levels respectively compared with normal tissues. *P* values are shown.

Gene ontology categories of hypermethylated and hypomethylated genes

We used gene ontology (GO) to analyze the biological functions of the hypermethylated and hypomethylated genes in thyroid cancer subtypes. A list of significantly enriched GO terms is provided in Supplemental Tables 7 and 8 (Fisher´s exact test, $P < .001$). Analysis of hypermethylated genes showed nonrandom distribution of GO terms in biological processes. These genes were mainly enriched for terms related to developmental processes in PTC, FTC, and ATC (Supplemental Table 7).

We also used the GO to investigate the biological functions of the hypomethylated genes (Supplemental Table 8). We identified significant enrichment of specific GO terms in poorly differentiated thyroid tumors (MTC and ATC), whereas the analysis did not detect any enriched terms in differentiated tumors (PTC and FTC). MTC and ATC hypomethylated genes were enriched for GO terms

Downloaded from https://academic.oup.com/jcem/article/98/7/2811/2537028 by guest on 18 April 2024

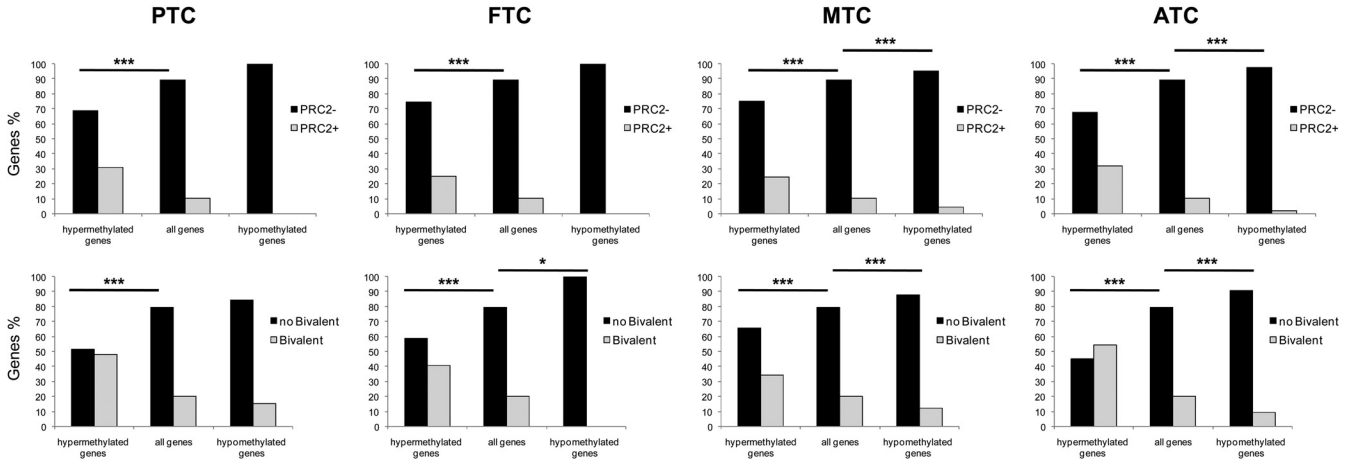

Figure 4. Bar plots for each thyroid cancer subtype displaying the percentage of genes enriched for polycomb repressor complex 2 (upper panels) and for 3mK4H3 and 3mK27H3 (lower panels) in embryonic stem cells. *, $P < .05$; **, $P < 0.01$; ***, $P < .001$.

mainly associated with the immune system (Supplemental Table 8).

Enrichment of PcG marks and bivalent histone domains of aberrantly methylated genes in thyroid cancer

To study genomic features that provide clues about the mechanisms underlying the aberrant methylation changes in thyroid cancer we investigated whether the differentially methylated genes were among those targeted by the polycomb repressive complex 2 (*PRC2*) (17) or bivalent histone domains (3mK4H3 + 3mK27H3) (18) in embryonic stem cells. As shown in Figure 4, compared with all the genes analyzed in the array, hypermethylated genes were highly enriched for loci targeted by PcG and bivalent histone marks in each thyroid cancer subtype (Fisher´s exact test, $P < .001$) (Figure 4). Conversely, hypomethylated genes showed a decrease in genes targeted by PcG and bivalent marks (Figure 4).

Hypermethylated tumor suppressor candidate genes in differentiated primary tumors

To further validate the results obtained with the methylation array for differentiated tumors, we selected 2 hypermethylated genes found in PTC, *ADAMTS8* and *HOXB4*, and another 2 hypermethylated genes in FTC, *ZIC1* and *KISS1R* (Figure 5). These selected genes have frequently been found hypermethylated, or been described as tumor suppressors, in other cancer types (19–22). We determined their promoter methylation profiles in 38 samples by bisulfite pyrosequencing. Results corroborated the methylation array data (Figure 5 and Supplemental Figure 3). We detected hypermethylation at the *ADAMTS8* and *HOXB4* promoters in 4 of the 22 PTC tumors (18%) and hypermethylation at the *ZIC1* and *KISS1R* promoters in 4 and 1 of the 6 FTC tumors, respectively (67% and 17%), confirming that this is a frequent event in vivo.

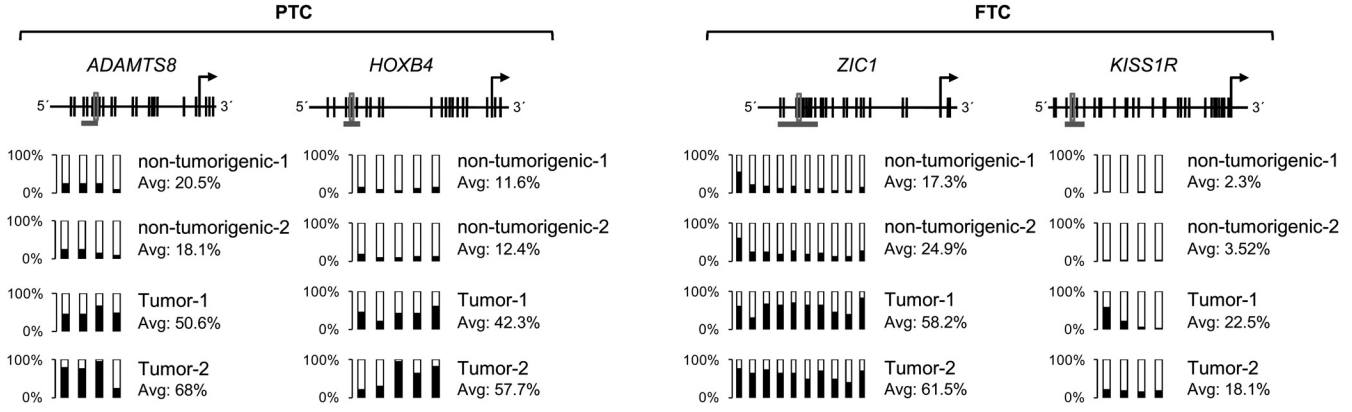

Figure 5. Examples of PTC and FTC hypermethylated CpG sites in particular genes further validated by pyrosequencing. A schematic representation of the studied region and the methylation values from 2 representative primary tumors and nontumorigenic (goiter) samples are shown. The location of each CpG site is represented by vertical lines, and the right angled arrow indicates the transcriptional start site. The analyzed region (blue line) and the CpG sites in the array (red box) are highlighted. Black depicts methylated CpG, and white indicates unmethylated CpG.

Downloaded from https://academic.oup.com/jcem/article/98/7/2811/2537028 by guest on 18 April 2024

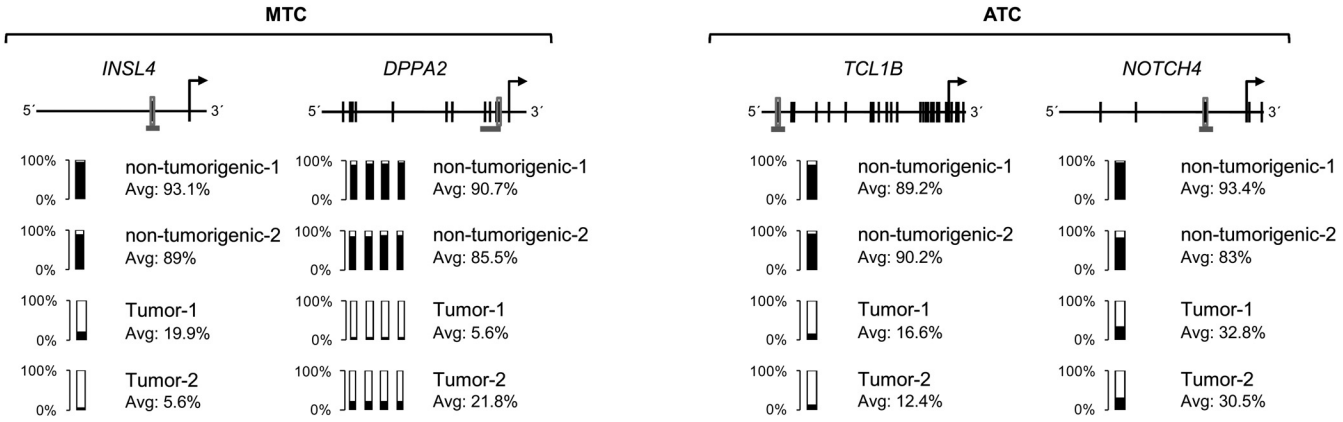

Figure 6. Examples of MTC and ATC hypomethylated CpG sites in particular genes further validated by pyrosequencing. A schematic representation of the studied region and the methylation values from 2 representative primary tumors and normal nontumorigenic (goiter) samples are shown. The location of each CpG site is represented by vertical lines, and the right angled arrow indicates the transcriptional start site. The analyzed region (blue line) and the CpG sites in the array (red box) are highlighted. Black depicts methylated CpG, and white indicates unmethylated CpG.

We also carried out a DNA methylation analysis of *ADAMTS8* and *HOXB4* promoters in an additional set of 12 paired papillary normal-tumor samples. The bisulfite pyrosequencing results corroborated our previous data (Supplemental Figure 4). To determine the role of aberrant promoter methylation in gene expression, we compared our methylation data for hypermethylated tumors with the available expression data of normal-tumor paired thyroid samples. We found that gene hypermethylation was not always associated with gene repression (Supplemental Figure 4). To further validate the DNA methylation associations with gene expression, we analyzed the papillary-derived cancer cell line K1 and found that *ADAMTS8* and *HOXB4* promoter hypermethylation was associated with gene repression (Supplemental Figure 5).

Hypomethylated candidate oncogenes in nondifferentiated primary tumors

To validate the results obtained with the methylation array for nondifferentiated tumors, we selected 2 genes found hypomethylated in MTC, *INSL4* and *DPPA2*, and another 2 genes hypomethylated in ATC, *TCL1B* and *NOTCH4* (Figure 6). These selected genes have been described as potential oncogenes in other cancer types (23–26). We determined their promoter methylation profiles in 35 samples by bisulfite pyrosequencing. Results corroborated the methylation array data (Supplemental Figure 3). We detected hypomethylation at the *INSL4* and *DPPA2* promoters in 12 and 6 of the 20 MTC tumors, respectively (60% and 30%) and hypomethylation at the *TCL1B* and *NOTCH4* promoters in 7 and 5 of the 11 ATC tumors, respectively (64% and 45%), evidencing the frequent occurrence of this event in vivo.

We also carried out a DNA methylation analysis of *TCL1B* and *NOTCH4* promoters in an additional set of 6 paired anaplastic normal-tumor samples. Again, the bisulfite pyrosequencing results corroborated our previous data (Supplemental Figure 4). The role of aberrant promoter methylation in gene expression was investigated by comparing our methylation data for hypomethylated tumors with expression data of normal-tumor paired thyroid samples. We found that, in some cases, gene hypomethylation was associated with gene activation (Supplemental Figure 4). To further study the associations between DNA methylation and gene expression, we analyzed the anaplastic-derived cancer cell line 8305C and found that *TCL1B* and *NOTCH4* hypomethylation was not associated with gene regulation (Supplemental Figure 5).

To study the functional role of aberrant methylation in thyroid cancer, we focused our attention on *MAP17*, which was less methylated in all the nondifferentiated tumors analyzed in this study as compared with nontumorigenic tissues (Supplemental Table 2) and which has been previously shown to be oncogenic in various types of tumors (27, 28).

To determine whether *MAP17* hypomethylation is a frequent in vivo event, we used bisulfite pyrosequencing to analyze its promoter methylation status in 35 nondifferentiated thyroid tumors and 6 normal thyroid samples. On average, DNA methylation of normal samples was 75% ± 12.4%. We detected hypomethylation (at least 20% less when comparing with normal tissue) at the *MAP17* promoter in 10 of the 26 medullary thyroid tumors (38%) and in 3 of the 9 anaplastic thyroid tumors (33%), which confirmed the data obtained with the methylation arrays (Supplemental Figure 6). To evaluate the functional role of aberrant DNA methylation of *MAP17* in thyroid cancer, we ectopically overexpressed *MAP17* in the thyroid can-

Downloaded from https://academic.oup.com/jcem/article/98/7/2811/2537028 by guest on 18 April 2024

cer cell line TT, which showed DNA methylation-associated *MAP17* repression (Supplemental Figure 6). Immunohistochemistry analysis showed that transfected cells expressed higher levels of MAP17 than parental cells (Supplemental Figure 7). In vitro, *MAP17* overexpression was associated with increased number of colonies, cell growth, and migration capability (Supplemental Figure 7). To further characterize the role of *MAP17* in vivo, we injected *MAP17*-overexpressing TT cells and control cells transfected with empty vector sc into immunodeficient nude mice. Over the same period, *MAP17*-expressing tumors were found to be larger than those lacking *MAP17* expression (Supplemental Figure 7), suggesting the potential oncogenicity of *MAP17*.

Discussion

Although aberrant genomic DNA methylation is a hallmark of cancer (29) and the genome-wide aberrant promoter DNA methylation patterns of many tumor types has been meticulously described (30–32), data on aberrant DNA methylation in thyroid cancer are mainly restricted to single-copy candidate genes in specific tumor subtypes (33–35). In this study, we used DNA methylation arrays to determine, for the first time, the genome-wide promoter methylation status of primary papillary, follicular, medullary, and anaplastic thyroid tumors, nontumorigenic thyroid tissues, and 4 thyroid cancer cells lines. Using this approach, we identified 262 genes hypermethylated and 13 hypomethylated in papillary tumors, 352 hypermethylated and 21 hypomethylated in follicular tumors, 86 hypermethylated and 280 hypomethylated in anaplastic tumors and, 131 hypermethylated and 393 hypomethylated in medullary tumors. As in other tissue types (36), healthy samples, cancer cell lines, and primary tumors presented specific DNA methylation signatures that permit their clustering within their corresponding category simply by noting their DNA methylation patterns. Papillary, follicular, medullary, and anaplastic thyroid tumor subtypes were also found to present specific DNA methylation signatures, which suggests that methylation profiling in thyroid cancer might be of clinical value in diagnosis, prognosis, and prediction of response to therapies (44). As in previous studies using the similar methylation arrays (31, 32), differentiated papillary and follicular tumors showed many more hypermethylated (526) than hypomethylated (33) genes. In contrast, nondifferentiated medullary and anaplastic tumors exhibited more hypomethylated than hypermethylated genes (546 vs 199). Because MTCs originate from parafollicular cells, we cannot completely rule out the possibility that some genes are methylated in a cell-type–specific fashion.

The role of promoter hypomethylation in cancer is still largely unknown. Previous studies have shown that hepatocellular tumors display more hypomethylated than hypermethylated gene promoters (38–40). However, these studies focused their attention mainly in the aberrant hypermethylated promoters. In the case of thyroid cancer, promoter hypomethylation might be even more relevant because it is specifically enriched in nondifferentiated medullary and anaplastic tumors. Because these tumor types are more aggressive and present poorer prognosis than differentiated tumors, further studies should be undertaken to determine the specific role of aberrant promoter hypomethylation in thyroid cancer.

The comparison of our methylation data with genome-wide gene expression data and the analysis of paired normal-tumor primary tissues showed that the role of aberrant promoter methylation in gene expression in thyroid cancer is complex. We found that regardless of methylation status, in nontumorigenic thyroid tissues, changes in DNA methylation do not necessarily involve changes in gene expression. This concurs with previous studies showing that changes in DNA methylation are not always associated with changes in gene expression, both in healthy tissues (41) and cancer (42, 43). Our data indicate that, in some cases, aberrant DNA methylation-associated gene regulation might play an important role in tumor growth. Indeed, we observed that hypomethylation and overexpression of the membrane-associated protein 17 (*MAP17*) promotes thyroid tumor growth in vitro and in vivo. Interestingly, *MAP17* has been previously shown to be oncogenic in a number of tumor types including lung, colon, ovarian, and skin cancer (27, 28). Thus, our observations in thyroid cancer suggest that aberrant promoter hypomethylation of *MAP17* might be a frequent event in cancer in general. Although our results show that, in certain genes, aberrant DNA methylation is not associated with gene expression changes, this does not imply that such molecular alterations are not important in cancer. For example, regardless of the functional implications, the aberrant patterns of DNA methylation in cancer might have important clinical applications because they can behave as diagnostic, prognostic, and/or response markers (44). Further studies are required to determine the role of nonfunctional aberrant methylation in thyroid cancer.

GO analysis indicated that gene promoter hypermethylation and hypomethylation in thyroid tumors tend to target different biological processes. Hypermethylated genes were mainly found to be related to developmental processes along the lines of/similar to those found in previous studies of other cancer types (45, 46). However,

Downloaded from https://academic.oup.com/jcem/article/98/7/2811/2537028 by guest on 18 April 2024

hypomethylated genes were enriched for GO terms associated with the immune system. Because this is associated with more aggressive tumors (MTC and ATC), more research is needed to clarify the role of the immune system in tumor progression in these subtypes of thyroid cancer.

We also confirmed previous observations that CpG hypermethylation events in cancer are significantly more likely to occur in the promoters of those genes with enriched polycomb occupancy and the presence of bivalent histone domains in embryonic stem cells (36, 47–49). This might link thyroid cancer pathogenesis to stem cells and suggests that thyroid tumors might originate from cells with stem-like features.

Using candidate gene approaches, recent works have identified a number of aberrantly methylated genes in thyroid cancer. For example, the cell cycle regulator p16 has been shown to be aberrantly hypermethylated in follicular and papillary thyroid tumors (50, 51). Other tumor suppressor genes frequently methylated in thyroid cancer are *RASFF1A* (32% PTCs, 100% FTCs, 40% MTCs, and 33% ATCs), *Rap1GAP* (72% PTCs and 38% FTCs), a GTPase-activating protein that inhibits *Rap1*, and whose downregulation is related with tumor invasion (52, 53). *PTEN* gene, a negative modulator of the PI3K/AKT pathway that is constitutively activated in tumors, has been found hypermethylated in 50% of PTCs and in 100% of FTCs (54). Aberrant methylation of other tumor suppressor genes such as *TIMP3* (53% PTC), *SLC5A8* (33% PTC), *DAPK* (34% PTC), and *RARβ2* (22% PTC) has been associated with mutation of the proto-oncogene *B-Raf* (*BRAF*) (13). Interestingly, thyroid tumors have also been shown to present aberrant promoter hypermethylation at endocrine-related genes such us the solute carrier family 5 (sodium iodide symporter), member 5 (*SLC5A5*) and the TSH receptor (*THSR*) has been shown to be frequently repressed by aberrant DNA promoter hypermethylation in thyroid cancer (8–10). Other examples of genes frequently hypermethylated in thyroid cancer include the apoptosis-related cysteine protease (*CASP8*) (11) and the ataxia telangiectasia mutated (*ATM*) (8). Several of these genes have also been identified in our study, which supports the reliability of our experimental design and confirms the notion that aberrant promoter methylation is a relevant molecular alteration in thyroid cancer.

Among the genes identified in our study, some have been previously shown to be oncogenic or to have tumor suppressor action in cancer. Although the percentage of aberrant methylation of these candidate genes in the primary tumors is not that high, it is comparable to the percentage of aberrant methylation of many tumor suppressor genes in cancer (44). For example, the member of the ZIC family (C2H2-type zinc finger proteins), *ZIC1*, that we found to be frequently hypermethylated in FTCs has also been shown to be a tumor suppressor gene frequently repressed by aberrant promoter hypermethylation in colorectal cancer (21). The other gene shown to be aberrantly hypermethylated in FTCs in our study, the galanin-like G protein-coupled receptor (*KISS1R* or *GPR54*) has been proposed as suppressing metastasis in melanoma and breast carcinoma (22). Moreover, *KISS1R* was found to be downregulated and to have a metastasis-suppressing role in follicular thyroid tumors (55), the tumor subtype in which we found this gene to be frequently hypermethylated. The 2 genes validated in papillary tumors, *HOXB4* and *ADAMTS8*, have also been proposed as possessing tumor-suppressing activity in cancer (19, 56) that in some cases is mediated by aberrant promoter hypermethylation (19, 20, 56–58). In nondifferentiated thyroid tumors, we validated 4 genes that become aberrantly hypomethylated (*NOTCH4* and *TCL1B* in ATCs and *INSL4* and *DPPA2* in MTCs), all of which have been proposed to have an oncogenic role in cancer: *NOTCH4* is frequently overexpressed in thyroid tumors (59) and has been shown to promote metastasis in salivary adenoid cystic carcinomas (60). *TCL1* is an oncogene frequently activated by reciprocal translocations t(14;14)(q11;q32), t(7;14)(q35;q32) or inversion inv(14) (q11;q32) in leukemia (25). It was also identified as an alternative mechanism of activation in B cell chronic lymphocytic leukemia mediated by promoter hypomethylation (61). Thus, hypomethylation-dependent oncogenic activation of *TCL1* might be a frequent event in cancer. *INSL4* and *DPPA2*, the 2 genes validated in MTCs, have also been reported to promote tumorigenesis in different types of cancer.

INSL4 (pro-EPIL) belongs to the insulin and IGF family and is expressed strongly during the first trimester of pregnancy by the differentiated syncytiotrophoblast (62). It has been shown to be overexpressed in breast tumors with an aggressive phenotype (23), but the underlying mechanisms are still unknown. The aberrant overexpression of *INSL4* in breast tumors together with the aberrant promoter hypomethylation reported in this study suggest that promoter demethylation might be a frequent mechanism of activation of *INSL4* oncogene activation in cancer. *DPPA2* (developmental pluripotency-associated 2) is expressed early in the embryo's development (37) but also in some tumor types (24). Although the underlying molecular mechanism has not been reported yet, our data indicate that promoter hypomethylation might play an important role. Collectively, these results suggest that aberrant promoter methylation is an important molecular mechanism underlying the aberrant regulation of tumor suppressor genes and oncogenes in thyroid cancer.

Downloaded from https://academic.oup.com/jcem/article/98/7/2811/2537028 by guest on 18 April 2024

In conclusion, our methylation profiling in thyroid cancer shows that thyroid cancer subtypes present differential promoter methylation signatures and that nondifferentiated subtypes are characterized by aberrant promoter hypomethylation rather than hypermethylation. Our results could have at least 2 types of clinical implications: the tumor subtype-specific DNA methylation signatures might be useful in diagnosis and/or prognosis, and the frequent promoter hypomethylation observed in nondifferentiated tumors might be relevant for treatment with demethylating drugs.

Acknowledgments

Address all correspondence and requests for reprints to: Mario F. Fraga, PhD, Centro Nacional de Biotecnología/Consejo Superior de Investigaciones Científicas-Departamento de Inmunología y Oncología, 28049 Madrid, Spain. E-mail: mffraga@cnb.csic.es.

This work has been financially supported by Fundación ASTURCOR (to S.R.R); the FIS/FEDER (PI11/01728 to A.F.F. and PI11/02795 to E.D.-A.); the ISCIII (CP11/00131 to A.F.F.); the FIS PI11/01359 to M.R.; the Spanish Ministry of Health (PS09/02454 and PI12/01080 to M.F.F.); the Spanish National Research Council (CSIC; 200820I172 to M.F.F.); Instituto Universitario de Oncología del Principado de Asturias (to C.F. and G.F.B.); Ramon Areces Foundation (to M.F.F. and G.F.B.); Fundacion Cientifica de la AECC (to R.G.U.); RD12/0036/0013, BFU-2010-16025, RD12/0036/0030 (to P.S.), RD12/0036/0015 and PI11/00929 (to C.S.), and S2011/BMD-2328 TIRONET (to P.S. and M.R.). Tumor samples were obtained with the support of Red de Biobancos ISCIII (RD09/0076/0059). V.M. is supported by a fellowship of "la Caixa"/Spanish National Cancer Research Center international PhD Program. The IUOPA is supported by the Obra Social Cajastur, Spain.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Cohen EE.** New approaches to thyroid cancer. *Clin Adv Hematol Oncol*. 2012;10:255–256.
2. **DeVita VT, Samuel A, Wells J.** Cancer of the endocrine system. In: Wilkins LW, ed. *Principles and Practice of Oncology*. 8th ed. Mexico City, Mexico: Wolters Kluwer Health Mexico; 2008
3. **Boichard A, Croux L, Al Ghuzlan A, et al.** Somatic RAS mutations occur in a large proportion of sporadic RET-negative medullary thyroid carcinomas and extend to a previously unidentified exon. *J Clin Endocrinol Metab*. 2012;97:E2031–E2035.
4. **Li C, Lee KC, Schneider EB, Zeiger MA.** BRAF V600E mutation and its association with clinicopathological features of papillary thyroid cancer: a meta-analysis. *J Clin Endocrinol Metab*. 2012;97:4559–4570.
5. **Miyake Y, Aratake Y, Sakaguchi T, et al.** Examination of CD26/DPPIV, p53, and PTEN expression in thyroid follicular adenoma. *Diagn Cytopathol*. 2012;40:1047–1053
6. **Romei C, Elisei R.** RET/PTC Translocations and Clinico-Pathological Features in Human Papillary Thyroid Carcinoma. *Front Endocrinol (Lausanne)*. 2012;3:54.
7. **A T, F S, G P, M B.** Genetic Alterations in Medullary Thyroid Cancer: Diagnostic and Prognostic Markers. *Curr Genom*. 2011;12:618–625.
8. **Smith JA, Fan CY, Zou C, Bodenner D, Kokoska MS.** Methylation status of genes in papillary thyroid carcinoma. *Arch Otolaryngol Head Neck Surg*. 2007;133:1006–1011.
9. **Venkataraman GM, Yatin M, Marcinek R, Ain KB.** Restoration of iodide uptake in dedifferentiated thyroid carcinoma: relationship to human Na^+/I^- symporter gene methylation status. *J Clin Endocrinol Metab*. 1999;84:2449–2457.
10. **Xing M, Usadel H, Cohen Y, et al.** Methylation of the thyroid-stimulating hormone receptor gene in epithelial thyroid tumors: a marker of malignancy and a cause of gene silencing. *Cancer Res*. 2003;63:2316–2321.
11. **Stephen JK, Chitale D, Narra V, Chen KM, Sawhney R, Worsham MJ.** DNA methylation in thyroid tumorigenesis. *Cancers (Basel)*. 2011;3:1732–1743.
12. **Anania MC, Sensi M, Radaelli E, et al.** TIMP3 regulates migration, invasion and in vivo tumorigenicity of thyroid tumor cells. *Oncogene*. 2011;30:3011–3023.
13. **Hu S, Liu D, Tufano RP, et al.** Association of aberrant methylation of tumor suppressor genes with tumor aggressiveness and BRAF mutation in papillary thyroid cancer. *Int J Cancer*. 2006;119:2322–2329.
14. **Xing M, Cohen Y, Mambo E, et al.** Early occurrence of RASSF1A hypermethylation and its mutual exclusion with BRAF mutation in thyroid tumorigenesis. *Cancer Res*. 2004;64:1664–1668.
15. **Gentleman RC, Carey VJ, Bates DM, et al.** Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol*. 2004;5:R80.
16. **Giordano TJ, Kuick R, Thomas DG, et al.** Molecular classification of papillary thyroid carcinoma: distinct BRAF, RAS, and RET/PTC mutation-specific gene expression profiles discovered by DNA microarray analysis. *Oncogene*. 2005;24:6646–6656.
17. **Lee TI, Jenner RG, Boyer LA, et al..** Control of developmental regulators by polycomb in human embryonic stem cells. *Cell*. 2006;125:301–313.
18. **Pan G, Tian S, Nie J, et al.** Whole-genome analysis of histone H3 lysine 4 and lysine 27 methylation in human embryonic stem cells. *Cell Stem Cell*. 2007;1:299–312.
19. **Dunn JR, Reed JE, du Plessis DG, et al.** Expression of ADAMTS-8, a secreted protease with antiangiogenic properties, is downregulated in brain tumours. *Br J Cancer*. 2006;94:1186–1193.
20. **Fackler MJ, Umbricht CB, Williams D, et al.** Genome-wide methylation analysis identifies genes specific to breast cancer hormone receptor status and risk of recurrence. *Cancer Res*. 2011;71:6195–6207.
21. **Gan L, Chen S, Zhong J, et al.** ZIC1 is downregulated through promoter hypermethylation, and functions as a tumor suppressor gene in colorectal cancer. *PLoS One*. 2011;6:e16916.
22. **Ohtaki T, Shintani Y, Honda S, et al.** Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature*. 2001;411:613–617.
23. **Brandt B, Kemming D, Packeisen J, et al.** Expression of early placenta insulin-like growth factor in breast cancer cells provides an autocrine loop that predominantly enhances invasiveness and motility. *Endocr Relat Cancer*. 2005;12:823–837.
24. **John T, Caballero OL, Svobodová SJ, et al.** ECSA/DPPA2 is an embryo-cancer antigen that is coexpressed with cancer-testis antigens in non-small cell lung cancer. *Clin Cancer Res*. 2008;14:3291–3298.
25. **Laine J, Künstle G, Obata T, Noguchi M.** Differential regulation of Akt kinase isoforms by the members of the TCL1 oncogene family. *J Biol Chem*. 2002;277:3743–3751.
26. **Xu K, Moghal N, Egan SE.** Notch signaling in lung development and disease. *Adv Exp Med Biol*. 2012;727:89–98.

Downloaded from https://academic.oup.com/jcem/article/98/7/2811/2537028 by guest on 18 April 2024

27. **Guijarro MV, Leal JF, Blanco-Aparicio C, et al.** MAP17 enhances the malignant behavior of tumor cells through ROS increase. *Carcinogenesis*. 2007;28:2096–2104.
28. **Guijarro MV, Leal JF, Fominaya J, et al.** MAP17 overexpression is a common characteristic of carcinomas. *Carcinogenesis*. 2007;28: 1646–1652.
29. **Esteller M.** Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet*. 2007;8:286–298.
30. **Calvisi DF, Ladu S, Gorden A, et al.** Mechanistic and prognostic significance of aberrant methylation in the molecular pathogenesis of human hepatocellular carcinoma. *J Clin Invest*. 2007;117:2713–2722.
31. **Davidsson J, Lilljebjörn H, Andersson A, et al.** The DNA methylome of pediatric acute lymphoblastic leukemia. *Hum Mol Genet*. 2009; 18:4054–4065.
32. **Omura N, Li CP, Li A, et al.** Genome-wide profiling of methylated promoters in pancreatic adenocarcinoma. *Cancer Biol Ther*. 2008; 7:1146–1156.
33. **Eze OP, Starker LF, Carling T.** The role of epigenetic alterations in papillary thyroid carcinogenesis. *J Thyroid Res*. 2011;2011: 895470.
34. **Hou P, Ji M, Xing M.** Association of PTEN gene methylation with genetic alterations in the phosphatidylinositol 3-kinase/AKT signaling pathway in thyroid tumors. *Cancer*. 2008;113:2440–2447.
35. **Ishida E, Nakamura M, Shimada K, et al.** DNA hypermethylation status of multiple genes in papillary thyroid carcinomas. *Pathobiology*. 2007;74:344–352.
36. **Fernandez AF, Assenov Y, Martin-Subero JI, et al.** A DNA methylation fingerprint of 1628 human samples. *Genome Res*. 2012;22: 407–419.
37. **Maldonado-Saldivia J, van den Bergen J, Krouskos M, et al.** Dppa2 and Dppa4 are closely linked SAP motif genes restricted to pluripotent cells and the germ line. *Stem Cells*. 2007;25:19–28.
38. **De Smet C, Loriot A.** DNA hypomethylation and activation of germline-specific genes in cancer. *Adv Exp Med Biol*. 2013;754:149–166.
39. **Neumann O, Kesselmeier M, Geffers R, et al.** Methylome analysis and integrative profiling of human HCCs identify novel protumorigenic factors. *Hepatology*. 2012;56:1817–1827
40. **Shen J, Wang S, Zhang YJ, et al.** Genome-wide DNA methylation profiles in hepatocellular carcinoma. *Hepatology*. 2012;55:1799–1808.
41. **Calvanese V, Fernández AF, Urdinguio RG, et al.** A promoter DNA demethylation landscape of human hematopoietic differentiation. *Nucleic Acids Res*. 2011;40:116–131.
42. **Gonzalez-Paz N, Chng WJ, McClure RF, et al.** Tumor suppressor p16 methylation in multiple myeloma: biological and clinical implications. *Blood*. 2007;109:1228–1232.
43. **Klug M, Heinz S, Gebhard C, et al.** Active DNA demethylation in human postmitotic cells correlates with activating histone modifications, but not transcription levels. *Genome Biol*. 2010;11:R63.
44. **Heyn H, Esteller M.** DNA methylation profiling in the clinic: applications and challenges. *Nat Rev Genet*. 2012;13:679–692.
45. **Helman E, Naxerova K, Kohane IS.** DNA hypermethylation in lung cancer is targeted at differentiation-associated genes. *Oncogene*. 2012;31:1181–1188.
46. **Shen X, He Z, Li H, et al.** Distinct functional patterns of gene promoter hypomethylation and hypermethylation in cancer genomes. *PLoS One*. 2012;7:e44822.
47. **Ohm JE, Baylin SB.** Stem cell chromatin patterns: an instructive mechanism for DNA hypermethylation? *Cell Cycle*. 2007;6:1040–1043.
48. **Schlesinger Y, Straussman R, Keshet I, et al.** Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. *Nat Genet*. 2007;39:232–236.
49. **Widschwendter M, Fiegl H, Egle D, et al.** Epigenetic stem cell signature in cancer. *Nat Genet*. 2007;39:157–158.
50. **Boltze C, Zack S, Quednow C, Bettge S, Roessner A, Schneider-Stock R.** Hypermethylation of the CDKN2/p16INK4A promotor in thyroid carcinogenesis. *Pathol Res Pract*. 2003;199:399–404.
51. **Lam AK, Lo CY, Leung P, Lang BH, Chan WF, Luk JM.** Clinicopathological roles of alterations of tumor suppressor gene p16 in papillary thyroid carcinoma. *Ann Surg Oncol*. 2007;14:1772–1779.
52. **Nakamura N, Carney JA, Jin L, et al.** RASSF1A and NORE1A methylation and BRAFV600E mutations in thyroid tumors. *Lab Invest*. 2005;85:1065–1075.
53. **Zuo H, Gandhi M, Edreira MM, et al.** Downregulation of Rap1GAP through epigenetic silencing and loss of heterozygosity promotes invasion and progression of thyroid tumors. *Cancer Res*. 2010;70: 1389–1397.
54. **Alvarez-Nuñez F, Bussaglia E, Mauricio D, et al.** PTEN promoter methylation in sporadic thyroid carcinomas. *Thyroid*. 2006;16:17–23.
55. **Ringel MD, Hardy E, Bernet VJ, et al.** Metastin receptor is overexpressed in papillary thyroid cancer and activates MAP kinase in thyroid cancer cells. *J Clin Endocrinol Metab*. 2002;87:2399.
56. **Gray S, Pandha HS, Michael A, Middleton G, Morgan R.** HOX genes in pancreatic development and cancer. *JOP*. 2011;12:216–219.
57. **Dunn JR, Panutsopulos D, Shaw MW, Het al.** METH-2 silencing and promoter hypermethylation in NSCLC. *Br J Cancer*. 2004;91: 1149–1154.
58. **Strathdee G, Holyoake TL, Sim A, et al.** Inactivation of HOXA genes by hypermethylation in myeloid and lymphoid malignancy is frequent and associated with poor prognosis. *Clin Cancer Res*. 2007; 13:5048–5055.
59. **Geers C, Colin IM, Gérard AC.** Delta-like 4/Notch pathway is differentially regulated in benign and malignant thyroid tissues. *Thyroid*. 2011;21:1323–1330.
60. **Ding LC, She L, Zheng DL, et al.** Notch-4 contributes to the metastasis of salivary adenoid cystic carcinoma. *Oncol Rep*. 2010;24: 363–368.
61. **Yuille MR, Condie A, Stone EM, et al.** TCL1 is activated by chromosomal rearrangement or by hypomethylation. *Genes Chromosomes Cancer*. 2001;30:336–341.
62. **Bellet D, Lavaissiere L, Mock P, et al.** Identification of pro-EPIL and EPIL peptides translated from insulin-like 4 (INSL4) mRNA in human placenta. *J Clin Endocrinol Metab*. 1997;82:3169–3172.

Downloaded from https://academic.oup.com/jcem/article/98/7/2811/2537028 by guest on 18 April 2024