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Correlation between *BRAF* mutation and promoter methylation of *TIMP3, RARβ2* and *RASSF1A* in thyroid cancer

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Our aim was to comprehensively analyze promoter hypermethylation of a panel of novel and known methylation markers for thyroid neoplasms and to establish their relationship with *BRAF* mutation and clinicopathologic parameters of thyroid cancer. A cohort of thyroid tumors, consisting of 44 cancers and 44 benign thyroid lesions, as well as 15 samples of adjacent normal thyroid tissue, was evaluated for *BRAF* mutation and promoter hypermethylation. Genes for quantitative methylation specific PCR (QMSP) were selected by a candidate gene approach. Twenty-two genes were tested: *TSHR*, *RASSF1A, RARβ2, DAPK, hMLH1, ATM, S100, p16, CTNNB1, GSTP1, CALCA, TIMP3, TGFβR2, THBS1, MINT1, CTNNB1, MT1G, PAK3, NISCH, DCC, AIM1* and *KIF1A*. The PCR-based "mutector assay" was used to detect *BRAF* mutation. All p values reported are two sided. Considerable overlap was seen in the methylation markers among the different tissue groups. Significantly higher methylation frequency and level were observed for *KIF1A* and *RARβ2* in cancer samples compared with benign tumors. A negative correlation between *BRAF* mutation and *RASSF1A* methylation, and a positive correlation with *RARβ2* methylation were observed in accordance with previous results. In addition, positive correlation with *TIMP3* and a marginal correlation with *DCC* methylation were observed. The present study constitutes a comprehensive promoter methylation profile of thyroid neoplasia and shows that results must be analyzed in a tissue-specific manner to identify clinically useful methylation markers. Integration of genetic and epigenetic changes in thyroid cancer will help identify relevant biologic pathways that drive its development.

Introduction

Genomic research has been able to identify cancer-specific genetic and epigenetic alterations. The field of classic genetics largely concentrates on the DNA sequence. Important mutations that lead to human cancer have been identified by genetics research. In contrast, the field of epigenetics refers to changes in the genome that alter gene expression without altering the DNA sequence itself. DNA methylation constitutes the most studied epigenetic event in cancer. The methylation of cytosine residues in the promoter region of genes inhibits transcriptional binding and hence gene expression.[1,2] Evidence points to a complex succession of critical molecular events that activates proto-oncogenes, and/or silences tumor suppressor genes (TSGs), leading to the development of cancer. Molecular biology techniques that detect these alterations could be powerful tools to potentially enhance diagnosis, understand tumor biology as well as characterize tumor's behavior, thereby providing new tools for cancer management.

The present study concentrates on two molecular phenomena in thyroid cancer: *BRAF* mutation and promoter DNA methylation. The *BRAF* activating mutation V600E constitutes a common oncogenic mechanism in up to 69% of papillary thyroid cancers (PTC).[3,4] Activation of the *RAF/MEK/MAPK* signaling pathway interferes with proliferation, differentiation and apoptosis.[5] Furthermore, *BRAF* mutation has been associated with poor prognosis in PTC patients.[6] DNA methylation in promoter regions of TSGs is a well-established event that has been described in virtually all tumor types. However, considerable variation exists between individual methylated genes among different tumor types.[7-10] In an effort to expand our knowledge of DNA methylation in thyroid cancer, a total of 22 cancer related

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Table 1. Demographic and clinical characteristics of study subjects (n = 103)

Characteristic	No. (%) Cancer patients (Total = 44)	No. (%) Benign thyroid pathology patients (Total = 44)	No. (%) Normal thyroid subjects (Total = 15)
Gender			
Female	33 (75%)	29 (65.9%)	12 (80%)
Male	10 (22.7%)	11 (25%)	1 (6.7%)
Unknown	1 (2.3%)	4 (9.1%)	2 (13.3%)
Median age (range)	47 (16–74)	50 (25–92)	50 (26–92)
Stage			
I	25 (56.8%)		
II	7 (15.9%)		
III	9 (20.5%)		
IV	2 (4.5%)		
Unknown	1 (2.3%)		

genes were selected for methylation analysis in adjacent normal thyroid, benign thyroid tumors and thyroid cancer. The genes studied were selected based on previous reported association with thyroid cancer as well as genes never evaluated in thyroid cancer with known tumor suppressor properties or promoter methylation in other cancer types.

The present study seeks to examine the methylation signatures of a panel of novel and known genes and to integrate methylation profiling with the most important genetic alteration (*BRAF* mutation) identified for thyroid cancer to date. Some studies have tested the methylation status only in thyroid cancer tissues and not in benign or normal controls.[11] We decided to test a comprehensive cohort of tissue samples that included normal tissues as well as benign neoplasias and thyroid cancers in an effort to molecularly differentiate these three groups. We have also tested *BRAF* mutations in all our thyroid cancer samples, and correlated *BRAF* mutation status with methylation profiling.

Results

We examined a cohort of 15 normal thyroid tissue samples, 44 benign thyroid lesions (6 hyperplastic nodules, 12 follicular adenomas, 6 adenomatoid nodules, 1 adenomatoid hyperplasia, 6 multinodular goiters, 1 multinodular hyperplasia, 12 Hürthle adenomas) and 44 thyroid cancers [27 papillary (10 of which were of the follicular variant of papillary thyroid cancer), 7 follicular, 2 Hürthle cell and 8 medullary carcinomas]. Demographic and clinicopathological characteristics are detailed in **Table 1**. No significant differences in demographic characteristic between sample groups were observed (data not shown). Staging for thyroid cancer was done according to the American Joint Committee on Cancer (AJCC) TNM system.

Frequency of methylation in different types of thyroid tissues. We examined 22 genes of diverse function, including cell cycle regulation, tumor suppression and DNA repair in thyroid tissues by QMSP. One would expect to see a trend of increasing methylation, across the three categories of samples: normal, benign and cancer. We did not find significant trends for the majority of the genes tested for promoter methylation, in either binary data (Cochran-Armitage tests) or continuous data (Cuzick tests) analyses.

The frequencies of individual gene methylation per tissue group are shown in **Table 2**. In our analysis of trends of increasing methylation across categories, *KIF1A* was the only marker with an increased probability of methylation in the tumor samples (14% in the cancer tissue and 0% in the normal and benign; Cochran-Armitage p value = 0.02). While the trend test across three categories for *RARβ2* was not significant, a standard Wilcoxon rank sum test directly comparing benign and malignant tumors confirmed a previously reported in reference 6, difference in methylation levels (p = 0.05, borderline significant). The frequencies of methylation for normal tissues, benign tissues and thyroid cancer were *CTNNB1* 20%, 3% and 16%; *GSTP1* 0%, 7%, 7%; *TIMP3* 27%, 42% and 51%. *AIM1* was methylated in one of the tumors but not in any other tissue (0%, 0% and 3%). No significant differences in methylation were seen for genes *DAPK* (71%, 64% and 65%), *CDH1* (67%, 66% and 56%) and *RARβ2* (7%, 2% and 14%). *NISCH* was methylated in all normal samples but not in all tumors (100% and 86%). To create a panel of genes that could distinguish the different categories of samples, we combined genes with high specificity: *GSTP1*, *P16*, *RARβ2* and *KIF1A* and analyzed the frequencies based on any one of these markers being methylated. This combination marker (COMBO) was positive in 2 (13%) normal samples, 5 (11%) benign samples and 12 (27%) thyroid cancers.

Figure 1 shows box plots of all the genes for the continuous methylation data in the three major sample categories (normal, benign and cancer). The box plots show overlap between the methylation ratios of almost all the genes in the different tissue groups. *KIF1A* methylation was detected in five cancer samples (14%) and in none of the normal or benign samples. Although very low frequency of methylation was observed for *KIF1A*, it can be used with other markers in the diagnosis of thyroid nodules due to its 100% specificity.

When making binary determinations of the presence or absence of methylation based on different cutoffs for methylation, such as zero, the 75th percentile or the 90th percentile of methylation level in the normal tissue group, there were no significant

Table 2. Frequencies of individual gene methylation per tissue group (Cochran-Armitage and Cuzick tests of trend across sample classes)

Marker	Missing n	Normal n (%)	Benign Adenoma n (%)	Carcinoma n (%)	C-A p value	Cuzick p value
RASSF1A	1	13 (87)	43 (98)	38 (88)	0.67	0.19
TSHR[1]	1	9 (60)	24 (55)	20 (45)	0.27	0.47
AIM1	19	0	0	1 (3)	0.32	0.85
ATM	19	8 (80)	19 (53)	27 (71)	0.74	0.63
CALCA	2	13 (87)	37 (84)	39 (93)	0.33	0.11
CDH1	1	10 (67)	29 (66)	24 (56)	0.34	0.36
DAPK	2	10 (71)	28 (64)	28 (65)	0.77	0.85
DCC	19	3 (30)	9 (25)	11 (29)	0.90	0.84
GSTP1	1	0	3 (7)	3 (7)	0.42	0.73
MINT1	19	7 (70)	24 (67)	25 (66)	0.82	0.47
hMLH1	19	7 (70)	19 (53)	27 (71)	0.44	0.22
MT1G	19	1 (10)	10 (28)	8 (21)	0.80	0.77
P16	1	1 (7)	1 (2)	2 (5)	0.94	0.98
RAR-β2	1	1 (7)	1 (2)	6 (14)	0.14	0.50
S100A2	1	4 (27)	24 (55)	21 (49)	0.32	0.77
TGFβR2	1	9 (60)	33 (75)	31 (72)	0.54	0.65
THBS1	19	4 (40)	10 (28)	6 (16)	0.08	0.17
TIMP3	2	4 (27)	18 (42)	22 (51)	0.10	0.21
PAK3	23	3 (33)	10 (29)	16 (44)	0.26	0.40
NISCH	22	8 (100)	31 (86)	32 (86)	0.46	0.12
KIF1A	23	0	0	5 (14)	0.02*	0.35
CTNNB1	19	2 (20)	1 (3)	6 (16)	0.60	0.80
COMBO[2]	0	2 (13)	5 (11)	12 (27)	0.09	NA

[1]*TSHR* is median dichotomized. All other markers are zero dichotomized. [2]*COMBO* is positive if any one of: *GSTP1*, *P16*, *RARβ2* or *KIF1A* is positive. No continuous data. Cochran-Armitage tests for trend were used for dichotomized methylation variables. The frequency and percent positive are shown for normal, benign and malignant categories. The Cuzick test for trend is a non-parametric test for the continuous data values (data not shown).

differences in the methylation status in the different groups. Due to the limited amounts of DNA not all the genes were tested for all samples (identified as missing in **Table 2**). Similarly we were not able to test the methylation status of all the potential relevant genes in these samples due to limited amount of DNA.

We performed a correlation analysis for all pairs of markers (Spearman correlation shown in **Table 3**). The strongest correlations ($r \geq 0.7$) were between *TGFβR2* and *TSHR* in normal and tumor samples. A strong correlation between *CDH1* and *TSHR* was observed in normal, while a moderate correlation was observed in tumors ($r \geq 0.5$). A moderate correlation between *TIMP3* and *THSR* was observed exclusively in the tumor samples' group.

The frequency of *DCC* methylation was 36% (8/22) in papillary and 18% (5/28) in follicular neoplasms (adenomas and carcinomas). None of the other markers showed significant correlation with demographic and clinicopathologic data such as age, sex or stage. Thyroid cancer patients were divided into two age groups, those older than 45 y and those younger than 45 y, to analyze the status of methylation given that AJCC determined that 45 y of age is a cut-off point for decreased prognosis. No significant correlation was found with methylation values and age groups by non-parametric Wilcoxon testing.

Frequency of *BRAF* mutation in thyroid tissues. We analyzed the *BRAF* mutation status in all thyroid cancer samples. Consistent with previous results, we found that 15 of the 25 papillary thyroid cancers were positive for the mutation (60%), and the mutation was not detectable in the other carcinomas tested, in agreement with previous findings showing that *BRAF* mutation is present in papillary thyroid cancers but not the other subtypes.[3] To our knowledge, there is no published report about germline mutation of *BRAF* in normal thyroid tissue. We therefore coded all nonmalignant samples as negative for the *BRAF* mutation for the purpose of the correlation analyses.

Correlation between *BRAF* mutation and methylation. In papillary thyroid cancer, we found significant correlations between the methylation status of four genes (*TIMP3*, *RASSF1A*, *RARβ2* and *DCC*) and the V600E *BRAF* mutation. The mutation was present in 15 of the papillary thyroid cancer tumors in our sample set. *RASSF1A* methylation decreased the probability of *BRAF* mutation, OR = 0.74 (95% CI: 0.56, 0.97), p = 0.035, while methylation in other genes increased the probability of *BRAF* mutation: *RARβ2* OR = 2.63 (95% CI: 1.00, 6.89), p = 0.05, *TIMP3*, OR = 2.04 (95% CI: 1.00, 4.12), p = 0.05, and *DCC*, OR = 1.32 (95% CI: 1.07, 1.63), p = 0.01. Multivariate logistic regression confirmed the negative association with

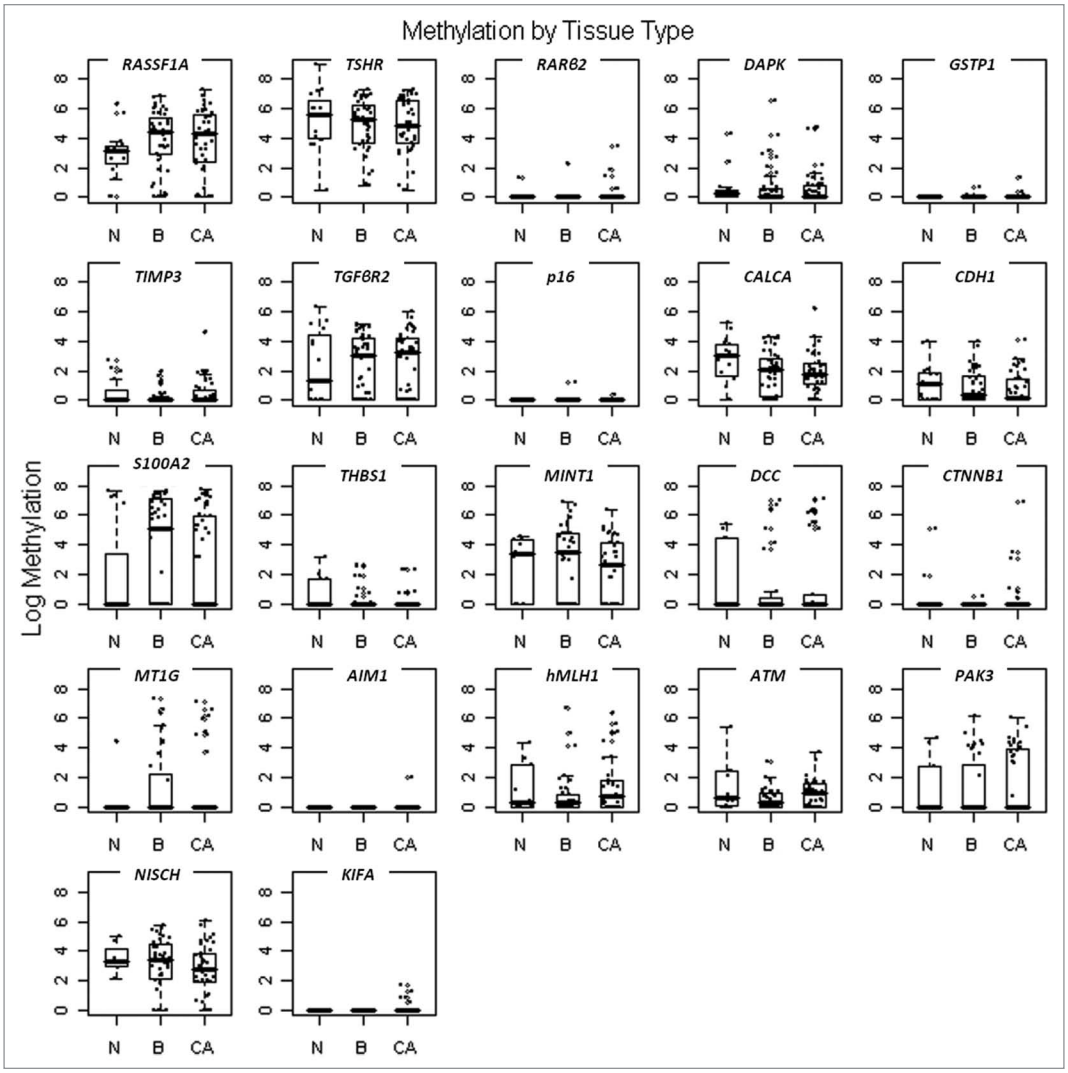

Figure 1. Promoter methylation levels for the different markers in the cancer patients (n = 44) [CA], the benign pathology patients (n = 44) (B), and the normal thyroid tissues (n = 15) (N). The quantity of methylation is expressed as the ratio of the PCR product for the gene of interest to that of β-*actin* multiplied by 1,000. Boxplots show the middle 50% of the data, the median with a bar in the center, and bars extending the median by 1.5 times the interquartile range.

RASSF1A, and the positive association with *RARβ2*, *TIMP3* as well as a marginal positive correlation with *DCC*. **Table 4** shows the odds ratios as well as the confidence intervals for these associations, in univariate and multivariate analyses.

Discussion

Dissecting all the genetic and epigenetic alterations involved in thyroid cancer is essential for our understanding of the pathogenesis of this disease, and hence, for more precise diagnosis, accurate prognosis prediction and appropriate management of patients. In this study, we evaluated the most common mutation found in papillary thyroid cancer as well as a comprehensive panel of candidate cancer methylation markers that include markers tested previously in thyroid cancer and cancer specific methylation markers that had not yet been tested in thyroid cancer. Nineteen of the genes we have tested in this study have been previously analyzed in thyroid cancer (*TSHR, RASSF1A, RARβ2, DAPK, TIMP3, hMLH1, p16, ATM, TGFβR2, PAK3, NISCH, KIF1A, CALCA, CDH1, S100A2, THBS1, GSTP1, CTNNB1* and *MT1G*).[6,8,11-18] Among these 19 genes, *PAK3, NISCH* and *KIF1A* were previously only tested in a small set of thyroid cancer samples by our group as a part of our comprehensive approach to discover methylated genes in cancer.[8] Three of the 22 genes, *MINT1, DCC* and *AIM1*, have not been tested in thyroid cancer to date.

In general, our analysis revealed considerable overlap between promoter hypermethylation of normal thyroid tissue, benign hyperplastic states and tumors, and malignant tumors. Although with very low frequency (14%), *KIF1A* methylation was found to be 100% cancer specific and this frequency is consistent with our previous findings.[8] The analysis of *BRAF* mutations confirmed the previously reported inverse relationship with *RASSF1A* methylation,[6,17,19] as well as the direct relationship with *RARβ2*

Table 3. Summary of spearman correlations for pair of genes (promoter methylation) in thyroid samples

THYROID CANCER

RASSF1A	0.56 *	0.41	0.06	0.23	0.48	0.22	-0.11	0	0.08	-0.18	-0.15	0.14	0.33	-0.1	0.08	-0.13	0.24	0.11	0.04	-0.11	-0.06
0.61 *	*TSHR*	0.63 *	0.6 *	0.52	0.77 *	0.47	0.15	-0.03	0.31	0.21	-0.04	0.04	0.36	-0.07	0.15	-0.18	0.1	0.26	0.1	0.15	-0.23
0.36	0.5 *	*CALCA*	0.68	0.37	0.45	0.25	0.27	0.34	0.33	0.36	-0.26	-0.1	0.36	0.06	0.04	-0.17	0.09	0.08	-0.21	0.02	-0.24
0.41	0.74 *	0.48	*CDH1*	0.39	0.41	0.45	0.36	0.23	0.42	0.58	-0.03	0	0.4	-0.03	0.19	0.09	0.15	0.14	-0.18	0.33	-0.18
0.23	0.35	0.23	0.49	*TIMP3*	0.36	0.26	0.19	0.25	0.22	0.05	-0.04	-0.01	0.29	-0.16	0.21	-0.23	0.18	0.18	-0.1	0.24	-0.16
0.6	0.84 *	0.49	0.66	0.27	*TGFβR2*	0.34	-0.07	-0.1	0.17	0.03	-0.08	0.06	0.32	-0.14	0.14	-0.27	0.02	0.51	0.06	0	-0.13
0.22	-0.05	-0.14	-0.16	0.1	-0.05	*MINT1*	-0.01	0.17	0.37	0.29	-0.02	0.05	0.37	0.1	0.43	-0.01	0	0.01	0.22	0.17	0.08
0.1	0.15	0.35	0.34	0.34	0.27	0.07	*DCC*	0.37	0.25	0.25	-0.01	0.18	0.2	0.17	-0.1	-0.13	0.08	-0.07	-0.09	0.09	-0.1
0.22	0.23	0.01	0.27	0.17	0.22	0.02	0.14	*MT1G*	0.12	0.22	-0.14	0.15	0.25	0.05	-0.04	-0.2	0.33	0.14	-0.04	0.18	-0.08
-0.07	-0.05	0.03	0.05	0.1	0.02	0.14	-0.09	-0.08	*RARβ2*	0.25	-0.11	-0.09	0.15	0.15	0.18	-0.08	0.08	0.05	-0.03	0.26	-0.07
-0.07	0.08	-0.01	0.16	0.29	-0.08	-0.35	-0.07	-0.04	-0.09	*DAPK*	0.07	0.1	0.19	0.18	0.09	0.07	-0.02	-0.06	-0.15	0.27	-0.18
-0.13	-0.19	-0.15	-0.14	-0.04	-0.24	-0.18	-0.09	0.32	-0.04	-0.12	*GSTP1*	-0.06	-0.13	-0.05	-0.13	0.15	0.08	0.11	0.15	0.17	-0.05
0.12	0.22	0.14	0.09	0.08	0.26	-0.18	-0.09	-0.08	-0.04	0.19	-0.04	*p16*	0.2	0.21	-0.1	-0.07	0.36	-0.01	-0.18	0.21	-0.04
0.09	0.16	0.04	0.12	0.13	0.21	0.13	-0.03	0.01	-0.1	0.09	-0.1	-0.1	*THBS1*	0.04	0.16	0.07	0.08	0.15	-0.17	0.03	-0.07
0.03	-0.11	-0.06	0.05	0.11	-0.08	0.16	0.19	0.06	-0.17	-0.16	0.01	-0.17	0.2	*S100A2*	0.06	-0.04	0.05	-0.1	-0.02	0.18	-0.14
-0.05	0.01	-0.01	-0.02	-0.22	0.06	-0.22	-0.15	-0.15	-0.04	-0.1	-0.04	-0.04	0.25	-0.24	*CTNNB1*	0.06	0.01	-0.05	0.09	0.03	-0.07
0.1	0.22	0.19	0.21	0	0.22	-0.22	-0.18	-0.04	-0.01	0.1	-0.15	0.24	0.18	-0.18	0.36	*hMLH1*	-0.07	-0.14	0.1	-0.04	-0.08
-0.03	0	0.1	-0.02	0.09	-0.03	0.1	-0.09	0.05	0.16	-0.17	-0.16	-0.16	0.03	0	0.32	0.01	*ATM*	0.04	-0.04	0.43	-0.2
-0.04	0.05	0.12	0.01	-0.21	0.04	-0.05	0.16	0.16	-0.1	-0.1	-0.1	.	-0.14	-0.15	0.01	0	0.24	*PAK3*	0.16	0.11	0.04
0.19	0.07	-0.13	-0.13	0.06	0.06	0.26	-0.25	0.1	0.2	-0.11	-0.2	.	0.23	0.2	0.05	0.17	0.16	0.13	*NISCH*	-0.06	-0.25
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*KIF1A*	-0.07
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	*AIM1*

THYROID NORMAL + BENIGN PATHOLOGY

The diagonal row of boxes lists the markers analyzed; correlations are indicated at the horizontal and vertical intersections of the markers. Light gray shading represents R > 0.5; dark gray shading represents R > 0.7. The samples were split into two groups: correlation between the pair of genes in tumors (n = 44). indicated in the upper half of the plot and correlations between normal (benign pathology and normal thyroid tissue, n = 59) indicated in the lower half of the plot. Black asterisk (*) indicates similar correlations observed in both groups. Black dot (.) indicates that the correlation could not be calculated due to values being mainly zero.

Table 4. Univariate and multivariate analyses of *BRAF* mutation and promoter methylation of the genes that showed statistically significant correlations

***BRAF mutation* versus promoter methylation**	**Chi Square p value**		**Odds ratio**		**95% CI**	
	Univariate/Multivariate		Univariate/Multivariate		Univariate/Multivariate	
DCC*	0.01	0.09	1.32	1.29	(1.07–1.63)	(0.97–1.72)
RARβ2*	0.05	0.01	2.63	4.35	(1.00–6.89)	(1.47–12.79)
RASSF1A*	0.035	0.005	0.74	0.53	(0.56–0.97)	(0.34–0.82)
TIMP3*	0.05	0.01	2.04	3.04	(1.0–4.12)	(1.26–7.34)

*Log transformed.

methylation[6] and with *TIMP3*.[16] Additionally we describe a positive correlation between *DCC* methylation and *BRAF* mutation. To our knowledge this is the first evidence indicating a relationship between these two genes and thyroid cancer. Interestingly, *DCC* was only methylated in the papillary subtype.

We were unable to confirm previously reported promising results for several potential methylation markers, possibly because the scope of previous methylation studies was too narrowly defined, particularly regarding samples tested and methylation detection methods used. Our group previously reported differential methylation in cancer and benign samples for the genes *TSHR* and *RARβ2*. Using conventional MSP we reported *TSHR* methylation was 59% (23/39) for papillary thyroid tumors and 47% (7/15) for follicular tumors and 0% (0/8) for the normal and benign tumors.[13] The current study used QMSP, a more sensitive and automated method that shows 45% (20/44) methylation in tumors, 55% (24/44) in benign tumors and 60% (9/15) in normal tissues. Although methylation levels are generally low in normal and benign tissues, the overlap between benign and malignant tumors precluded reaching statistical significance in this cohort, and will complicate the use of this marker in a diagnostic and/or prognostic setting. *RARβ2* had shown significantly higher methylation in thyroid cancer in comparison to benign thyroid (22% of the papillary and follicular thyroid

carcinomas, and only 4% of the adenomas) in our prior QMSP study.[6] However, in this study we found methylation in only 14% of thyroid cancers, compared with 2% in the benign tumors, and 7% of the normal. The source of *RARβ2* methylation in the normal thyroid is unclear at this time, and also has the potential to confound diagnostic testing, although the statistically significant difference between benign lesions and malignant tumors found in our previous study was confirmed. Larger sample sizes are needed to confirm the frequency of methylation of *RARβ2* in thyroid cancer and various benign thyroid neoplasms and normal thyroid tissue. Hu et al. reported the methylation of *DAPK, RARβ2* and *TIMP3* in papillary thyroid cancer through the use of QMSP.[16] Their frequency of methylation for *DAPK* and *TIMP3* in thyroid cancer is similar to ours (64 vs. 65% for *DAPK* and 55 vs. 51% for *TIMP3*), while different for *RARβ2* (58 vs. 14%). Unfortunately, in the present study as well as a previous one from our group, we have shown that normal thyroid samples and cancer samples have overlapping frequencies of methylation for *DAPK*, *TIMP3* and *RARβ2 (DAPK* 65% tumors vs. 63% benign vs. 71% in normal; *TIMP3* 51% tumors vs. 42% benign vs. 27% normal; *RARβ2* mentioned above).[6] These findings limit the use of these particular genes as diagnostic markers for cancer in thyroid specimens and question the neoplastic relevance of the hypermethylation present in the tumors.

The present results raise concerns regarding the laboratory techniques and tissue samples selection in the study of methylation. Quantitative Methylation Specific PCR (known as QMSP or methylight) is based on a real time PCR and is more sensitive than conventional methylation specific PCR (MSP). Eads et al. reported a sensitivity of detection of 1 methylated allele in a background of 10,000 unmethylated alleles (1:10,000). Herman et al.[9] reported when first reporting conventional MSP a sensitivity of 1:1,000, which represents a 10-fold difference. QMSP, being a real time PCR assay is more objective to be analyzed than conventional PCR, with reduced cross-contamination, as there is no post-PCR analysis (gel electrophoresis). The quantitative type of assay is more specific due to the incorporation of the methylation specific probe, in addition to the pair of primers. On the other hand, QMSP is able to detect only fully methylated molecules, while MSP can also detect partially methylated molecules. Virmani et al. compared their results of *APC* promoter methylation in lung cancer cell lines using conventional MSP and QMSP, and observed higher percentages when using the quantitative method. MSP was used to test the majority of the genes previously reported as hypermethylated in thyroid cancer (*MT1G, TSHR, CTNNB1, DAPK, ATM, p16* and *hMLH1*).[12-15] For most of the genes, we were unable to establish cutoff levels that could reliably distinguish methylation frequencies in the normal and benign groups from the cancer group. Overall, the high levels of methylation in normal and benign thyroid tumors reported in this study are consistent with our previous QMSP study in thyroid tissues.[6] An additional difference among various studies is the methodology to process tissues, which is also a factor that can change the detected levels of methylation. Furthermore, some of the previous studies did not use appropriate normal controls for the determination of thyroid cancer specific methylated genes.[11] Of significant concern to our group is the evaluation of the methylation markers on comprehensive sample sets that not only include cancer cases, but also normal and benign tumors that are a common part of the clinical differential diagnosis.

Methylation of *TSHR* often occurred together with *TGFβR2* and *CDH1* in normal, benign and tumor samples. These associations could have happened by chance alone and should be interpreted with caution. A possible interaction of these genes in thyroid physiology will need to be further assessed. There was only one interaction, between *TIMP3* and *THSR,* which was observed exclusively in cancer. None of these genes showed a cancer specific hypermethylation pattern in our study. Although both were frequently methylated in all samples, further studies should be conducted to elucidate the role of this coordinated methylation. A lack of inhibition of TSHR by TIMPs has been previously reported in reference 22. It is possible that pathways related to both TIMP3 and TSHR signaling need to be altered for developing subsets of thyroid cancers.

Genes studied here are putative TSGs that are active in a wide variety of normal tissues and have been reported to be hypermethylated across many tumor types (some examples are *p16*, *CTNNB1* and *DAPK*). Reddy et al. demonstrated, however, that *DAPK* promoter methylation was present in lymphocytes from normal individuals, specifically in B lymphocytes.[23] This is an example of a tissue specific methylation of a gene that has been reported as a cancer specific methylated gene. Similarly, we have recently reported *RARβ2* methylation presence in cancer-free patients (29%; 46/157).[24] In the later study, *RARβ2* promoter methylation was observed in 45% of subjects who had a high-fat diet. Many groups are investigating how exogenous as well as endogenous factors (like hormones) participate in promoter methylation.[25,26] Thyroid being an endocrine organ, exposure to variations of hormone levels could lead to specific promoter methylation patterns like *RARβ2*. In contrast, there are TSGs that are specifically affected in particular tumor types like *GSTP1* in prostate cancer.[27] We believe each potential TSG needs to be evaluated in the context of the tissue type being examined. In this context, it should come as no surprise that an endocrine organ such as the thyroid may show significant differences in pathway regulation and TSG-dependency compared with non-endocrine epithelial organs, from which most currently known cancer methylation markers have been developed.

The relevance for the RAF/MEK/MAPK kinase pathway in thyroid cancer being upregulated either by a *BRAF* activating mutation or *RASSF1A* methylation silencing is further confirmed by our study. *RASSF1A* methylation has been reported in several cancer types.[6,28-30] This gene contains a Ras-binding domain, and its association to Ras activation has been demonstrated in vitro, as well as its ability to induce apoptosis.[31-33] Accumulated data suggested its direct correlation to *RAF/MEK/MAPK* oncogenic pathway. It would be interesting to see how *DCC* plays a role in this pathway for papillary tumors, in a larger cohort of samples. *DCC* is a postulated TSG initially identified in colon cancer that mediates apoptosis by a mechanism requiring receptor ligand activity.[34,35] *TIMP3* proteins are inhibitors of the matrix metalloproteinases, a group of peptidases involved in degradation

of the extracellular matrix. TIMP3 is of particular interest for therapeutic purposes due to its characteristics of inhibiting different aspects of tumor development, mainly because it is a potent angiogenesis inhibitor. The positive association of *TIMP3* methylation with *BRAF* mutations suggests that methylation of this metastasis suppressor gene may play a role in the aggressiveness of PTC conferred by *BRAF* mutation. Further studies are necessary to understand the biological relevance of the positive correlation between *BRAF* mutation and promoter methylation of *TIMP3*. After establishing biological relevance, specific therapy may be developed for *BRAF* mutated PTC patients with or without *TIMP3* methylation. The methylation markers associated with *BRAF* mutations may well have specific roles in thyroid carcinomas, but since only a minority of thyroid carcinomas in this cohort were mutated in *BRAF*, it is likely that our cohort lacked the statistical power to show their association with specific clinical behavior of thyroid carcinoma.

The present study constitutes a comprehensive profile of hypermethylated genes previously reported as altered in thyroid cancer as well as an extended panel of genes never evaluated in thyroid tissues. Moreover, we also analyzed *BRAF* mutations, a common genetic event in papillary thyroid cancer. Methylation profiling of thyroid tissues to date has concentrated on a candidate gene approach for known TSGs in epithelial cancers. To our knowledge, no genes have been identified as thyroid cancer specific methylated genes using appropriate controls and sufficient numbers of benign tumor samples for comparison. It is possible that methylation is not a common event for thyroid cancer, but since epigenetic control seems otherwise ubiquitous, it is more likely that the relevant methylation markers remain unidentified. Alternatively, it is possible that the identification of combinations of markers, both genetic and epigenetic, reflecting alterations in several regulatory and metabolic pathways may be required to achieve acceptable positive and negative predictive values in a clinical diagnostic test. Correct diagnosis of thyroid nodules from FNAs specimens and prognostic profiling of thyroid cancers constitute pressing challenges in the current medical practice. Additional systematic genome-wide approaches using primary tumors and appropriate controls will be necessary to develop new panels of biomarkers for thyroid cancer. The planned comprehensive effort by the National Cancer Institute's The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov) thyroid consortium should provide valuable insights in thyroid cancer-specific methylome changes.

Materials and Methods

Tissue samples. We selected 109 patients who underwent surgical resection for a thyroid tumor from 2000–2003 at The Johns Hopkins Hospital from whom a frozen tumor sample was available for DNA extraction. Collection of tissue and demographic data was performed in accordance to the guidelines of The Johns Hopkins University Institutional Review Board under protocol NA_00018307. The molecular studies were performed under protocol 03-11-12-06e. Tissue was routinely obtained from the center of the lesions and from uninvolved adjacent thyroid tissue and snap frozen in liquid nitrogen. To avoid field-cancerization effects (the increased risk of the adjacent epithelial surface of the tumor for the development of malignant lesions due to the potential presence of multiple molecular alterations in the entire region), only normal thyroid tissue sampled adjacent to benign nodules was used in this study. All cases were classified according to the clinical pathology report and cryosections were obtained from experimental tissue samples to verify the presence of tumor tissue and scored for the presence of inflammatory cell infiltrates suggestive of thyroiditis. Cases with chronic lymphocytic (Hashimoto) thyroiditis as a secondary diagnosis were excluded from the study, since extensive inflammation has been shown to induce aberrant DNA methylation.[36]

DNA extraction and sodium bisulfite treatment. DNA was extracted from frozen thyroid tissue, and subsequently subjected to bisulfite treatment, as described previously in reference 6.

Methylation analysis. Bisulfite treated DNA was used as a template for the fluorogenic gene-specific QMSP reactions. Quantitative PCR was performed in a TaqMan 7900HT Applied Biosystem and analyzed by a sequence detector system (SDS 2.3; Applied Biosystems), as previously described in reference 37. **Table 5** shows the primer and probe sequences used. Each plate included studied DNA samples, positive (in-vitro methylated leukocyte DNA) and negative (normal leukocyte DNA or DNA from a known unmethylated cell line) controls and multiple water blanks (molecular grade water was used as a non-template control). Leukocyte DNA from a healthy individual was methylated in vitro with excess SssI methyltransferase (New England Biolabs Inc.) to generate completely methylated DNA, and serial dilutions (90–0.009 ng) of this DNA were used to construct a calibration curve for each plate. The β-*actin* gene was used to normalize the fluorescence emission as well as an internal loading control, β-*actin* primers and probe were designed to amplify a region that is devoid of CpG nucleotides which allows amplification independent of its methylation status. The methylation ratio is defined as the ratio of the fluorescence emission intensity values for the gene-specific PCR products to those of the β-actin (reference gene) and then multiplied by 1,000 for easier tabulation. All samples were within the assay's range of sensitivity and reproducibility, based on the amplification of the internal reference standard [threshold cycle (CT) value for β-actin of 40].

Gene selection. The thyrotropin receptor (*TSHR*) gene plays an important role in thyroid function by initiating thyroid hormone synthesis. *TSH*R has been reported as hypermethylated in thyroid cancers and unmethylated in benign and normal thyroid tissues.[6,12,13] *RASSF1A* is a direct player in the RAF/MEK/MAPK pathway and is considered a negative regulator of the cell cycle progression.[38] *RAR*β*2* is a retinoic acid receptor commonly silenced in cancer that was found by our group to be hypermethylated in thyroid cancer and not in benign thyroid tissues.[6,19] *p16, DAPK, hMLH1, ATM, MT1G, TIMP3, TGF*β*R2* and *CTNNB1* have been previously found to be hypermethylated in thyroid cancers.[6,11,12,14,19,39] The role of p16 as an important cell cycle regulator has been widely described in reference 40. DAPK is involved in apoptosis.[41] hMLH1 is involved in DNA repair[42] while ATM regulates cell cycle in DNA damage scenarios.[43] S100A2, GSTP1,

Table 5. Primers and probes used in QMSP assay

Gene	Forward primer 5'-3'	Probe 5'-3' (6-FAM-5'-3'-6-TAMRA)	Reverse primer 5'-3'	Amplicon location (Genbank numbering)	Accession number
β-*actin*	TGG TGA TGG AGG AGG TTT AGT AAG T	ACC ACC ACC CAA CAC ACA ATA ACA AAC ACA	AAC CAA TAA AAC CTA CTC CTC CCT TAA	390–522	Y00474
TSHR	GGT GTA GAG TTG AGA ATG AGG TGA TTT C	ACA ACA CCA ACT ACA ACA AAT CCG CCG A	GCC CAA ATC CCT AAA CAA ATC G	188–310	BC024205
RASSF1A	GCG TTG AAG TCG GGG TTC	ACA AAC GCG AAC CGA ACG AAA CCA	CCC GTA CTT CGC TAA CTT TAA ACG	45–119	NM_007182
*RAR*β*2*	GGG ATT AGA ATT TTT TAT GCG AGT TGT	TGT CGA GAA CGC GAG CGA TTC G	TAC CCC GAC GAT ACC CAA AC	907–999	X56849
DAPK	GGA TAG TCG GAT CGA GTT AAC GTC	TTC GGT AAT TCG TAG CGG TAG GGT TTG G	CCC TCC CAA ACG CCG A	4–102	X76104
hMLH1	CGT TAT ATA TCG TTC GTA GTA TTC GTG TTT	CGC GAC GTC AAA CGC CAC TAC G	CTA TCG CCG CCT CAT CGT	254–341	U26559
ATM	CGG GTC GAA TGT TTT GGG G	ATC CAA TAT CAC GCG ATC TCC GC	GCA AAA CAC GAT ATA CCC ATA C	82–160	NM_000051.3
S100A2	TGG TTT CGA TTT TTT GAT TTC G	CGA CCG AAC GCG ATA ACT TAC TCC TA	CGA CCG AAC GCG ATA ACT TAC TCC TA	5075–5317	Y07755
p16	TTA TTA GAG GGT GGG GCG GAT CGC	AGT AGT ATG GAG TCG GCG GCG GG	GAC CCC GAA CCG CGA CCG TAA	25–174	U12818
CDH1	AAT TTT AGG TTA GAG GGT TAT CGC GT	CGC CCA CCC GAC CTC GCA T	TCC CCA AAA CGA AAC TAA CGA C	842–911	L34545
GSTP1	AGT TGC GCG GCG ATT TC	CGG TCG ACG TTC GGG GTG TAG CG	GCC CCA ATA CTA AAT CAC GAC G	1033–1172	M24485
CALCA	GTT TTG GAA GTA TGA GGG TGA CG	ATT CCG CCA ATA CAC AAC AAC CAA TAA ACG	TTC CCG CCG CTA TAA ATC G	1706–1806	X15943
TIMP3	GCG TCG GAG GTT AAG GTT GTT	AAC TCG CTC GCC CGC CGA A	CTC TCC AAA ATT ACC GTA CGC G	1051–1143	U33110
*TGF*β*R2*	GAG GGG AGG CGG TAG AT	CGA CGT CCA ACC CCT AAC TCT C	CAA CTT CAA CTC AAC GCT ACG	(-)224–91	NM_001024847.2
THBS1	CGA CGC ACC AAC CTA CCG	ACG CCG CGC TCA CCT CCC T	GTT TTG AGT TGG TTT TAC GTT CGT T	1642–1716	J04835
MINT1	ATT TTC GAA GCG TTT GTT TGG C	GCG AAA CTC CCC TAC TCT CCA AC	ACA AAA AAC CTC AAC CCC GC	63970–64053	AC026774.7
CTNNB1	GGA AAG GCG CGT CGA GT	CGC GCG TTT CCC GAA CCG	TCC CCT ATC CCA AAC CCG	583–664	X89448
MT1G	TGC GAA AGG GGT CGT TTT GC	GCG ATC CCG ACC TAA ACT ATA CG	AAC CCG CTA AAT CCG CAC C	120–235	J03910
PAK3	TTA CGG TCG TCG TTA TTA TCG	AAC CAA AAA AAA TAA AAA ATC ACA ACC G	ACC GAA AAT TCT ACC CTT CG	943–1065	NM_002578
NISCH	TTT TTT TCG TAT AGA GTT CGT	CGC GAC CCA ACA CGC AAT AAT ACT C	CTA AAC CTC TCT AAA ATT CG	361–517	NM_007184
DCC	TTG TTC GCG ATT TTT GGT TTC	GCG CTA AAC AAA AAA ACT CCG AAA A	ACC GAT TAC TTA AAA ATA CGC G	549–680	NM_005215
AIM1	CGC GGG TAT TGG ATG TTA GT	GGG AGC GTT GCG GAT TAT TCG TAG	CCG ACC CAC CTA TAC GAA AA	6862–6982	AL359292.12
KIF1A	GCG CGA TAA ATT AGT TGG CGA TT	CCT CCC GAA ACG CTA ATT AAC TAC GCG	CTC GAC GAC TAC TCT ACG CTA T	870–1010	NM_004321

CALCA, THBS1, MINT1, CTNNB1, PAK3, DCC, AIM1 and KIF1A are other genes known to be methylated in cancer.[8,27,37,44]

***BRAF* mutation detection.** Samples were analyzed for the thymine (T)-adenine (A) miss-sense mutation at nucleotide 1,796 in the BRAF gene. Briefly, PCR primer sequences were designed to amplify a 102-bp fragment of exon 15 (5'-GAA GAC CTC ACA GTA AAA ATA GGT GA-3' and 5'-CCA CAA AAT GGA TCC AGA CA-3'). PCR amplification was performed using 100 ng of genomic DNA as template. Cycling conditions were as follows: a denaturation step at 95°C for 5 min was followed by two cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, primer extension at 72°C for 1 min, two cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, primer extension at 72°C for 1 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, primer extension at 72°C for 1 min, and one final extension at 72°C for 5 min. Amplified fragments were separated on an agarose gel and visualized by ethidium bromide staining. Analysis of the products was performed using the colorimetric Mutector assay according to the manufacturer's instructions (TrimGen). A detection primer was designed that does not permit primer extension when the target base is wild-type. When the target base is mutated, primer extension continues and a color reaction is observed. The assay was preformed according to the manufacturer's instructions. The melanoma cell line HTB 72 was used as positive control for the BRAF T1796A mutation, and the cervical cancer cell line ME180, known to be wild type for BRAF at T1796, served as a negative control. This assay is reported to have sensitivity and specificity nearly of 100%, and it is capable of detecting as low as 1% mutated DNA in a wild-type background.[45]

Statistical analysis. The primary objective of this study was to describe the methylation patterns of 22 genes in normal, benign and cancer thyroid tissue. Continuous distributions of QMSP ratios are often distinctly non-normal with a clump of zeros in the lower tail of a distribution of continuous values. To evaluate the two parts of these distributions for a trend of increasing methylation across the categories of samples: normal, benign and cancer, two types of analyses were performed. In the first, methylation was considered binary and the presence or absence of methylation was analyzed with an exact version of the Cochran-Armitage trend test. In the second, the continuous methylation distributions of each gene were evaluated across the tissue categories with the non-parametric Cuzick test for trend.

A secondary objective of this study was to determine if methylation of these genes was associated with an increased probability of BRAF mutation. Genes associated with this outcome were selected based on univariate and multivariate logistic regression modeling. All statistical computations were performed using the SAS system[46] StatXact or R. All p values reported are two sided. p values of < 0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest

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