Gene-specific promoter hypermethylation without global hypomethylation in follicular thyroid cancer

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Abstract. Genome-wide hypomethylation and hypermethylation at CpG promoters are common in cancer. To date, little is known about global methylation changes in follicular thyroid cancer (FTC). Two independent quantitative methods, bisulphite Pyrosequencing of Long Interspersed Nucleotide Elements-1 (LINE-1) and LUminometric Methylation Assay (LUMA) were used to quantify genome-wide methylation in 21 FTC and corresponding normal thyroid tissues. Unexpectedly global methylation was not found significantly altered in tumors compared to normal thyroid by either LINE-1 (p=0.57) or LUMA (p=0.42), whilst the promoter of a tumor suppressor that is often epigenetically dysregulated, *RASSF1A* was found to be significantly hypermethylated by Pyrosequencing (p=0.0001). Moreover, allelic imbalance at the *RASSF1A* locus was observed in 15/21 of the tumors. mRNA expression of *RASSF1A* was significantly lower in tumors compared to corresponding normal tissues (p=0.0002). In summary, the epigenetic inactivation of *RASSF1A* is a frequent event in FTC, but is not coupled to changes in global methylation.

Introduction

Epigenetic alterations such as DNA methylation are common in human cancers and include both promoter-specific hypermethylation and global hypomethylation (1). Aberrant promoter methylation at the CpG islands leads to inappropriate silencing of genes. In addition, global hypomethylation, i.e. an overall decrease in 5-methylcytosine content in the genome, is also associated with tumor development (2). Both *in vitro* and mouse model studies have shown that global hypomethylation leads to chromosomal instability and increased tumor frequency, as well as oncogene activation (3). Global hypomethylation occurs in genome-wide repetitive sequences such as retroelements and CpG-rich satellites and leads to activation of transposons (4).

Genome-wide methylation studies of follicular thyroid cancer (FTC) are sparse in the literature. Galusca *et al* evaluated several types of thyroid tumors using a monoclonal antibody specific for 5-methylcytosine (5-mc) (5). Five-mc immunostaining was significantly lower in thyroid cancer as compared to benign tumors or adjacent normal thyroid parenchyma, suggesting the possibility of hypomethylation in thyroid cancer (5).

On the other hand, several tumor suppressor genes have been reported to be silenced by promoter methylation in thyroid cancer (6). Promoter methylation of the tumor suppressor *RASSF1A*, a member of the RalGDS/AF-6 domain family of Ras effectors, is frequently seen in all histological types of thyroid tumors and is regarded as an early event in thyroid tumorigenesis (7-11). We and others have found reduced expression and promoter methylation of *RASSF1A* in thyroid cancer cell lines and primary tumors (6,12). Although *RASSF1A* is located in 3p21.3, a region frequently deleted in FTC, allelic imbalance (AI) of *RASSF1A* has not been studied as a mechanism of *RASSF1A* inactivation in these tumors.

In the present study, global methylation was assessed in FTCs and matched corresponding normal thyroid by applying bisulphite Pyrosequencing of Long Interspersed Nucleotide Elements-1 (LINE-1) and LUminometric methylation assay (LUMA). LINE-1 analysis and LUMA are expected to give complementary information, where the former targets CpGs whilst the latter recognizes CCGG sequences. For a comparison, gene-specific methylation of *RASSF1A*, a tumor suppressor that is frequently methylated in FTCs was assessed by Pyrosequencing. Furthermore its mRNA expression and AI status were investigated in the same material.

Materials and methods

Tissues. Frozen tissues of primary FTC and corresponding normal thyroid tissues were obtained from 21 patients who underwent total thyroidectomy at the Karolinska University

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Hospital, Stockholm, Sweden. All samples were collected with informed consent from the patients and the study was approved by the local ethics committee. A representative section was cut and subjected to histopathological evaluation to confirm high purity of tumor cells (>70%) in the tumor samples and lack of neoplastic cells in the normal thyroid tissues. The clinical data are given in Table I. RAS mutation status has been previously presented by Foukakis *et al* (12).

DNA extraction and sodium bisulfite modification. High-molecular weight genomic DNA was extracted from all tissues by conventional phenol purification methodology. Sodium bisulfite modification was performed using the EZ DNA Methylation Kit™ , Zymo Research, Orange, CA, USA according to the manufacturer's instructions.

Global methylation analysis by LINE-1. LINE-1 is retro-transposon that comprises ~17% of the human genome. These sequences contain a disproportionately high fraction of methylcytosine compared to the rest of the genome. Low levels of DNA methylation have been found in CpGs of LINE-1 elements dispersed throughout the genome in cancer cells (13). Global DNA hypomethylation analysis was performed using the PyroMark™ LINE-1 assay (Biotage AB, Uppsala, Sweden). The PCR conditions and primers applied were according to the manufacturer's instructions. In brief, 100 ng of bisulfite treated gDNA was set up in a 25 μl PCR reaction using the Pyro Gold Reagents (Biotage) with LINE-1 forward and reverse primers provided in the reagent. Subsequently, methylation of the PCR product was quantified by Pyrosequencing performed at Biotage.

Global methylation analysis by LUMA. Global methylation was quantified by LUMA according to previously described methodology (14). Briefly, 500 ng genomic DNA was cleaved with *Hpa*II + *Eco*RI or *Msp*I + *Eco*RI in separate reactions. After the digestion step, the extent of cleavage was quantified by Pyrosequencing. *Hpa*II and *Msp*I are methylation sensitive isochizomers. DNA methylation was defined from the *Hpa*II/*Msp*I ratio, whereby fully methylated DNA gives a ratio that approaches 0. The assay was carried out on two separate occasions.

Promoter methylation analyses of the RASSF1A promoter. Methylation of the *RASSF1A* promoter was investigated by Pyrosequencing targeting the same promoter sequence and validated by MSP. Bisulphite-treated DNA from tumors and their corresponding normal thyroid tissues were used for PCR amplifications. MSP reactions were carried out using 100 ng treated DNA in a 25 μl of 1 U AmpliTaq Gold Polymerase (Applied Biosystems, Foster City, CA), 200 μM dNTP, 2.5 μM of $MgCl_2$ and 25 pmol of each specific primer (Proligo, France). Primers applied for the MSP were as described (15). The following cycling conditions were applied: 95˚C for 10 min, 35X (94˚C for 30 sec, 62˚C for 30 sec, 72˚C for 30 sec), 72˚C for 10 min. Pyrosequencing was performed at Biotage, using the PSQHS96 system (Biotage). Primers and conditions for the reactions were performed as previously described (16). *Sss*I methylase treated lymphocytic DNA from healthy individuals was used as a positive control, while lymphocytic DNA source without SssI treatment served as unmethylated assay control.

Real-time quantitative PCR (qRT-PCR). Total RNA was isolated from all tumors and normal thyroid tissues using TRIzol reagent (Invitrogen Life Technologies, Inc.), column purified by RNeasy kit (Qiagen, Valencia, CA) and used for cDNA synthesis and expression quantifications as previously described (12,16). The gene expression levels of *RASSF1A* and the control gene *18S* were quantified using TaqMan technology on an ABI PRISM 7700 sequence detection system (Applied Biosystems). Gene specific primers and probes were available as TaqMan Gene Expression Assays for *RASSF1A* (Hs00945257_ml) and for *18S* (Hs99999901_sl) (Applied Biosystems). Values were normalized against the standard curve established from pooled normal thyroids, and subsequently against *18S*. qRT-PCR results for *RASSF1A* were previously published for some of the cases in Foukakis *et al* (12) and were re-analyzed here.

Genotyping of microsatellites and AI analysis. Matched tumor and normal DNA samples were genotyped by three markers close to the *RASSF1A* gene locus: D3S4597/P1.5; D3S4604/LUCA19.1; *RASSF1A*, and D3S4614/LUCA8.2 (17). Primers were end-labeled with fluorochromes FAM, HEX, or TET. The PCR reaction was carried out in GeneAmp® PCR System 9700 (PE, Applied Biosystems) employing 50 ng genomic DNA in a 20 μl volume containing 1 U AmpliTaq Gold Polymerase (Applied Biosystems), 200 μM dNTP, 2.5 μM of $MgCl_2$ and 25 pmol of each specific primer (Proligo, France). Cycling conditions to generate PCR products included an initial phase of 10 min at 95˚C, followed by 35 cycles of 1 min at 95˚C, 1 min at 60˚C and 1 min at 72˚C and a final extension step of 10 min at 72˚C. PCR products were analyzed on an ABI377 DNA sequencer (Applied Biosystems) with TAMRA-labeled internal lane standard. AI was analyzed by Genotyper 2.0 (Applied Biosystems).

Statistical analysis. Paired t-tests were used to compare global methylation status, *RASSF1A* expression and promoter methylation status between tumors and corresponding normal tissues. Non-parametric Mann-Whitney U test was used to assess the relationship between global methylation as well as *RASSF1A* methylation, expression and AI with different clinical and histopathological tumor features. Spearman's rank order was utilized to determine the relationship between the continuous variables global methylation, *RASSF1A* methylation, and mRNA expression. P-values <0.05 were considered significant. All statistical tests were performed using Statistica version 6 (StatSoft Inc., Johannesburg, South Africa).

Results

Lack of genome-wide hypomethylation in FTCs. The mean LINE-1 methylation in tumor samples was 71.3±2.6 compared with 71.8±3.4 in their corresponding normal tissues. In two tumors LINE-1 methylation was decreased by >10% and in 11 tumors by <10%. The remaining eight had slight relative

Table I. Clinical and genetic information for the FTC cases studied.

Case no.	Age at surgery	Gender M/F	Subtype MI/WI	Capsular penetration	Vascular invasion	Locally advanced	Metastasis at surgery	Size (cm)	Local recurrence	Metastases post-operartively	Follow-up Outcome	Follow-up Time (years)	*RAS* mutation
1	32	F	MI	Yes	-	-	-	1.5	-	-	NED	21.0	No
2	47	M	WI	Yes	Yes	Yes	-	8.0	Yes 2 years	Lung, brain 0.2 years	DoD	2.6	No
3	11	F	MI	Yes	Yes	-	-	2.0	-	-	NED	18.0	No
4	31	M	MI	Yes	-	-	-	3.5	-	-	NED	17.0	*NRAS*
5	49	M	MI	-	Yes	-	-	5.0	-	-	NED	16.0	No
6	57	F	MI	Yes	-	-	-	2.0	-	-	NED	15.0	No
7	30	F	MI	Yes	-	-	-	3.5	-	-	NED	3.0	No
8	45	M	MI	Yes	Yes	-	-	6.0	-	-	NED	12.0	No
9	26	M	MI	Yes	-	-	-	2.5	-	-	NED	10.0	No
10	46	F	MI	Yes	Yes	-	-	3.0	-	-	NED	12.0	No
11	75	F	MI	Yes	Yes	-	-	3.5	-	-	NED	12.0	*KRAS*
12	44	F	MI	Yes	-	-	-	4.0	-	-	NED	12.0	No
13	61	F	WI	Yes	Yes	-	-	4.0	Yes 1 year	Skeletal 1.2 years	DoD	2.8	*NRAS*
14	41	F	MI	-	Yes	-	-	4.0	-	-	NED	8.0	No
15	31	F	MI	-	Yes	-	-	2.0	-	-	NED	7.0	No
16	56	F	MI	Yes	Yes	-	-	2.5	-	-	NED	8.0	No
17	24	F	MI	Yes	-	-	-	3.5	-	-	NED	7.0	No
18	72	F	MI	Yes	Yes	-	Skeletal	3.5	-	Skeletal	AwD	7.0	No
19	45	F	MI	Yes	-	-	-	3.5	-	-	NED	6.0	No
20	57	M	MI	Yes	-	-	Skeletal	5.0	-	Skeletal	NED	5.0	No
21	42	F	MI	Yes	-	-	-	2.5	-	-	NED	3.5	No

F, female; M, male; MI, minimally invasive; WI, widely invasive; -, no; NED, no evidence of disease; DoD, died of disease; AwD, alive with disease. *RAS* mutations status according to Foukakis *et al* (12).

hypermethylation (Tables II and III and Fig. 1A). Overall there was no significant difference in the LINE-1 methylation level of the tumor samples compared with the normal tissues (p=0.57, by paired t-test) (Fig. 1B).

LUMA revealed no significant difference of the *Hpa*II/*Msp*I ratio between the tumor samples and their corresponding normal tissues (Table II and Fig. 1C) (p=0.42, by paired t-test). When 15 female and 6 male patients were compared, the *Hpa*II/*Msp*I ratio was higher in the tumor samples obtained from female patients (0.34±0.05 vs. 0.26±0.04; p<0.05, by Mann-Whitney U test).

RASSF1A promoter hypermethylation in FTCs. The methylation status of *RASSF1A* was determined in 21 pairs of FTC with its corresponding normal thyroid tissues (Fig. 2A and Table II). Methylation density at five CpG sites within the *RASSF1A* promoter was quantified using bisulfite Pyrosequencing (Fig. 2A and Tables II-IV), which was further validated by the qualitative method MSP (Table II). In general normal thyroid tissues demonstrated low levels of methylation at the CpG sites examined and six normal thyroids exhibited methylation between 10 and 19%, while one case was heavily methylated. Significantly increased methylation was seen in tumors as compared to their corresponding normal tissues (p=0.0001; paired t-test; Fig. 2B). Interestingly, an inverse correlation was observed between tumor size and *RASSF1A* promoter methylation (R=-0.424, by Spearman's rank-order correlation). There was weak correlation between *RASSF1A* methylation Pyrosequencing results with LINE-1 and LUMA global methylation results (Spearman's rank correlation; -0.08 and -0.05 respectively).

Reduced expression and AI of the RASSF1A gene in FTCs. *RASSF1A* mRNA levels were significantly reduced in primary FTCs when compared with matched non-neoplastic thyroid tissue (p=0.0002, by paired t-test; Table II and Fig. 2C). *RASSF1A* mRNA levels remain unchanged for 4 of 21 (19%) cases.

Table II. Results from analyses of the *RASSF1A* locus and genome-wide methylation.

		RASSF1A analyses						Genome-wide methylation			
		qRT-PCR				Pyrosequencing mean (range)		LUMA		LINE-1 analysis mean (range)	
Case no.	Allelic imbalance	Normal	Tumor	mRNA fold change	Methylation by MSP	Normal (%)	Tumor (%)	Normal	Tumor	Normal (%)	Tumor (%)
1	Yes	5.7	6.6	1.2	Yes	55 (47-61)	5 (3-6)	1.0	0.31	68 (56-79)	73 (64-78)
2	Yes	1.9	0.9	0.5	Yes	6.5 (5-8)	42 (36-47)	0.31	0.25	71 (62-77)	71 (63-79)
3	Yes	0.9	1.3	1.4	Yes	6 (3-9)	47 (19-61)	ND	0.42	81 (73-84)	68 (61-71)
4	Yes	2.0	0.3	0.2	Yes	13 (7-19)	81 (75-90)	0.26	0.24	74 (67-80)	73 (67-79)
5	Yes	1.1	1.1	1.0	No	9 (4-13)	1 (0-1)	0.50	0.34	73 (67-79)	72 (66-78)
6	No	4.7	3.9	0.8	Yes	5 (3-8)	56 (39-23)	ND	0.33	71 (65-75)	71 (69-75)
7	No	3.2	0.8	0.3	Yes	5 (3-7)	16 (9-24)	0.26	0.29	68 (58-79)	74 (66-79)
8	Yes	1.5	1.0	0.7	Yes	10 (5-13)	32 (18-41)	ND	0.28	73 (66-79)	72 (67-75)
9	Yes	1.5	0.3	0.2	Yes	7 (4-11)	69 (44-83)	0.29	0.24	73 (64-78)	72 (63-80)
10	Yes	2.2	0.5	0.2	No	7 (3-10)	25 (16-34)	ND	0.38	70 (61-79)	68 (62-73)
11	No	2.5	0.8	0.3	Yes	9 (7-10)	56 (32-67)	0.25	0.39	74 (66-80)	74 (66-81)
12	Yes	2.2	0.4	0.2	Yes	5 (3-8)	14 (5-27)	0.41	ND	74 (69-80)	72 (65-81)
13	No	2.0	0.4	0.2	Yes	13 (6-17)	53 (32-70)	ND	0.37	68 (59-72)	75 (67-80)
14	No	3.4	1.2	0.4	Yes	10 (4-14)	54 (21-73)	ND	0.35	68 (62-72)	65 (56-69)
15	No	7.9	1.7	0.2	Yes	6 (3-8)	34 (15-44)	0.26	0.3	74 (68-79)	72 (65-76)
16	Yes	4.4	1.1	0.3	Yes	6 (3-8)	92 (83-99)	0.35	0.28	74 (67-79)	73 (67-78)
17	Yes	2.3	0.9	0.4	No	7 (4-10)	0 (0-0)	ND	0.3	72 (64-76)	69 (59-73)
18	Yes	1.9	0.5	0.3	Yes	3 (2-4)	10 (6-11)	0.77	0.31	67 (62-71)	74 (67-81)
19	Yes	3.1	0.8	0.3	Yes	17 (9-21)	54 (28-69)	ND	ND	67 (60-72)	69 (63-74)
20	Yes	0.9	2.0	2.2	Yes	19 (10-26)	58 (38-68)	0.26	0.24	72 (63-79)	72 (65-79)
21	Yes	1.2	0.4	0.3	Yes	9 (5-13)	59 (48-67)	0.34	0.39	76 (67-84)	67 (59-72)

Summary of allelic loss based on four satellite markers (D3S4597, D3S4604 and D3S4614). ND, not determined. *RASSF1A* mRNA expression values represent *RASSF1A*/18S ratios for each sample. *RASSF1A* mRNA expression fold change was determined by the *RASSF1A* mRNA expression of tumor/*RASSF1A* mRNA expression from the corresponding normal thyroid tissues.

AI at chromosome 3p21.3 was examined in 21 FTCs. Representative results are shown in Fig. 4. AI at these loci were 6 of 21 (29%) for D3S4597, 9 of 21 (43%) for D3S4604, and 14 of 21 (67%) for D3S4614, respectively. In total, 16 of 21 (76%) cases were informative for the allelic status of 3p21.3, and the frequency of AI for *RASSF1A* was 15 of 21 (71%). There was a significant correlation between AI for D3S4597 with tumor size, whereby AI in this region was associated with bigger tumor (p=0.0002 by Mann-Whitney U test; Fig. 4C).

Discussion

Alterations of DNA methylation are important events in tumorigenesis of human cancer. Genomic hypomethylation may lead to both genome instability and hypomethylation of proto-oncogenes, which in turn leads to their over-expression (13). On the contrary, local promoter hypermethylation is often associated with silencing of tumor suppressor genes (3). While genome-wide demethylation and single-copy CpG island hypermethylation occur in cancer, it is not known whether these two alterations are linked. In this study we demonstrate that *RASSF1A* promoter hypermethylation occurs independently of genome-wide methylation changes.

Two independent methods revealed no global hypomethylation, while by contrast hypermethylation of *RASSF1A* promoter was frequently seen in FTCs as compared to corresponding normal thyroid tissues. It has been hypothesized that hypomethylation generally arises earlier and is strongly linked to chromosomal instability and loss of imprinting, whereas hypermethylation occurs in promoters, and is usually a later event (3). This notion was supported by several studies in many carcinomas including breast, colon, lung, head and neck, bladder, esophagus, liver, prostate, and stomach, which revealed frequent hypomethylation compared to their normal tissue counterparts (18-20). Although we observed elevated levels of *RASSF1A* methylation, no global hypomethylation was seen in the tumors as compared to matched normal

Table III. Detailed Pyrosequencing results from LINE-1 genome-wide methylation analysis.

Case no.	Normal (% methylation at CpG sites)					Tumor (% methylation at CpG sites)					Ratio Tumor/normal
	CpG1	CpG2	CpG3	CpG4	Mean 1-4	CpG1	CpG2	CpG3	CpG4	Mean 1-4	
1	70.9	67.5	56.4	78.9	68.4	78.5	73.3	64.4	75.7	73.0	1.1
2	77.3	70.9	62.0	73.1	70.8	78.7	70.7	63.2	72.7	71.3	1.0
3	84.4	82.2	73.1	83.5	80.8	71.3	69.6	60.9	70.8	68.2	0.8
4	79.6	73.0	67.0	75.6	73.8	79.4	72.5	66.6	74.8	73.3	1.0
5	78.8	74.3	66.9	73.6	73.4	78.2	72.5	66.2	72.7	72.4	1.0
6	74.9	70.9	64.9	72.8	70.9	74.5	68.7	65.5	74.8	70.9	1.0
7	78.5	62.4	57.5	75.4	68.5	78.7	72.9	66.3	76.2	73.5	1.1
8	78.5	73.0	65.9	73.7	72.8	75.1	71.1	66.5	74.5	71.8	1.0
9	77.6	72.7	64.2	77.5	73.0	80.4	71.2	62.8	73.1	71.9	1.0
10	79.3	60.5	64.4	76.5	70.2	73.4	69.1	61.9	68.9	68.3	1.0
11	80.0	73.7	66.0	77.1	74.2	80.5	71.9	66.2	76.1	73.7	1.0
12	79.7	73.9	68.6	75.7	74.5	80.7	70.1	65.0	72.8	72.2	1.0
13	72.4	70.3	58.6	70.3	67.9	80.2	74.1	67.1	77.0	74.6	1.1
14	72.3	71.2	62.0	68.1	68.4	69.1	66.5	56.4	68.0	65.0	1.0
15	78.8	74.7	67.5	74.7	73.9	76.0	72.5	65.1	73.8	71.9	1.0
16	78.6	75.1	66.7	75.3	73.9	76.3	72.5	67.3	77.5	73.4	1.0
17	75.8	74.0	64.1	74.4	72.1	73.2	70.4	58.7	72.2	68.6	1.0
18	71.1	66.6	61.6	69.1	67.1	80.7	72.8	67.2	75.6	74.1	0.9
19	71.9	67.0	60.3	68.3	66.9	74.4	69.3	62.6	70.5	69.2	1.1
20	78.6	71.9	62.8	72.7	71.5	78.8	71.5	64.8	74.3	72.4	1.0
21	83.9	75.4	67.0	75.6	75.5	72.4	67.9	59.3	69.7	67.3	0.9

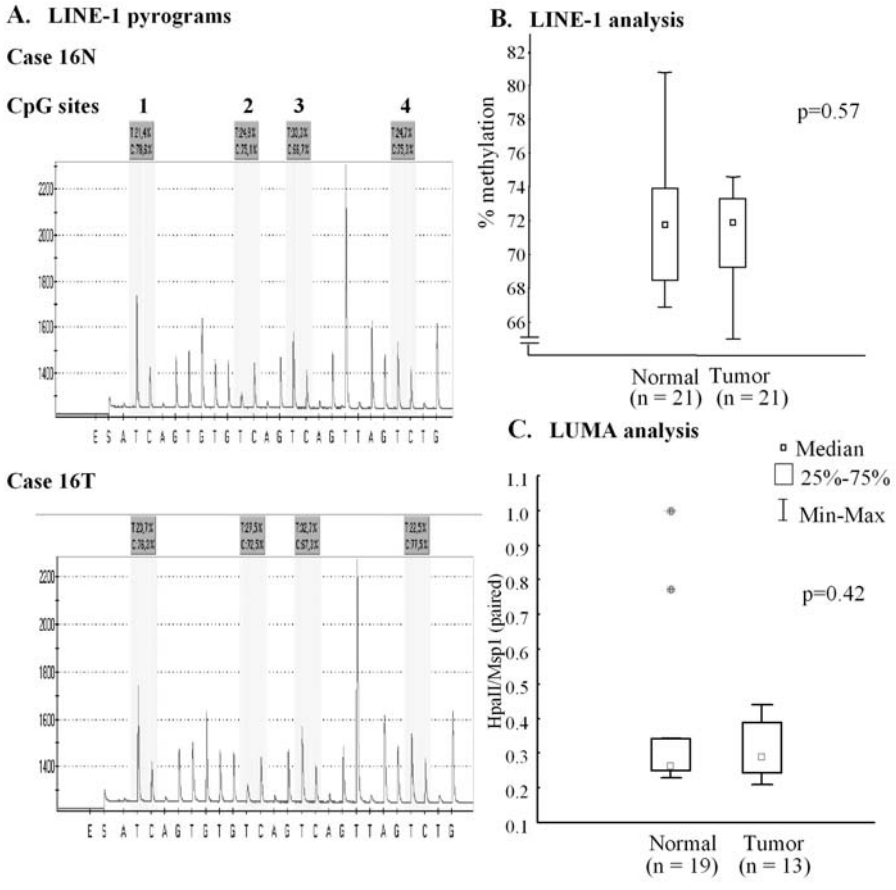


Figure 1. (A) LINE-1 methylation by Pyrosequencing at CpG sites 1-4 for tumor (16T) compared to its corresponding normal tissue (16N). FTCs and corresponding normal thyroids have similar levels of global methylation by: (B) LINE-1 analysis and (C) LUMA.

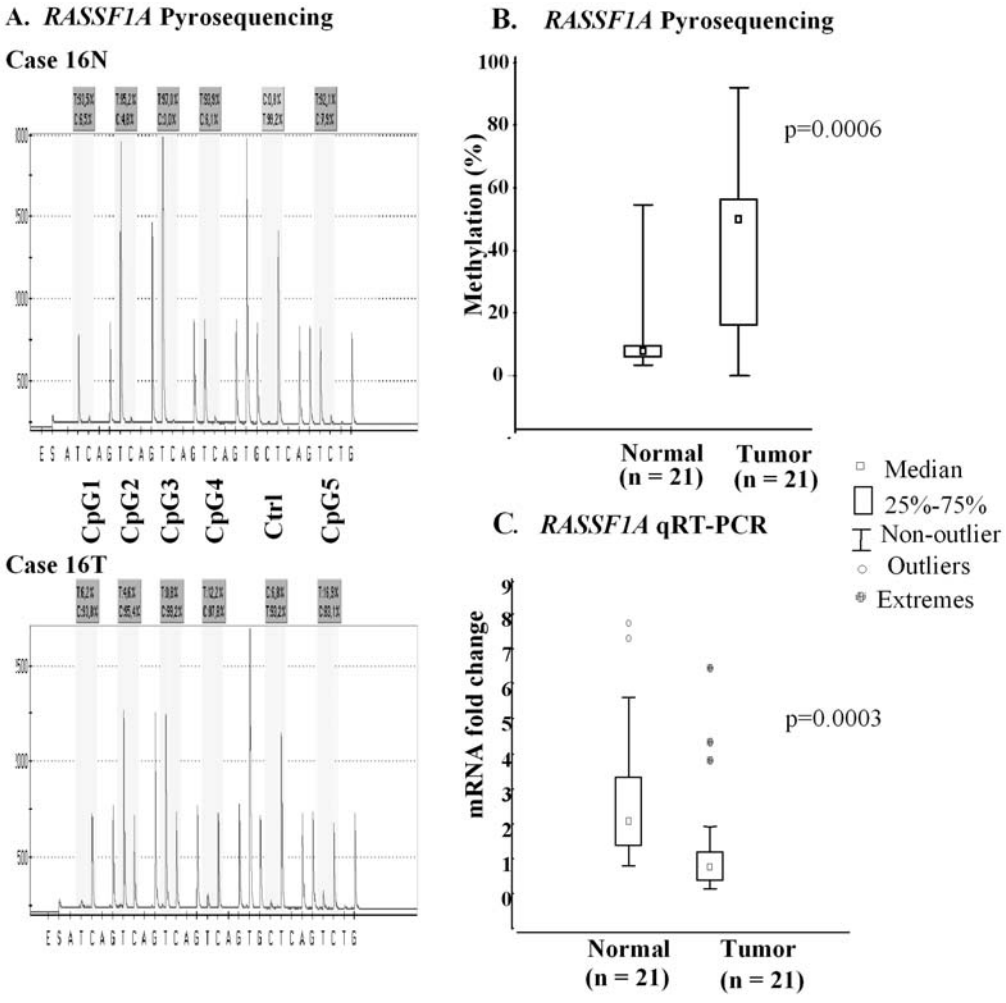


Figure 2. Comparison of *RASSF1A* promoter methylation and mRNA expression levels in follicular thyroid cancer and matching normal thyroids. (A) Pyrograms from quantification of *RASSF1A* promoter methylation for case 16, showing hypermethylation in the tumor tissue (16T). The proportion of C:T reflects the degree of methylation at a particular site assessed. In this designed assay, five specific CpG sites were assessed, whereas a sixth site was served as an internal quality control of the assay. (B) The mean methylation of the five CpG sites assessed was significantly increased in tumors as compared to their normal tissue counterparts. (C) *RASSF1A* mRNA levels were significantly reduced in tumors as compared with their normal tissue counterparts.

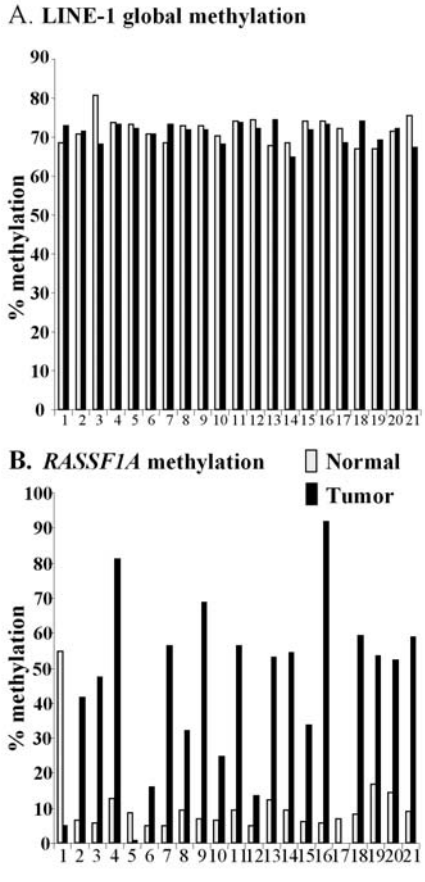
thyroid tissues. Our observation is concordant with previous reports sharing lack of global hypomethylation e.g. in ovarian cancer, lymphoma, renal cell carcinoma and another thyroid epithelial cancer, papillary thyroid cancer (2,18,21). Collectively, these findings suggest that gene-specific hypermethylation and global hypomethylation could be independent processes.

Galusca *et al* showed a significantly lower level of global methylation in thyroid cancer tissues when compared with benign tumors or adjacent normal thyroid by employing 5-methylcytidine antibodies (5). Differences in methodology and patient materials may have contributed to the differences between our and Chalitchagorn *et al* findings (18) with Galusca *et al* (5). We and Chalitchgorn *et al* (18) investigated the repetitive element, LINE-1 using quantitative or qualitative (18) assessments. On the other hand, Galusca *et al* (5) utilised an immunochemical approach by detecting the presence of a methyl group on the carbon 5 of cytosine of the DNA on interphase nuclei. LINE-1 is now known as a common surrogate for global methylation because much of the effects of global DNA hypomethylation are thought to occur through activation of normally dormant transposons and endogenous retroviruses present in the human genome (2). We also assessed methylation by a novel method, referred to as LUMA, which targets CCGG sequences throughout the entire genome. Both LINE-1 and LUMA methodologies resulted in similar observations, indicating a lack of hypomethylation between FTCs and their normal counterparts.

RASSF1A is hypermethylated in various human tumors, including thyroid adenomas and cancers (22,23). Although there are several reports on *RASSF1A* promoter hypermethylation in thyroid tumors, comparisons between FTCs and their corresponding normal tissues are few. Most studies utilized non-quantitative methods such as MSP (10). Two studies determined *RASSF1A* promoter methylation quantitatively in thyroid tumors. Using quantitative MSP, Hoque *et al* detected 15% methylation in a series of thyroid tumors (7) and Xing *et al* observed *RASSF1A* methylation in 44% of thyroid adenoma, 75% of FTCs and 20% of PTCs (9). In the present study, *RASSF1A* methylation was determined by MSP, although the results from MSP gave no precise methylation value (Table II), the majority of FTCs (86%) had an elevated level of *RASSF1A* methylation as compared with their corresponding normal tissues. Interestingly, 33% (7 out

Table IV. Detailed results from *RASSF1A* methylation analyses by Pyrosequencing.

Case no.	Normal (% methylation at CpG sites 1-5)						Tumor (% methylation at CpG sites 1-5)						Ratio Tumor/normal
	CpG1	CpG2	CpG3	CpG4	CpG5	Mean 1-5	CpG1	CpG2	CpG3	CpG4	CpG5	Mean 1-5	
1	58.7	60.9	47.3	54.8	51.2	54.6	6.3	4.9	3.1	4.2	5.9	4.9	0.1
2	7.1	7.7	6.7	5.6	5.3	6.5	41.3	47.0	46.6	37.7	35.6	41.6	6.4
3	6.6	4.0	3.1	6.1	8.6	5.7	61.3	50.1	19.2	54.1	52.2	47.4	8.3
4	14.9	10.0	6.8	13.8	18.7	12.8	90.4	78.1	74.6	80.7	82.2	81.2	6.3
5	10.3	7.0	3.9	8.9	12.7	8.6	1.1	1.2	1.4	1.0	0.0	0.94	0.1
6	5.7	4.3	3.0	5.4	6.9	5.1	18.2	12.2	8.6	16.7	23.6	15.9	3.1
7	6.2	4.0	2.6	5.1	7.8	5.1	57.3	60.6	39.1	52.0	73.2	56.4	0.1
8	11.9	7.9	5.4	9.7	12.9	9.6	36.0	29.2	18.2	40.7	35.4	31.9	11.1
9	8.8	5.6	3.5	7.6	10.6	7.2	83.1	66.9	44.3	73.3	76.0	68.7	9.5
10	7.9	5.6	3.3	7.2	9.7	6.7	27.7	15.8	16.9	28.8	33.9	24.6	3.7
11	10.3	10.3	7.4	9.0	9.6	9.3	64.4	66.5	32.0	60.4	57.9	56.2	6.0
12	5.9	4.0	2.9	5.0	7.5	5.1	27.0	6.4	5.3	14.3	14.3	13.5	2.6
13	15.4	10.8	5.7	13.6	17.1	12.5	69.7	47.9	32.0	49.9	65.1	52.9	4.2
14	11.7	7.9	3.7	10.8	14.0	9.6	72.8	50.3	21.1	67.4	59.9	54.3	5.7
15	7.7	5.2	3.1	6.4	8.4	6.2	43.8	34.1	15.0	34.0	41.9	33.8	5.5
16	6.5	4.8	3.0	6.1	7.9	5.7	93.8	95.4	99.2	87.8	83.1	91.9	16.0
17	8.3	5.3	3.6	7.7	10.4	7.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
18	9.9	6.2	4.3	8.8	11.5	8.1	65.9	57.6	47.7	62.8	62.3	59.3	7.3
19	20.7	17.8	9.1	18.8	17.8	16.8	67.1	57.2	27.8	69.2	46.1	53.5	3.2
20	21.0	16.5	10.1	20.5	25.9	18.8	66.2	50.4	37.9	68.1	64.9	57.5	3.1
21	10.6	6.9	5.0	10.2	12.7	9.1	59.3	67.0	48.1	53.4	66.3	58.8	6.5



of 21) of the matched normal thyroids were found to have methylation ≥10%. This observation coincides with previous studies in other human cancers whereby modest hypermethylation of tumor suppressor genes has been observed in the normal tissues of patients in which the same gene is hypermethylated in the tumor tissue (3,24).

Silencing by epigenetic modification is a fundamental mechanism for cancer-related genes inactivation in the pathogenesis of human cancer, in addition to mutations and allelic loss. Mutations in *RASSF1A* are rarely detected in human tumors (25). *RASSF1A* resides at 3p21.3, and allelic loss of this region has been commonly observed in cancers of the lung, head and neck, kidney, bladder, female genital tract and breast (23,25). Although there are reports on reduced *RASSF1A* expression and hypermethylation of *RASSF1A* in thyroid cancer, its allelic loss was not previously investigated. We found that 71% of FTCs showed an allelic loss for *RASSF1A*. Furthermore, *RASSF1A* expression was significantly reduced in FTCs compared to matched normal thyroid tissues (Table II and Fig. 2C). However, we did not find an obvious

Figure 3. Comparison between global LINE-1 and *RASSF1A* promoter methylations in FTCs with their corresponding normal thyroid tissues. (A) No significant difference in LINE-1 methylation between FTCs and normal thyroid tissues while (B) hypermethylation of *RASSF1A* promoter was significantly increased in tumors.

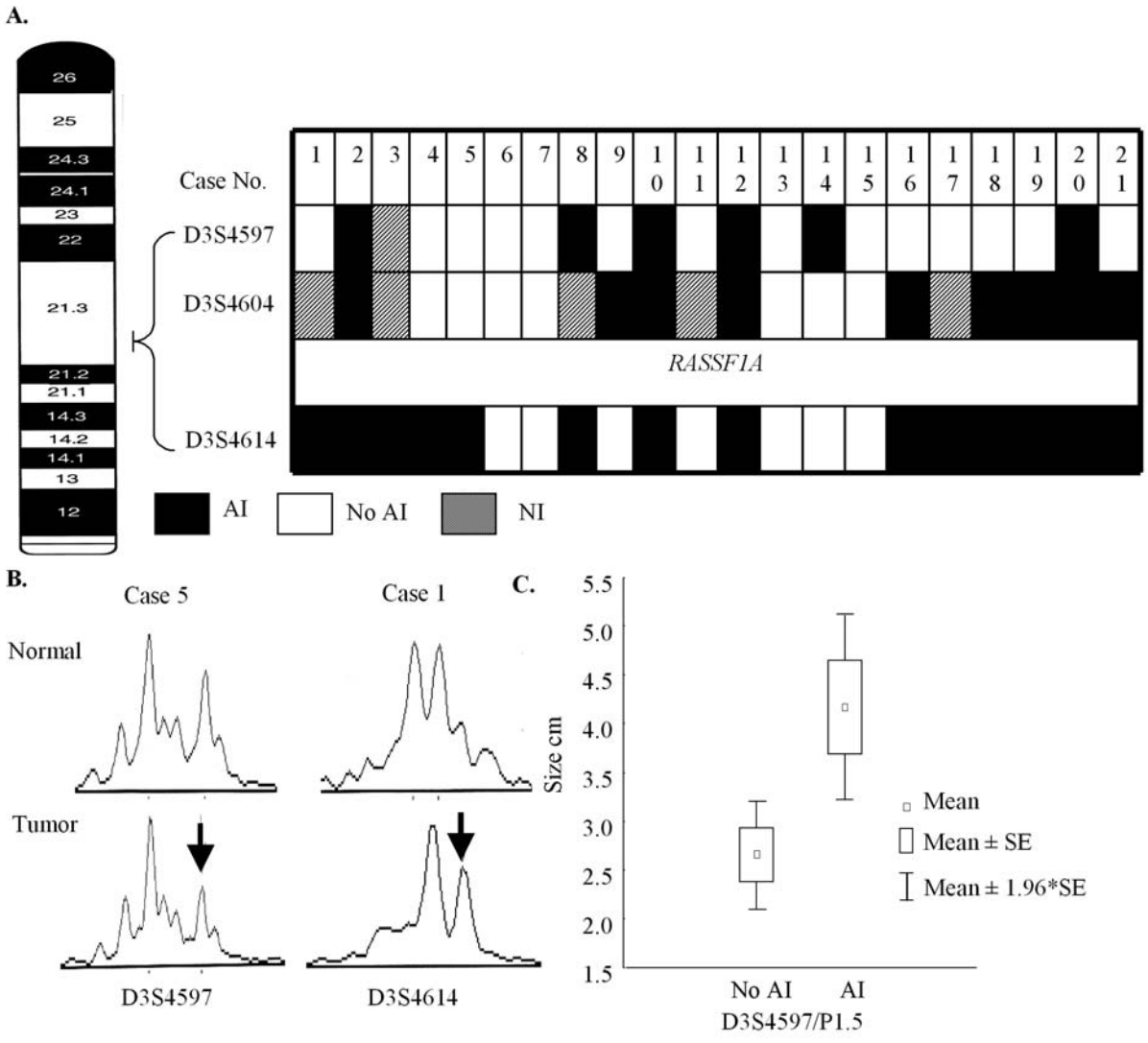


Figure 4. (A) Allelic imbalance (AI) at the *RASSF1A* locus was assessed in three positions (markers). (B) AI profiles for cases 1 and 5. (C) Smaller tumor size is correlated with AI at D3S4957 (Mann-Whitney U test; p=0.0002). NI, non-informative.

correlation between *RASSF1A* hypermethylation, *RASSF1A* allelic loss and *RASSF1A* mRNA reduction in our series.

In conclusion, our results show that genome-wide methylation changes are not observed between FTCs and their corresponding normal thyroid tissues, although *RASSF1A* promoter methylation is observed in the tumors. This suggests that genome-wide methylation changes are not coupled with promoter hypermethylation of *RASSF1A* in FTCs. As expected, *RASSF1A* is frequently inactivated in FTC both by promoter hypermethylation and allelic loss.

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