BRAF Mutation and RASSF1A Expression in Thyroid Carcinoma of Southern Italy

Angela Santoro,[1] Giuseppe Pannone,[1] Maria Antonia Carosi,[2] Arianna Francesconi,[2] Edoardo Pescarmona,[2] Giuseppe Maria Russo,[3] Antonia Feola,[4] Simona Losito,[5] Renato Franco,[5] Luigi Nappi,[6] Gabriella Aquino,[5] Gaetano De Rosa,[7] Marina Di Domenico,[4,8*] and Pantaleo Bufo[9]

[1]Department of Clinical and Experimental Medicine, Section of Anatomic Pathology, University of Foggia, Foggia, Italy

[2]Division of Medical Oncology, Regina Elena National Cancer Institute, Rome, Italy

[3]Department of Otorhinolaryngology, University of Foggia, Foggia, Italy

[4]Department of Biochemistry, Biophysics and General Pathology, Second Univesity of Naples, Naples, Italy

[5]Section of Pathological Anatomy, Fourth Section of Maxillofacial Surgery, Istituto Nazionale per lo Studio e la Cura dei Tumori, Fondazione 'G.Pascale', Naples, Italy

[6]Department of Surgical Sciences, Institute of Obstetrics and Gynecology, University of Foggia, Foggia, Italy

[7]Department of Biomorphological and Functional Sciences, Pathological Section, University of Naples, Italy

[8]Center for Biotechnology, Sbarro Institute for Cancer Research and Molecular Medicine, Temple University, Philadelphia, Pennsylvania

[9]Department of Clinical and Experimental Medicine, Section of Anatomic Pathology and Cytopathology, University of Foggia, Foggia, Italy and IRCCS CROB−Centro di Riferimento Oncologico di Basilicata, Rionero in Vulture, Potenza, Italy

ABSTRACT

Aim of this work is to provide a detailed comparison of clinical-pathologic features between well-differentiated and poorly differentiated tumors according to their BRAF and RASSF1A status. We analyzed RASSF1A methylation by MSP and BRAF mutation by LCRT-PCR with LightMix® kit BRAF V600E in neoplastic thyroid tissues. Immunohistochemical evaluation of RASSF1A expression was also performed by standard automated LSAB-HRP technique. An overall higher degree of RASSF1A over-expression than normal thyroid parenchyma surrounding tumors ($P < 0.05$) has been found in all malignant well-differentiated lesions. Moreover, statistically significant higher levels of RASSF1A expression were observed in differentiated cancers associated to an inflammatory autoimmune background ($P = 0.01$). Amplifiable DNA for LC PCR with LightMix® kit BRAF V600E was obtained in nine PTCs, four FVPTCs, five ATCs, and one control. The V600E mutation was found in 13 of 18 (72%) tumors. BRAF was mutated in 6 of 9 (66%) classical PTC, in 2 of 4 (50%) follicular variant PTC and in all ACs (100%). The overall frequency of RASSF1A promoter methylation observed was 20.5% (9 cases out 44). Hypermethylation of RASSF1A in primary tumors was variable according to histotypes ranging from100% (5/5) in ACs to only 12.5% (4/32) in PTCs. We show a correlation between RASSF1A methylation status and RASSF1A protein expression. Finally, we conclude that BRAF V600E mutation and RASSF1A methylation were pathogenetic event restricted to a subgroup of PTC/FVPTCs in early stage and to clinically aggressive ATCs. J. Cell. Biochem. 114: 1174–1182, 2013. © 2012 Wiley Periodicals, Inc.

KEY WORDS: BRAF; RASSF1A; IMMUNOHISTOCHEMISTRY; PROMOTER METHYLATION

Four types of thyroid carcinomas comprise 98% of all thyroid malignancies: (a) PTC; (b) FTC; (c) UTC; and (d) MTC [Figge, 2006]. PTC, FTC, and UTC originate from the thyroid follicular epithelial cells. In contrast, MTC derives from the para-follicular C cells and it is the main type of hereditary thyroid cancer [Figge, 2006]. The overall incidence is rising for reasons that remain

unclear, but in part, could be related to improved diagnostic procedures. Recurrence is a common event in thyroid carcinomas (15–30% of patients), even in early-stage disease [Hay et al., 1993; Mazzaferri and Jhiang, 1994; Fonseca et al., 1997; Gilliland et al., 1997; Sherman et al., 1998; LiVolsi et al., 2000]. Therefore, it is crucial to identify patients at higher risk of recurrence so that more aggressive therapy and monitoring can be realized. In this way, molecular investigation on cancerous genetic and epigenetic background could also assume a prognostic role. Genetic alterations are common in thyroid cancers, including BRAF gene mutation [Cohen et al., 2003; Fukushima et al., 2003; Kimura et al., 2003; Namba et al., 2003; Soares et al., 2003; Xu et al., 2003] and RET/PTC rearrangements [Moretti et al., 2000] in PTC, and Ras proto-oncogene mutations [Moretti et al., 2000] and PAX8–PPAR fusions [Kroll et al., 2000] in FTC.

In vitro studies have demonstrated the important role for BRAF as a central regulator of thyroid-specific protein expression (i.e., differentiation) and proliferative capacity [Mitsutake et al., 2005]. The BRAF gene codes a cytoplasmic serine/threonine kinase regulated by binding RAS. Recently, a somatic point mutation in the BRAF gene has been identified as the most common genetic event in papillary thyroid carcinoma [Kimura et al., 2003]. More than 80% of the reported BRAF mutations affect nucleotide 1,799 in exon 15, resulting in a thymidine to adenine transversion (T1799A), which translates into valine-to-glutamate substitution at residue 600 (V600E). Mutations that result in a V600E substitution in BRAF and consequent constitutive activation occur in approximately 45% of PTCs in adults, making BRAF mutations the most common defined genetic abnormality in thyroid cancers [Xing et al., 2005]. It remains unclear whether this gene is restricted to papillary carcinoma or it also mutated in poorly differentiated and anaplastic thyroid carcinomas. In several studies, the presence of a BRAF V600E mutation has been also associated with a more aggressive clinical course [Namba et al., 2003; Nikiforova et al., 2003; Xing, 2005; Riesco-Eizaguirre et al., 2006].

More than half of PCs also harbor one of several chimeric oncogenes called RET/PTC, which result from gene rearrangements involving the RET proto-oncogene on chromosome 10, as early demonstrated in Italy [Grieco et al., 1990]. These novel fusion transcripts can be regarded as molecular marker that is specific for PC [Moretti et al., 2000].

Though PAX8-PPARγ rearrangement and RAS mutations are found in up to 80% of all follicular carcinomas, until recently, identifiable genetic alterations were observed in only 30–40% of papillary carcinomas, suggesting that additional genetic defect remained undiscovered [Kroll et al., 2000].

Epigenetic alterations of genes, such as aberrant promoter methylation, are common and important mechanisms involved in thyroidal carcinogenesis [Baylin, 2002], and may be alternative mechanisms to gene mutations for thyroid cancers. An example is the tumor suppressor gene RASSF1A, which is ubiquitously expressed in normal tissues and silenced in numerous cancers through promoter hyper-methylation [Dammann et al., 2000; Pfeifer et al., 2002]. It is well known that both alleles of a tumor suppressor gene need to be inactivated in the classical and revised two-hit hypothesis. Recent theories have shown that RASSF1A may

belong to the class of haplo-insufficient tumor suppressor genes that promotes tumor formation through the inactivation of only one allele. RASSF1A hypermethylation was also reported recently in thyroid cancers [Schagdarsurengin et al., 2002]. Some Authors have declared that primary thyroid carcinomas (71%) and all thyroid cancer cell lines showed RASSF1A inactivation; the highest methylation (80%) was found in the more aggressive forms (UTC and MTC) of thyroid carcinoma, whereas in the less aggressive PTC, RASSF1A hypermethylation (62%) was less pronounced [Schagdarsurengin et al., 2002]. Therefore, RASSF1A inactivation correlated with the malignancy of the primary tumors.

In this study, we analyzed a series of thyroid carcinomas and the surrounding parenchyma for BRAF mutation, RASSF1A immuno-histochemical expression, and epigenetic promoter methylation. Aim of the work has been to provide a detailed comparison of clinical-pathologic features between thyroid tumors according to their BRAF and RASSF1A status.

MATERIALS AND METHODS

STUDY POPULATION AND CLINICOPATHOLOGICAL DATA

Upon approval by the Ethical Committee of the all Institutions, 44 thyroid carcinomas (32 PTCs, 5 ATCs, 3 HTCs, and 4 MTCs), with the respective controls of normal thyroidal parenchyma surrounding tumor were obtained from the archive files of the Department of Clinical and Experimental Medicine, Section of Anatomic Pathology, University of Foggia, Foggia−Italy. Study population were represented by patients having undergone therapeutic surgery for single or multiple thyroidal nodules (2002–2009). All patients or their relatives gave their informed written consent. All tumors were examined and classified according to the criteria of the World Health Organization.

At least two H&E-stained sections for each case were examined by pathologists (GP, AS, PB) with expertise in thyroidal oncology for confirmation of histology. Clinical findings, and pathological data of all patients are shown in Table I.

IMMUNOHISTOCHEMISTRY

All cases were analyzed by immunohistochemistry for RASSF1A expression to establish its role in thyroidal carcinogenesis (Table II). Immunohistochemical analysis on 4-μm serial sections was performed by using Ventana Benchmark XT autostainer and/or manual standard linked streptavidin–biotin horseradish peroxidase technique (LSAB-HRP) [De Maria et al., 2009; Pannone et al., 2012], according to the best protocol for the antibody used in our laboratory, the anti-RASSF1A (RASSF1-3F3−Santa Cruz Biotech-nology, Inc., mouse monoclonal antibody) diluted 1:40 with EDTA, at room temperature. Negative control slides without primary antibody were included for each staining. Slight nuclear counter-staining was realized with Mayer' hematoxylin. The results of the immunohistochemical staining were evaluated separately by two observers. Immunostained cells were counted in at least 10 high power field (HPF) analyzed at optical microscope (OLYMPUS BX41, at 40×). For each case, the cumulative percentage of positive cells among all sections examined was determined. Inter-rate reliability between the two investigators blindly and independently examining

TABLE I. Demographical and Clinicopathological Features of Enrolled Patients

Age			
Range	31–81	Mean	56.95
Gender			
Male	18	Female	26
Histotype			
Papillary thyroid cancers (16 PTC +16 FV-PTC)		32	
Anaplastic thyroid cancers (ATC)		5	
Hürtle cell tumors (HCT)		3	
Medullary thyroid cancers (MTC)		4	
Total		44	

TNM Staging			
T	N0	N1	Total
T1	21	0	21
T2	2	1	3
T3	5	2 (1a + 1b)	7
T4	4	9	13
Total	32	12	44

Stage			
St1	St2	St3	St4
21	2	7	14

No. of cases with lymph node metastases at diagnosis according to histotype

PTC, n (%)	FV-PTC, n (%)	HCT, n (%)	ATC, n (%)	MTC, n (%)
4 (25%)	3 (18.7%)	0	3 (60%)	2 (50%)

Pattern of primitive tumors	
Multicentric	Solitary
14	30

Thyroid background			
Nodular goiter (thyroid hyperplasia)		Hashimoto's thyroiditis	
21 present	23 absent	11 present	33 absent

PTC, papillary thyroid cancer; FV-PTC, follicular variant of papillary thyroid cancer.
Table summarizes clinical data referring to patients' sex and age and histological characteristics of the tumors (such as histotypes, TNM staging, pattern of distribution of primitive tumors in thyroid and thyroid pathological background, frequency of metastases according to histotype).

the immunostained sections was assessed by the Cohen's K test, yielding K values higher than 0.70 in almost all instances.

GENOMIC DNA EXTRACTION

After careful examination of H&E-stained slides, we selected tumor sections with the greatest proportion of malignant tissue. DNA was isolated from five consecutive 10-μM sections of each formalin-fixed, paraffin-embedded tissue sample. Genomic DNA was extracted using a proteinase K (Qiagen, Valencia, CA) digestion followed by DNA isolation using the Wizard DNA clean-up kit (Promega, Madison, WI) according to the manufacturer's protocols. DNA concentration was quantified by A 260 absorbance with a BioPhotometer (Eppendorf, Hamburg, Germany). Genomic DNA (50–100 ng/sample) was used as a template. The isolated DNA has been used for real-time LightCycler PCR (LC PCR) with LightMix® kit BRAF V600E, a new assay method for BRAF mutation detection, and MSP analyses.

LC PCR WITH LIGHTMIX® KIT BRAF V600E

LightMix® kit for the detection of BRAF V600E DNA provides a fast, easy, and accurate system to identify the genotype of the target in a nucleic acid extract according to the manufactures, illustrated in datasheet, of Roche Diagnostic. LightMix® kit includes a mixture of primers that amplify a 212 bp fragment. The resulting PCR fragments are analyzed with hybridization probes labeled with LightCycler® Red 640. The genotype is identified by running a melting curve with specific melting points (T_m). If not clamped, the wild-type BRAF V600E exhibits a T_m of 55°C in channel 640. The mutant BRAF V600E exhibits a Tm of 59°C in channel 640. The supplied control DNA allows the accuracy and the effectiveness of the technique.

LC PCR with LightMix® kit BRAF V600E and melting curve analysis were performed only on selected cases, when amplifiable DNA was obtained. In this way only nine PTCs, four FVPTCs, five ATCs, and one control were analyzed.

A total of 20 μl of PCR mixture containing 2–5 μl of sample DNA according to Roche's datasheet of LightCycler® FastStart DNA Master Plus HybProbe, was used. The LC PCR assay was performed on the LightCycler thermal cycler (Roche), with an initial denaturation at 95°C for 10 min, followed by 50 cycles with denaturation at 95°C for 10 s, 58°C for 15 s, and 72°C for 10 s.

After amplification cycles, the reaction mixture was denatured at 95°C for 20 s, held at 58°C for 20 s followed by one step at 40°C for 20 s, and gradually heated to 85°C at a rate of 0.2°C/s. The melting curves were converted to melting peaks by plotting the negative derivative of the fluorescent signal with respect to temperature $[-d(F2)/dT]$. In this way, the presence of a mutant heteroduplex (containing the wild-type sequences and the mutant allele) is easily detectable because of its low melting temperatures.

SODIUM BISULFITE CONVERSION

Sodium bisulfite modification of the DNA was performed using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) following the manufacturer's protocol, with the addition of a 5-min initial incubation at 95°C prior to denaturation. The de-cross-linking steps in the extraction as well as the 95°C incubation ensure more complete melting of the DNA and thus more complete sodium bisulfite conversion for these highly cross-linked formalin-fixed specimens.

METHYLATION–SPECIFIC PCR (MSP) ASSAY

All cases were analyzed by methylation-specific PCR (MSP) to establish the role of epigenetic phenomena in thyroidal carcinogenesis. Sodium bisulfite-modified DNA was used as the template for MSP as previously described [Herman et al., 1996], using primers specific for the methylated promoters of RASSF1A. All MSPs are optimized to detect >5% methylated substrate in each sample. To test the integrity of isolated DNA the wide hemoglobin gene was amplified by PCR and visualized by gel electrophoresis for both control and pathological samples. The hemoglobin gene primers used were: *forward*, 5′-GAA GAG CCA AGG ACA GGT A-3′, and *reverse*, 5′-GGA AAA TAG ACC AAT AGG CAG 3′. The primers used for Nested-PCR to flank methylated/unmethylated (M/U) study genes have been reported in Table III.

STATISTICS

The data were analyzed by the Stanton Glantz statistical software (version 6, Mc Graw Hill, 2007) and GraphPad Prism software version 4.00 for Windows (Graph Pad software San Diego, CA, www.graphpad.com). Differences between the groups were determined using the one-way analysis of variance (ANOVA) and the

TABLE II. Statistical Univariate Analysis of RASSF1A IHC Expression and Associated Clinico-Pathological Findings of 44 Neoplastic Thyroids

Variables	N	Mean	SD	SEM	P value (ANOVA/Student–Newman–Keuls)
Normal tissue	44	3.57	7.59	1.17	**0.000**
Neoplastic tissue	44	60.12	38.15	5.89	
Age					
≤50 years	17	65.59	30.82	7.47	>0.05
>50 years	27	56.40	42.64	8.53	
Sex					
Male ♂	18	62.50	36.99	8.72	>0.05
Female ♀	26	58.33	39.69	8.10	
Histotype					
Papillary	32	65.16	34.72	6.14	**0.010** (*Hürtle Cell* vs. *Anaplastic*) (*Papillary* vs. *Anaplastic*)
Anaplastic	5	6.66	7.64	4.41	
Hürtle cell	3	93.33	11.55	6.67	
Medullary	4	35	47.26	23.63	
TNM staging					
I	21	65.71	37.46	8.17	>0.05
II–III–IV	23	52.25	38.47	8.60	
Pathological background					
Thyroid hyperplasia	21	65	34.75	7.58	>0.05
No thyroid hyperplasia	23	55.24	41.55	9.07	
Hashimoto's thyroiditis	11	88.33	19.69	6.56	**0.010**
No Hashimoto's thyroiditis	33	52.42	38.51	6.7	
Metastases					
Yes	16	71.07	36.17	9.67	>0.05
No	28	51.43	38.39	7.26	
Multicentric pattern of distribution in thyroid					
Yes	14	74.58	36.15	10.43	>0.05
No	30	54.33	37.96	6.93	

SEM, standard error of mean.
Mean: mean percent of cells stained positive as evaluated in at least 10 high power field (HPF) analyzed at optical microscope.
The statistical evaluation of immunohistochemical data has been compared to clinical reports and pathological findings. An overall higher degree of protein over-expression than normal thyroid parenchyma ($P < 0.05$) has been found in all tumors. Then, we have observed significant correlations between RASSF1A protein over-expression and histotype (*Hürtle Cell* vs. *Anaplastic* and *Papillary* vs. *Anaplastic*) and presence of a Hashimoto's thyroiditis ($P < 0.05$). No statistically significant correlations between protein over-expression and sex, age, TNM stage, presence of metastases and pattern of distribution in thyroid have been noted.
The bold values mean the statistical significant results ($P < 0.05$).

Student–Newman–Keuls test. Only P-values <0.05 were considered significant.

RESULTS

IMMUNOHISTOCHEMISTRY

The statistical evaluation of immunohistochemical data has been compared to clinical reports and pathological findings (Table II). An overall higher degree of protein over-expression than normal thyroid parenchyma ($P < 0.05$) has been found in almost all tumors (Fig. 1). Then, we have observed significant correlations between RASSF1A protein over-expression and histotype (*Hürtle Cell* vs. *Anaplastic* and *Papillary* vs. *Anaplastic*) and presence of Hashimoto's thyroiditis ($P < 0.05$). Synthetically, higher levels of RASSF1A expression were observed in well-differentiated thyroid cancers (WD-TC), especially in those associated to an inflammatory autoimmune background. No statistically significant correlations between protein over-expression and other clinicopathologic parameters (sex, age, TNM stage, presence of metastases, and pattern of distribution in thyroid) have been noted.

BRAF MUTATION IN PTC SAMPLES

The V600E mutation was found in 13 of 18 (72%) tumors (Table IV). BRAF was mutated in 6 of 9 (66%) classical PTC, in 2 of 4 (50%) follicular variant PTC and in all ACs (100%), supporting the importance of the BRAF pathway alterations in PTC and ATC pathogenesis. Moreover, we found V600E mutation in a group of PTC/FVPTCs in early stage and in more advanced ATCs (Table V).

Some mutant genotypes from paraffin specimens were heterozygous, as detected by the presence of a wild-type melting curve and a downward shift in the mutant melting curve. The wild-type specimens had only a wild-type melting curve (Fig. 2).

TABLE III. Table Reports the Specific Primers used for Nested-PCR to Flank Methylated/Unmethylated (M/U) Study Genes

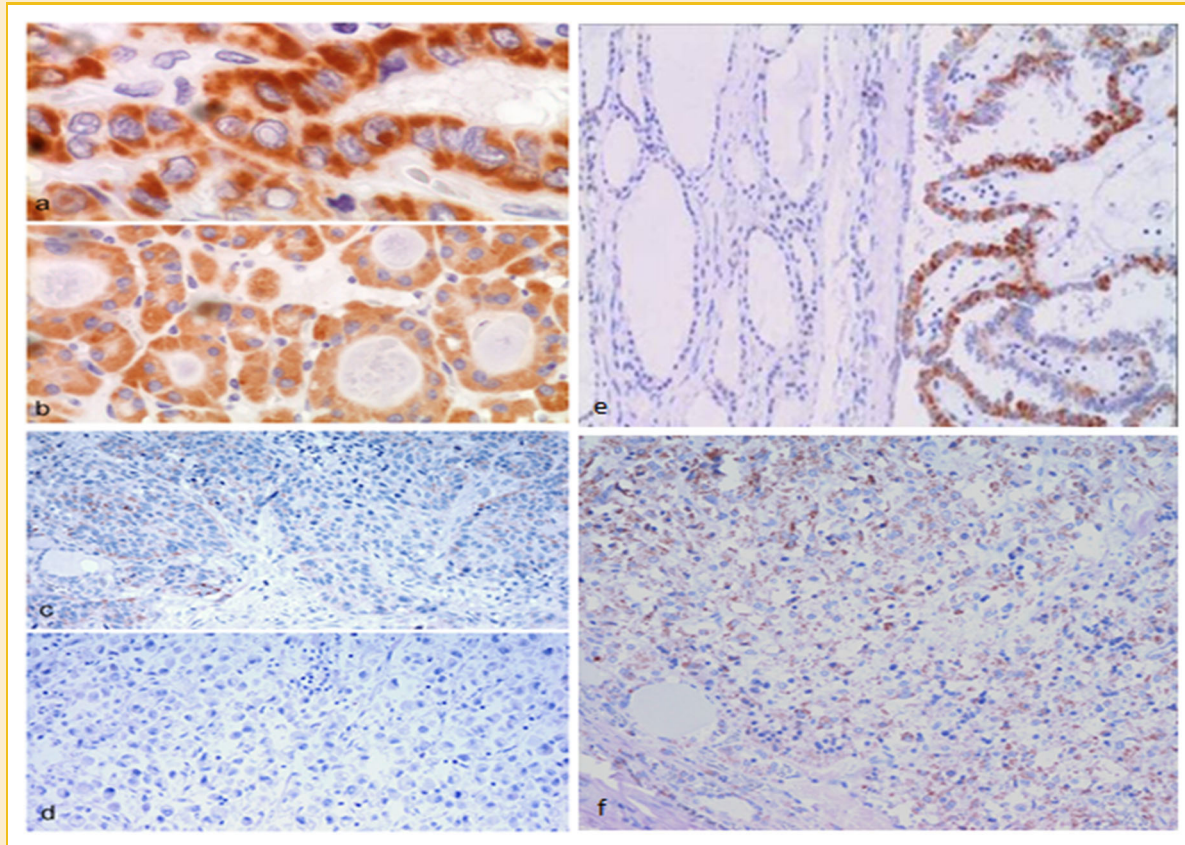| Gene	Genbank no.		Sequences (5′–3′)	Annealing temp. (°C)	Product size (bp)	Ref.
RASSF1A-Methylated	NM_007182	F	GTGTTAACGCGTTGCGTATC	64–60	96	Nakamura et al. [2005]
		R	AACCCCGCGAACTAAAAACGA			
RASSF1A-Unmethylated	NM_007182	F	TTTGGTTGGAGTGTGTTAATGTG	64–60	108	
		R	CAAACCCCACAAACTAAAAACAA			

Fig. 1. Immunohistochemical expression of RASSF1A in thyroid cancer and in normal thyroid. High immunohistochemical expression of RASSF1A in the follicular variant of papillary cancer (a, 40×), in a representative case affected by Hürthle tumor (b, 20×) and in a classical papillary cancer (e, 10×); in (e), the peritumoral gland parenchyma does not stain with RASSF1A. Note the faint staining (c, 4×) or negative (d, 10×) RASSF1A staining in cases of anaplastic carcinomas. A representative medullary cancer shows moderate staining for RASSF1A staining (f, 10×) (LSAB–HRP technique, nuclear counterstaining with hematoxylin).

RASSF1A PROMOTER METHYLATION

The overall frequency of RASSF1A promoter methylation observed was 20.5% (9 cases out 44). The MSP analysis has shown that hypermethylation of RASSF1A in primary tumors was variable and particularly evident in 5/5 ACs (100%) (all cases positive for the BRAF V600E mutation) and in only 4/32 PTCs (12.5%), two of which mutant for BRAF V600E. Among the subtypes of PTC, only a percentage of the classical PTC (4/16, 25‰) were methylated (Fig. 3).

TABLE IV. LC PCR With LightMix® Kit BRAF V600E Analysis of BRAF

N	Sex	Age	Diagnosis	TNM	Stage	RASSF1A methylation status	BRAF^V600E
1	M	61	PTC	T3N1M0	III	U	Positive
2	F	50	PTC	T1N0M0	I	U	Positive
3	F	48	PTC	T4aN0M0	IVA	U	Positive
4	M	38	PTC	T1N0M0	I	U	Positive
5	M	63	PTC	T3N0M0	III	U	Negative
6	F	46	PTC	T1N0M0	I	M	Negative
7	F	35	PTC	T1N0M0	I	M	Positive
8	F	45	PTC	T1N0M0	I	M	Positive
9	M	34	PTC	T2N0M0	II	M	Negative
10	F	48	FVPTC	T2N0M0	II	U	Positive
11	F	76	FVPTC	T3N1M0	III	U	Negative
12	M	43	FVPTC	T1N0M0	I	U	Positive
13	M	48	FVPTC	T3N1M0	III	U	Negative
14	F	48	ATC	T3N1M0	III	M	Positive
15	F	61	ATC	T4aN0M0	IVA	M	Positive
16	M	68	ATC	T3N0M0	III	M	Positive
17	F	74	ATC	T3N0M0	III	M	Positive
18	F	103	ATC	T3N0M0	III	M	Positive
19	M	56	Control: goiter	−	−	U	Negative

Mutation on selected cases and correlation with RASSF1A promoter methylation status.

TABLE V. BRAF Mutation Frequency According to Histotype and Stage of Thyroidal Cancer

Tumor Stage	N.	No. of mutated cases	BRAF mutation frequency
Well-differentiated thyroid tumors			
Early cancer, *Stage I*	6	5	83%
Advanced cancer, *Stage II–III–IV*	7	3	42%
Poorly differentiated thyroid tumors (ATCs)			
Early cancer, *Stage I*	0	–	–
Advanced cancer, *Stage II–III–IV*	5	5	100%

DISCUSSION

Recently significant progress has been achieved in the understanding of molecular events in thyroid carcinogenesis. Researchers have tried to relate any histological variant of cancer to a specific molecular event. RET/PTC and TRK rearrangement have been identified as specific events in papillary thyroid carcinoma. PAX8-PPARγ rearrangement has been found predominantly in the genetic background of follicular carcinomas, Activating point mutations of the RAS genes have been discovered in follicular carcinomas and
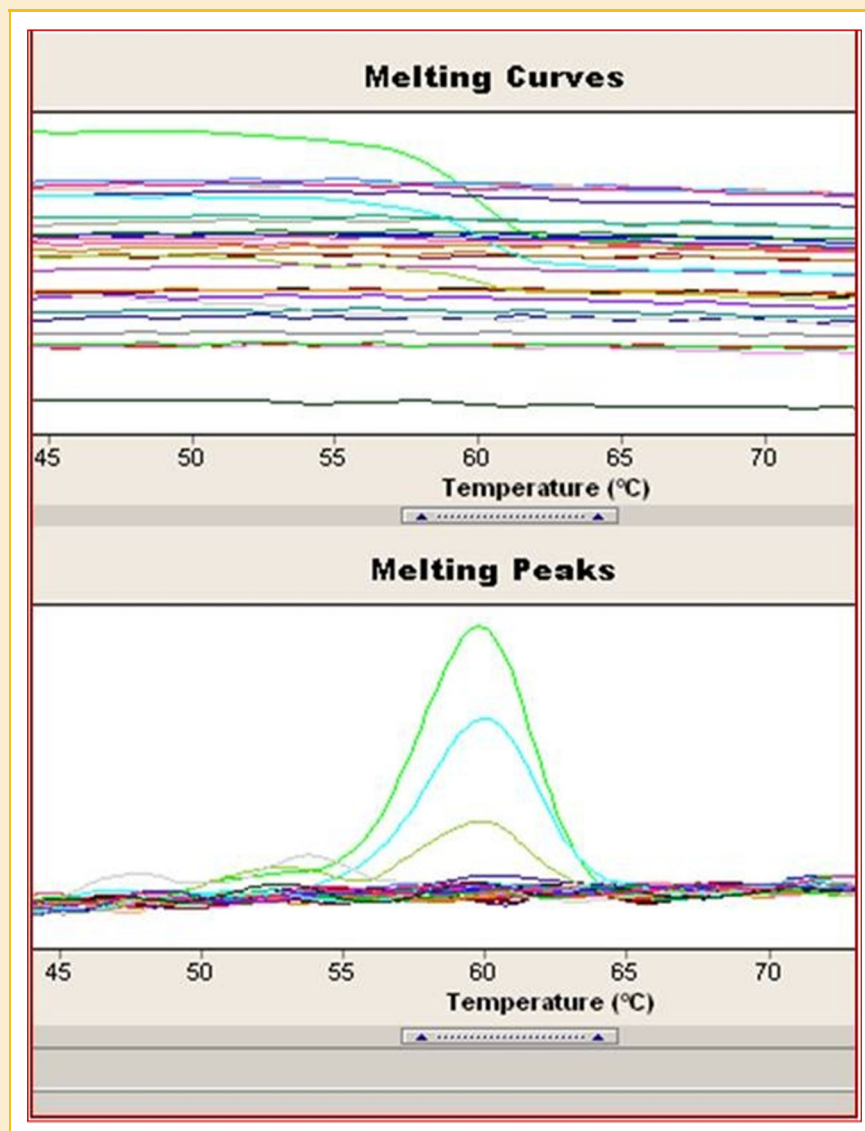

Fig. 2. Melting curves and melting peaks in mutant and wild–type PTC. LC PCR with LightMix® kit BRAF V600E and melting curve analysis for BRAF mutation on thyroid PTC specimens. Melting curves were converted to melting peaks by plotting the negative derivative of the fluorescence [−d(F2)/dT]. A melting peak at 61°C indicates the presence of the mutation, the wild–type samples do not show a peak. Moreover the assay displays a peak at 53°C due to incomplete clamping of the wild type DNA. So the presence both of the peak at 61 and 53°C indicate heterozygous samples.
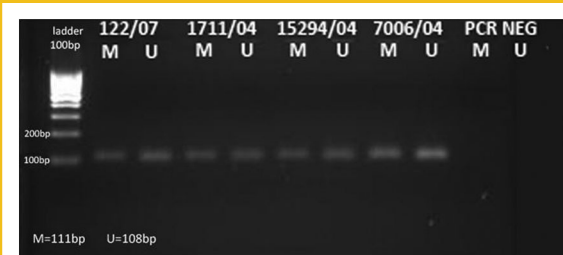
Fig. 3. MSP analysis of RASSF1A promoter methylation status in representative cases of papillary cancer (classical variant). Representative cases of classical PTC showing promoter methylation of RASSF1A as evaluated by MSP. On electrophoretic gel, note the promoter methylation status for RASSF1A. For further details see Material and Methods Section. M, methylated; U, unmethylated.

adenomas, poorly differentiated and anaplastic carcinomas, and, also, in few cases of papillary carcinoma, predominantly of the follicular variant.

The role of aberrant tumor suppressor gene methylation in thyroid cancer has been recently investigated and documented. For example, authors have shown that methylation of TIMP3, SLC5A8, and DAPK are significantly associated with BRAF mutation and several aggressive features of PTC, including the tall-cell phenotype, extra-thyroidal invasion, lymph node metastasis, multifocal localization, and advanced tumor stages [Hu et al., 2006]. These results suggest that the aberrant methylation may be an important step in thyroid carcinogenesis and progression.

In our work, a detailed evaluation of the association between clinical-pathologic features of the thyroid cancers and their RASSF1A status has been provided.

RASSF1A is a member of the RAS association domain family gene, located at the chromosome 3p21 [Dammann et al., 2000; Lerman and Minna, 2000]. The biological function of RASSF1 and all its isoforms (A, B, C, D, E, F, and G) is still not well known. However, the RASSF1A form is transcribed from distinct CpG island promoters and is able to reduce colony formation and to inhibit cancer formation in vitro and in vivo. Mutational inactivation of the coding sequence of this gene is very rare (2%), and the main mechanisms of its inactivation are through promoter methylation and LOH. RASSF1A isoform is epigenetically inactivated in a variety of human primary tumors, including lung (30–40% of non-small cell cancers), breast (62%), nasopharyngeal (67%), kidney (56%), and ovarian carcinoma (30–40%) [Dammann et al., 2000, 2001; Agathanggelou et al., 2001; Burbee et al., 2001; Lo et al., 2001; Yoon et al., 2001].

A recent study has demonstrated that RASSF1A silencing is a common event in thyroid carcinomas and that the RASSF1A inactivation is correlated with the malignancy of the primary tumors: in fact the highest levels of methylation are associated with the more aggressive forms (UTC and MTC) of thyroid carcinoma, whereas in the PTC, RASSF1A hypermethylation is less pronounced [Schagdarsurengin et al., 2002].

Our results have shown that there was generally good agreement between RASSF1A methylation status and RASSF1A protein expression. The MSP analysis has shown that the overall frequency

of methylation observed was 20.5%. Overall, only 9 out 44 analyzed thyroid tumors were methylated with consequent decreased expression of RASSF1A. In particular, the MSP analysis has revealed that the epigenetic methylation for RASSF1A was evident only in 4/32 PTCs (in stages I–II and two of which mutant for BRAF V600E) and in 5/5 ATCs (all cases characterized by BRAF V600E mutation and advanced tumor stage). No methylation was found in control thyroidal parenchyma.

Considering the well-differentiated subgroup of thyroid cancer (classical PTC), we found RASSF1A promoter methylation more frequently in early stage, as previously observed by Nakamura et al. [2005]. Similarly, also in other cancer models, as primarily described in colon cancer, gene methylation occurs in the early stage of cancer development [Li et al., 2003]. On the other hand, the undifferentiated subgroup of thyroid cancer (ATCs) revealed the epigenetic event in advanced stage. We could hypothesize that in ATC, methylation of RASSF1A is related to later stages of progression and probably occurs as a secondary event to BRAF-mutated initiation of the MAPK pathway, as also reported for methylation of other thyroid genes, that is, SLC5A8 [Rusinek et al., 2011].

According to the ANOVA analysis, RASSF1A was expressed in cancer more than in the normal thyroid parenchyma ($P < 0.05$). Significant correlations between RASSF1A protein over-expression and histotype (Hürtle Cell vs. Anaplastic and Papillary vs. Anaplastic) and presence of Hashimoto's thyroiditis ($P < 0.05$) have been observed. Briefly, higher levels of RASSF1A expression were observed in well-differentiated cancers, also associated to an inflammatory autoimmune background.

The somatic point mutation in the BRAF gene at the nucleotide position 1799 has been defined as the most common genetic event in papillary thyroid carcinoma [Kimura et al., 2003; Xing, 2005]. Harboring a thymine-to-adenine transversion at nucleotide position 1,799, the neoplastic cells result in a valine-to-glutamate substitution at residue 600 (V600E). In our study, we have investigated for the presence of BRAF mutation, the V600E, precisely at the exon 15, by using LC PCR with LightMix® kit. The V600E mutation was found in 13 of 18 (72%) tumors.

Generally, we can confirm the high frequency of BRAF mutation, according to the recent literature, but in our series we also reported that BRAF mutations in thyroid tumors are, probably, not restricted to PC. In addition, it has been important to determine whether BRAF mutation confers to PC distinct phenotypical, histological, and biological properties. In particular, in the selected cases with valuable DNA, BRAF was mutated in 6 of 9 (66%) classical PTC, in 2 of 4 (50%) follicular variant PTC (with an overall mutation frequency of 61.5% for papillary cancer) and in all ATCs (100%). Considering the well-differentiated subgroup of thyroid cancer (classical PTC/FVPTC), we found V600E mutation more frequently (80%) in early cancer. More advanced tumors revealed BRAF mutation in 42% of cases. On the other hand, the poorly differentiated subgroup of thyroid cancer (ATCs) was represented by advanced tumors, all of them characterized by BRAF mutation (Table V).

Therefore, in our work, among well-differentiated tumors, BRAF mutation as well as RASSF1A epigenetic changes seems to be early molecular events in thyroid tumor pathogenesis, that can
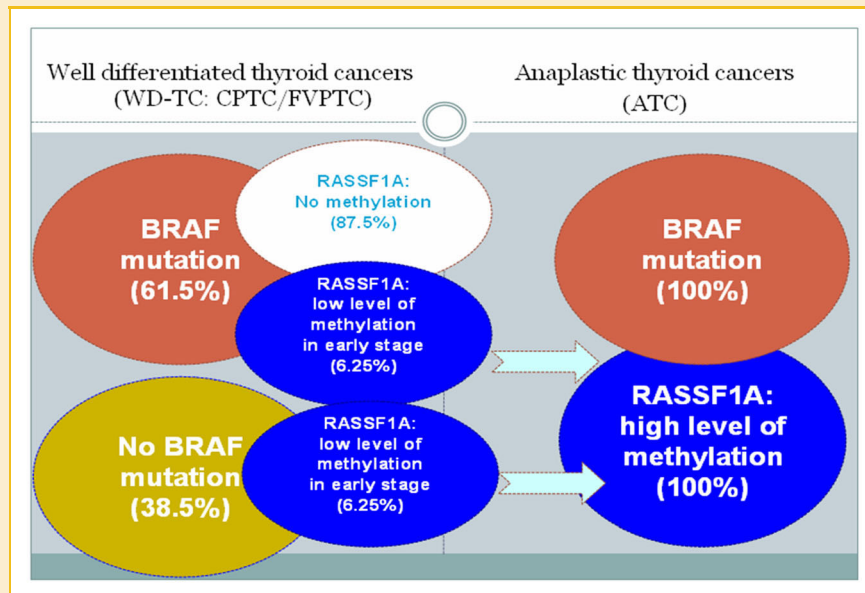
Fig. 4. Thyroid carcinogenesis: a no mutually exclusive relationship between BRAF mutation and RASSF1A expression in thyroid carcinoma. BRAF mutation as well as RASSF1A epigenetic changes seems to be early molecular events in thyroid tumor pathogenesis, that can prematurely predispose to the progression versus poorly differentiated cancer. BRAF V600E mutation and RASSF1A methylation select a subgroup of well–differentiated thyroid cancer with more aggressive clinical course. In our study, although an inverse relationship between BRAFV600E mutation and hypermethylation of RASSF1A was mostly present, we did not always observe it, demonstrating that a no mutually exclusive relationship could also exists between these genetic/epigenetic alterations in thyroid carcinogenesis.

prematurely predispose to the progression versus poorly differentiated cancer (Fig. 4). These results agree with studies showing the association of BRAF V600E mutation and RASSF1A methylation with a more aggressive clinical course of the papillary carcinoma [Schagdarsurengin et al., 2002; Namba et al., 2003; Nikiforova et al., 2003; Nakamura et al., 2005; Xing, 2005; Riesco-Eizaguirre et al., 2006].

CONCLUSION

The gene expression profile differences between papillary thyroid cancer and poorly differentiated thyroid cancer mainly reflect the differences in the degree of tumor differentiation. However, the observed profound differences between BRAF-positive and BRAF-negative PTCs, and RASSF1A methylated and RASSF1A unmethylated PTCs are indicative of profound aggressive changes in thyroidal cancerous biology related to the appearance of these type of molecular events.

Although an inverse relationship between BRAF V600E mutation and hyper-methylation of RASSF1A was suggested by Xing et al. [2004], we did not observe this relationship in our study, demonstrating that a no mutually exclusive relationship can also exists between these genetic/epigenetic alterations in thyroid carcinogenesis. However, we have to consider that the statistical reported data should be considered appropriately in relation to the low number of representative cases selected for this study, characterized by valuable DNA. Further studies on larger series should be performed in order to quantify the weight of these results.

In conclusion, our work confirms and underlines the importance of the precise knowledge about the molecular background in the study of the thyroid malignancies, in order to identify potentially important markers for tumor diagnosis, prognosis, and planning appropriate therapy.

REFERENCES

Agathanggelou A, Honorio S, Macartney DP, Martinez A, Dallol A, Rader J, Fullwood P, Chauhan A, Walker R, Shaw JA, Hosoe S, Lerman MI, Minna JD, Maher ER, Latif F. 2001. Methylation associated inactivation of RASSF1A from region 3p21.3 in lung, breast and ovarian tumours. Oncogene 20:1509–1518.

Baylin SB. 2002. Mechanisms underlying epigenetically mediated gene silencing in cancer. Semin Cancer Biol 12:331–337.

Burbee DG, Forgacs E, Zochbauer-Muller S, Shivakumar L, Fong K, Gao B, Randle D, Kondo M, Virmani A, Bader S, Sekido Y, Latif F, Milchgrub S, Toyooka S, Gazdar AF, Lerman MI, Zabarovsky E, White M, Minna JD. 2001. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. J Natl Cancer Inst 93:691–699.

Cohen Y, Xing M, Mambo E, Guo Z, Wu G, Trink B, Beller U, Westra WH, Ladenson PW, Sidransky D. 2003. BRAF mutation in papillary thyroid carcinoma. J Natl Cancer Inst 95:625–627.

Dammann R, Li C, Yoon JH, Chin PL, Bates S, Pfeifer GP. 2000. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. Nat Genet 25:315–319.

Dammann R, Yang G, Pfeifer GP. 2001. Hypermethylation of the CpG island of Ras association domain family 1A (RASSF1A), a putative tumor suppressor gene from the 3p21.3 locus, occurs in a large percentage of human breast cancers. Cancer Res 61:3105–3109.

De Maria S, Pannone G, Bufo P, Santoro A, Serpico R, Metafora S, Rubini C, Pasquali D, Papagerakis SM, Staibano S, De Rosa G, Farina E, Emanuelli M, Santarelli A, Mariggiò MA, Lo Russo L, Lo Muzio L. 2009. Survivin gene-expression and splicing isoforms in oral squamous cell carcinoma. J Cancer Res Clin Oncol 135(1):107–116.

Figge JJ. 2006. Epidemiology of thyroid cancer. In Thyroid cancer: A Comprehensive Guide to Clinical Management, 2nd edition. New Jersey: Humana press. pp 9–14.

Fonseca E, Soares P, Rossi S, Sobrinho-Simoes M. 1997. Prognostic factors in thyroid carcinomas. Verhndlungen der deutschen gesellschaft fur pathologie 1:82–96.

Fukushima T, Suzuki S, Mashiko M, Ohtake T, Endo Y, Takebayashi Y, Sekikawa K, Hagiwara K, Takenoshita S. 2003. BRAF mutations in papillary carcinomas of the thyroid. Oncogene 22:6455–6457.

Gilliland FD, Hunt WC, Morris DM, Key CR. 1997. Prognostic factors for thyroid carcinoma. A population-based study of 15,698 cases from the Surveillance, Epidemiology and End Results (SEER) program 1973–1991. Cancer 79:564–657.

Grieco M, Santoro M, Berlingieri MT, Melillo RM, Donghi R, Bongarzone I, Pierotti MA, Della Porta G, Fusco A, Vecchio G. 1990. PTC is a novel rearranged form of the ret proto-oncogene and is frequently detected in vivo in human thyroid papillary carcinomas. Cell 60:557–563.

Hay ID, Bergstralh EJ, Goellner JR, Ebersold JR, Grant CS. 1993. Predicting outcome in papillary thyroid carcinoma: Development of a reliable prognostic scoring system in a cohort of 1779 patients surgically treated at one institution during 1940 through 1989. Surgery 114:1050–1105.

Herman JG, Graff JR, Myöhänen S, Nelkin BD, Baylin SB. 1996. Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93:9821–9866.

Hu S, Liu D, Tufano RP, Carson KA, Rosenbaum E, Cohen Y, Holt EH, Kiseljak-Vassiliades K, Rhoden KJ, Tolaney S, Condouris S, Tallini G, Westra WH, Umbricht CB, Zeiger MA, Califano JA, Vasko V, Xing M. 2006. Association of aberrant methylation of tumor suppressor genes with tumor aggressiveness and BRAF mutation in papillary thyroid cancer. Int J Cancer 119:2322–2329.

Kimura ET, Nikiforova MN, Zhu Z, Knauf JA, Nikiforov YE, Fagin JA. 2003. High prevalence of BRAF mutations in thyroid cancer: Genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. Cancer Res 63:1454–1457.

Kroll TG, Sarraf P, Pecciarini L, Chen CJ, Mueller E, Spiegelman BM, Fletcher JA. 2000. PAX8-PPARgamma1 fusion oncogene in human thyroid carcinoma. Science 289:1357–1360.

Lerman MI, Minna JD. 2000. The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: Identification and evaluation of the resident candidate tumor suppressor genes. The International Lung Cancer Chromosome 3p21.3 Tumor Suppressor Gene Consortium. Cancer Res 60:6116–6133.

Li H, Myeroff L, Smiraglia D, Romero MF, Pretlow TP, Kasturi L, Lutterbaugh J, Rerko RM, Casey G, Issa JP, Willis J, Willson JKV, Plass C, Markowitz SD. 2003. SLC5A8, a sodium transporter, is a tumor suppressor gene silenced by methylation in human colon aberrant crypt foci and cancers. PNAS 100:8412–8417.

LiVolsi VA, Fadda G, Baloch ZW. 2000. Prognostic factors in well-differentiated thyroid cancer. Rays 25:163–175.

Lo KW, Kwong J, Hui AB, Chan SY, To KF, Chan AS, Chow LS, Teo PM, Johnson PJ, Huang DP. 2001. High frequency of promoter hypermethylation of RASSF1A in nasopharyngeal carcinoma. Cancer Res 61:3877–3881.

Mazzaferri EL, Jhiang SM. 1994. Long-term impact of initial surgical and medical therapy on papillary and follicular thyroid cancer. Am J Med 97:418–428.

Mitsutake N, Knauf JA, Mitsutake S, Mesa C, Jr., Zhang L, Fagin JA. 2005. Conditional BRAFV600E expression induces DNA synthesis, apoptosis, dedifferentiation, and chromosomal instability in thyroid PCCL3 cells. Cancer Res 65:2465–2473.

Moretti F, Nanni S, Pontecorvi A. 2000. Molecular pathogenesis of thyroid nodules and cancer. Baillieres Best Pract Res Clin Endocrinol Metab 14:517–539.

Nakamura N, Carney JA, Jin L, Kajita S, Pallares J, Zhang H, Qian X, Sebo TJ, Erickson LA, Lloyd RV. 2005. RASSF1A and NORE1A methylation and BRAFV600E mutations in thyroid tumors. Lab Invest 85:1065–1075.

Namba H, Nakashima M, Hayashi T, Hayashida N, Maeda S, Rogounovitch TI, Ohtsuru A, Saenko VA, Kanematsu T, Yamashita S. 2003. Clinical implication of hot spot BRAF mutation, V599E, in papillary thyroid cancers. J Clin Endocrinol Metab 88:4393–4397.

Nikiforova MN, Kimura ET, Gandhi M, Biddinger PW, Knauf JA, Basolo F, Zhu Z, Giannini R, Salvatore G, Fusco A, Santoro M, Fagin JA, Nikiforov YE. 2003. BRAF mutations in thyroid tumors are restricted to papillary carcinomas and anaplastic or poorly differentiated carcinomas arising from papillary carcinomas. J Clin Endocrinol Metab 88:5399–5404.

Pannone G, Rodolico V, Santoro A, Lo Muzio L, Franco R, Botti G, Aquino G, Pedicillo MC, Cagiano S, Campisi G, Rubini C, Papagerakis S, De Rosa G, Tornesello ML, Buonaguro FM, Staibano S, Bufo P. 2012. Evaluation of a combined triple method to detect causative HPV in oral and oropharyngeal squamous cell carcinomas: p16 Immunohistochemistry, Consensus PCR HPV-DNA, and In Situ Hybridization. Infect Agent Cancer 7:4.

Pfeifer GP, Yoon JH, Liu L, Tommasi S, Wilczynski SP, Dammann R. 2002. Methylation of the RASSF1A gene in human cancers. Biol Chem 383:907–914.

Riesco-Eizaguirre G, Gutierrez-Martinez P, Garcia-Cabezas MA, Nistal M, Santisteban P. 2006. The oncogene BRAF V600E is associated with a high risk of recurrence and less differentiated papillary thyroid carcinoma due to the impairment of Na+/I− targeting to the membrane. Endocr Relat Cancer 13:257–269.

Rusinek D, Szpak-Ulczok S, Jarzab B. 2011. Gene expression profile of human thyroid cancer in relation to its mutational status. J Mol Endocrinol 47:R91–R103.

Schagdarsurengin U, Gimm O, Hoang-Vu C, Dralle H, Pfeifer GP, Dammann R. 2002. Frequent epigenetic silencing of the CpG island promoter of RASSF1A in thyroid carcinoma. Cancer Res 62:3698–3701.

Sherman SI, Brierley JD, Sperling M, Ain KB, Bigos ST, Cooper DS, Haugen BR, Ho M, Klein I, Ladenson PW, Robbins J, Ross DS, Specker B, Taylor T, Maxon HR. 1998. Prospective multicenter study of thyroid carcinoma treatment: Initial analysis of staging and outcome. National thyroid cancer treatment cooperative study registry group. Cancer 83:1012–1021.

Soares P, Trovisco V, Rocha AS, Lima J, Castro P, Preto A, Maximo V, Botelho T, Seruca R, Sobrinho-Simoes M. 2003. BRAF mutations and RET/PTC rearrangements are alternative events in the etiopathogenesis of PTC. Oncogene 22:4578–4580.

Xing M. 2005. BRAF mutation in thyroid cancer. Endocr Relat Cancer 12:245–262.

Xing M, Cohen Y, Mambo E, Tallini G, Udelsman R, Ladenson PW, Sidransky D. 2004. Early occurrence of RASSF1A hypermethylation and its mutual exclusion with BRAF mutation in thyroid tumorigenesis. Cancer Res 64:1664–1668.

Xing M, Westra WH, Tufano RP, Cohen Y, Rosenbaum E, Rhoden KJ, Carson KA, Vasko V, Larin A, Tallini G, Tolaney S, Holt EH, Hui P, Umbricht CB, Basaria S, Ewertz M, Tufaro AP, Califano JA, Ringel MD, Zeiger MA, Sidransky D, Ladenson PW. 2005. BRAF mutation predicts a poorer clinical prognosis for papillary thyroid cancer. J Clin Endocrinol Metab 90:6373–6379.

Xu X, Quiros RM, Gattuso P, Ain KB, Prinz RA. 2003. High prevalence of BRAF gene mutation in papillary thyroid carcinomas and thyroid tumor cell lines. Cancer Res 63:4561–4567.

Yoon JH, Dammann R, Pfeifer GP. 2001. Hypermethylation of the CpG island of the RASSF1A gene in ovarian and renal cell carcinomas. Int J Cancer 94:212–217.