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RASSF1A and *NORE1A* methylation and *BRAF*^{V600E} mutations in thyroid tumors

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We analyzed *RASSF1A* and *NORE1A* methylation and *BRAF* mutation in 89 thyroid tumors, 42 non-neoplastic thyroid tissues and three thyroid tumor cell lines using polymerase chain reaction (PCR), methylation-specific PCR, Western blotting and DNA sequencing in order to study thyroid tumor pathogenesis and progression. *RASSF1A* promoter methylation was present in all three thyroid cell lines and in 27/78 (35%) of benign and malignant thyroid tumors. We showed for the first time that there was generally good agreement between *RASSF1A* methylation status and *RASSF1A* protein expression. We also examined for the first time *NORE1A* promoter region methylation in thyroid cell lines and primary tumors and showed that two of three thyroid cell lines were methylated in the *NORE1A* promoter region, while all primary thyroid tumors analyzed ($n = 51$) were unmethylated. *BRAF* mutation was present in 38% of papillary thyroid carcinomas (PTC), including 20% of PTC with a follicular variant pattern and 67% of the tall cell variant of PTC. Hyalinizing trabecular tumors ($n = 23$), which had nuclear features similar to PTC, did not have *BRAF* mutations, indicating that the presence of *BRAF* mutations can help to separate these two tumor types. Phospho-MEK expression was increased in the NPA cell line, which had a *BRAF* mutation, supporting the importance of the *BRAF* pathway alterations in PTC pathogenesis. These results indicate that *RASSF1A* epigenetic changes are an early event in thyroid tumor pathogenesis and progression and that *NORE1A methylation* is uncommon in primary thyroid tumors. *BRAF* mutation occurs later in thyroid tumor progression and is restricted mainly to PTC and anaplastic thyroid carcinoma.
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Thyroid tumor development and progression is regulated by various oncogenes and tumor suppressor genes, only a few of which have been identified to date.[1,2] RAS association domain family protein 1 (*RASSF1*) gene, located on chromosome 3p21.3,[3] is a recently described tumor suppressor gene whose expression is regulated epigenetically. *RASSF1A* and *RASSF1C*, two major transcript variants, are expressed in a wide variety of normal tissues.[3] Exogenous expression of *RASSF1A* in a cell line lacking expression decreased *in vitro* colony formation and *in vivo* tumorigenicity.[4] Mutant *RASSF1A* had significantly reduced growth suppression activity.[5] The reintroduction of *RASSF1A* expression suppressed the growth of various tumor cell lines, including those from the prostate and lungs.[3,6] *RASSF1A* has a role as a tumor suppressor gene, since this gene blocked cell cycle progression and inhibited cyclin D1 accumulation.[7] Loss of *RASSF1* expression has been correlated with methylation of the CpG-island promoter sequence of *RASSF1* gene.[3–17] *RASSF1A* has been reported to be inactivated in lung, breast, kidney, prostate, ovary, colon, thyroid and other cancers.[3–15] A few groups have examined the inactivation of *RASSF1A* in thyroid tumors and found inactivation of *RASSF1A* in many types of thyroid cancer.[15–17] However, *RASSF1A* protein expression has not been correlated with *RASSF1A* gene methylation, an analysis that should provide insight into the functional consequences of *RASSF1A* methylation.

Vavvas *et al*[18] identified a novel potential Ras effector target Nore1 and showed that Nore1 directly interacted with Ras *in vitro* in a GTP-dependent manner, an interaction that required an intact Ras effector domain. *NORE1A* and *NORE1B* are two spliced variants, which are expressed in most normal tissues.[19] Methylation of the CpG islands of *NORE1A* and *NORE1B* has been examined in only a few primary tumors.[19] *NORE1A* promoter was

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methylated in some breast, lung, colorectal and kidney cell lines, while the *NORE1B* promoter was unmethylated in the same tumor cell lines.[20] *NORE1* methylation has not been examined in thyroid cell lines or tissues.

Three Raf families of serine/threonine kinases have been identified as raf-1 (c-RAF, A-raf and B-raf) in human.[21–30] They function in the transduction of mitogenic signals from the cell membrane to the nucleus and mediate cell proliferation and differentiation.[31] RAS function is not required for the growth of cancer cell lines with the *BRAF* V600E T1799A (previously reported as V599E T1796A) mutation.[32] Kumar *et al*[33] reported that the mutational hot spot codon and nucleotide incorrectly reported in earlier reports as 599 and 1796 were correctly located at codon 600 and nucleotide 1799. *BRAF*^{V600E} mutation has been detected in various tumors, including malignant melanoma, nevi, colon cancer and thyroid cancers. Although papillary thyroid carcinoma (PTC) and hyalinizing trabecular tumor (HTT) have common nuclear cytologic features and can be confused, the role of *BRAF* mutation, if any, in the development of HTT is unknown.[32–36]

In this study, we analyzed normal thyroid tissues and thyroid tumors for methylation of *RASSF1A* and *NORE1A* genes and for *BRAF*^{V600E} gene mutation to determine the role of these genes in thyroid cancer pathogenesis and progression.

Materials and methods

Human Tissues and Cell Lines

In all, 89 thyroid tumors and 42 non-neoplastic thyroid tissues were used in this study (Table 1). Non-neoplastic tissues adjacent to the tumor areas of PTC ($n=34$), follicular thyroid carcinomas (FTC) ($n=3$) and medullary thyroid carcinomas (MTC) ($n=5$) were analyzed. The tumors included 42 PTC, four FTC, five MTC, 12 anaplastic thyroid carcinomas (ATC), 23 HTT and three follicular adenomas (FA). There were 45 formalin-fixed paraffin-embedded tissues among a total of 89 tumors (Table 1). Approval of the Mayo Clinic Institutional Review Board was obtained for this study.

Table 1 Types of thyroid tissues analyzed

Diagnosis	*Cases (total no.)*	*Frozen tissues (no. of cases)*	*Paraffin sections (no. of cases)*
Non-neoplastic tissue	42	42	0
PTC	42	34	8
FTC	4	3	1
MTC	5	5	0
ATC	12	2	10
FA	3	0	3
HTT	23	0	23

PTC: papillary thyroid carcinoma, FTC: follicular thyroid carcinoma, MTC: medullary thyroid carcinoma, ATC: anaplastic thyroid carcinoma, FA: follicular adenoma, HTT: hyalinizing trabecular tumor. No.—number.

Three human thyroid cancer cell lines were used. NPA and TPC1 cells derived from PTCs were kindly provided by Dr Yuri E Nikiforov (Department of Pathology, University of Cincinnati College of Medicine, Cincinnati, OH, USA). TT1 was derived from an FTC and established in our laboratory as a cell line. NPA was used as a positive control for *BRAF*^{V600E} mutation.[36–38] TPC1 had a *RET/PTC-1* rearrangement.[37] NPA, TPC1 and TT1 cells were grown at 37°C in a humidified incubator with 5% CO_2. NPA was cultured in RPMI Medium1640 supplemented with 10% fetal bovine serum, 1% antibiotic–antimycotic and 1 mg/l insulin. TPC1 and TT1 were cultured in Dulbecco's modified Eagle medium, 15% horse serum heated inactivated, 2.5% fetal bovine serum, 1% antibiotic–antimycotic and 1 mg/l insulin. All cell culture reagents were obtained from Life Technologies, Inc., Grand Island, NY, USA.

Genomic DNA Extraction

Genomic DNA was extracted from the thyroid tissues using the following procedure: frozen tissues were cut into small pieces with a scalpel, mixed with 2 ml of TEN buffer (10 mM Tris-HCl, pH 7.4, 25 mM EDTA, 10 mM NaCl) and 100 μl of 20% sodium dodecyl sulfate (SDS, Sigma, St Louis, MO, USA). Proteinase K (Roche Diagnostics Corporation, Indianapolis, IN, USA) was added to achieve a final concentration of 0.2 mg/ml and the tissues were incubated overnight at 50°C. After incubation, 1 volume of phenol/chloroform/isoamyl alcohol (Invitrogen, Carlsbad, CA, USA) was added and thoroughly mixed well. The tissues were centrifuged and the aqueous layer containing the DNA was transferred to fresh tubes. One volume of chloroform/isoamyl alcohol (1:1) was added and well mixed. After centrifugation the aqueous layers were transferred to a fresh tube. Genomic DNA was precipitated by the addition of 2.5 volumes of 100% ethanol and the samples were centrifuged. The DNA pellets were washed by adding 70% ethanol, centrifuged and re-suspended in Molecular Biology Grade water (Eppendorf, Westbury, NY, USA). The concentrations of genomic DNA were measured by optical density with a spectrophotometer (Spectronic Genesys 8 Thermo Spectronic, Rochester, NY, USA).

Cultured cells were trypsinized, collected by centrifugation and washed with phosphate-buffered saline (PBS) twice. Genomic DNA from the cell lines was extracted using the same procedure. Genomic DNA from paraffin-embedded specimens was prepared from four to six 10-μm-thick sections. The sections were deparaffinized and gradually dehy-

drated and air-dried. DNA was isolated using the procedure described above.

Methylation-Specific Polymerase Chain Reaction (MS-PCR)

The bisulfite method was used to detect DNA methylation of *RASSF1A* and *NORE1A*.[39,40] Genomic DNA (2 μg) was treated with bisulfite, which converts unmethylated cytosine to uracil, from an EZ DNA Methylation Kit, according to the manufacturer's suggestions (Zymo Research, Orange, CA, USA). The CpGenome Universal Methylated DNA (1 μg) (Chemicon International, Inc., Temecula, CA, USA) was used as positive control for methylated DNA and as negative control for unmethylated DNA. Genomic DNA (2 μg) of the HeLa cell line was used as positive control for unmethylated DNA.

Four primer sets for *RASSF1A* and *NORE1A* were prepared for methylated DNA and unmethylated DNA (Table 2). The PCR mix included: 0.2 mM dNTPs, 0.2 μM of each primer, 1 $\times$ Easy-A Reaction Buffer and 0.75 U Easy-A High-Fidelity PCR Cloning Enzyme (Stratagene, La Jolla, CA, USA). For MS-PCR, 1 μl of the bisulfite-modified DNA was used for each sample. Step-down PCR was performed to improve the specificity for *RASSF1A* gene. A step-down PCR protocol for methylated DNA of *RASSF1A* was used as follows: 95°C for 2 min, $\times$1 cycle; 95°C for 30 s, 64°C for 30 s and 72°C for 30 s, $\times$2 cycles; 95°C for 30 s, 62°C for 30 s and 72°C for 30 s, $\times$2 cycles; 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, $\times$20 cycles for DNA from frozen tissues, or $\times$30 cycles for DNA from paraffin sections and a final extension at 72°C for 10 min. A step-down PCR protocol for unmethylated DNA of *RASSF1A* was used similar to that for methylated DNA of *RASSF1A*, except that 28 cycles of amplification were used for DNA from paraffin sections. For *NORE1A* gene, MS-PCR protocol for methylated DNA was used as follows: 95°C for 10 min, $\times$1 cycle; 95°C for 1 min, 62°C for 1 min and 72°C for 2 min, $\times$30 cycles for DNA from frozen tissues, or $\times$46 cycles for DNA from paraffin sections and a final extension at 72°C for 10 min. MS-PCR protocol for unmethylated DNA of *NORE1A* gene was used same as above, except that the annealing temperature was 64°C. Molecular Biology Grade Water was used as negative control of both MS-PCR reactions.

In all, 10 μl of each PCR product was electrophoresed on Ready Gels of 10% TBE for polyacrylamide electrophoresis (Bio-Rad Laboratories, Hercules, CA, USA), stained with ethidium bromide and visualized under UV light.

Polymerase Chain Reaction (PCR)

PCR primers to amplify *BRAF* exon 15 (*BRAF* set 1) are shown in Table 2. PCR was performed in 50 μl of reaction mixture using 100 ng of genomic DNA for frozen tissues or cultured cells, and 500–2000 ng of genomic DNA from paraffin sections. The reaction mixture contained 100 pM of each primer, 0.2 mM of dNTPs (Roche), 2 mM $MgCl_2$, 1 $\times$ reaction buffer and 1.25 U Taq DNA polymerase (Promega, Madison, WI, USA). The reaction mixture was subjected to initial denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min on the GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, CA, USA). Molecular Biology Grade Water (Eppendorf) was used as negative control.

BRAF Mutation Analysis by Direct Sequencing

PCR products (20 μl) were electrophoresed on a 1.2% agarose gel (Invitrogen life technologies), stained with ethidium bromide and visualized under UV light. The PCR product obtained with

Table 2 Primers used for *BRAF* mutation analysis, MS-PCR for *RASSF1A* and *NORE1A*

Gene	*Genbank no.*	*Sequences (5′–3′)*	*Annealing TM (°C)*	*Product size (bp)*	*Reference no.*
RASSF1A-M	NM_007182	F GTGTTAACGCGTTGCGTATC	64–60	96	15
		R AACCCCGCGAACTAAAAACGA			
RASSF1A-U	NM_007182	F TTTGGTTGGAGTGTGTTAATGTG	64–60	108	15
		R CAAACCCCACAAACTAAAAACAA			
NORE1A-M	NM_031437	F CGTCGTTTGGTACGGATTTTATTTTTTTCGGTTC	62	202	41
		R GACAACTTTAACAACGACGACTTTAACGACTACG			
NORE1A-U	NM_031437	F ATTTATATTTGTGTAGATGTTGTTTGGTAT	64	215	41
		R ACTTTAACAACAACAACTTTAACAACTACA			
BRAF set 1	NM_004333	F TCATAATGCTTGCTCTGATAGGA	56	224	37
		R GGCCAAAAATTTAATCAGTGGA			
BRAF set 2	NM_004333	F TTTCCTTTACTTACTACACCTCA			
		R GAAAAATAGCCTCAATTCTTAC			

BRAF set 2 used for sequencing only. *RASSF1A*-M and *NORE1A*-M: primers used for methylated DNA, *RASSF1A*-U and *NORE1A*-U: primers used for unmethylated DNA. F: forward primer, R: reverse primer.

BRAF primer set 1 was extracted with an Ultrafree-DA (Millipore Corporation, Bedford, MA, USA) and purified with a Microcon YM-100 (Millipore Corporation). The same primers were used for DNA sequencing. The cases with *BRAF* mutation were re-sequenced using the *BRAF* set 2 primers (Table 2). DNA sequence was analyzed in the Molecular Biology Core Facility at Mayo Clinic with an ABI Prism 377 DNA Sequencer (PE Applied Biosystems).[42]

Western Blot Analysis

Proteins were extracted from thyroid tissues and tumor cell lines. In all, 25 μg of total protein in each lane was used for one-dimensional SDS-polyacrylamide gel electrophoresis and subsequently transferred to a polyvinylidene difluoride membrane (Bio-Rad) for immunoblotting. The antibodies used for Western blotting included MEK1/2 antibody (1:2000 dilution, Cell Signaling Technology, Inc., Beverly, MA, USA), phospho-MEK1/2 (Ser217/221) antibody (1:2000 dilution, Cell Signaling Technology, Inc.), p44/42 MAP kinase antibody (1:2000 dilution, Cell Signaling Technology, Inc.), anti-phospho-MAPK antibody (1:5000 dilution, Promega) and anti-*RASSF1A* antibody (1:500 dilution, eBioscience, San Diego, CA, USA). Anti-β-actin antibody (1:1000 dilution, Sigma) was used to check for equal loading on the gel as previously reported.[43–45] The reaction products were detected with enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA). Densitometric analysis of the bands was carried out with a Fluor-S multimager (Bio-Rad). Results were expressed as relative densitometry units, and all data were normalized with β-actin. A linear response was obtained using different amounts of proteins on the gel.

Results

RASSF1A Methylation and Protein Expression

Analysis of the methylation status of the *RASSF1A* promoter region showed hypermethylation of the *RASSF1A* gene in all three thyroid tumor cell lines (NPA, TPC1 and TT1) (Table 3 and Figure 1a).

Table 3 Analysis of *RASSF1A* and *NORE1A* methylation and *BRAF* mutation in thyroid tumor cell lines

Cell line	*Diagnosis*	*Hypermethylated* RASSF1A	*Hypermethylated* NORE1A	BRAF *mutation*
NPA	PTC	Yes	Yes	Yes
TPC1	PTC	Yes	Yes	No
TT1	FTC	Yes	No	No

PTC: papillary thyroid carcinoma, FTC: follicular thyroid carcinoma.

Analysis of *RASSF1A* methylation in PTC and matching normal tissues showed that 32% of PTC was methylated (Figure 1a and Table 4). There was generally good agreement between unmethylated *RASSF1A* and protein expression detected by Western blotting. The 39 kDa *RASSF1A* protein was detected in all non-neoplastic thyroids and in most unmethylated tumors (Figure 1b). However, the amount of *RASSF1A* protein in different samples was variable. Normal thyroid tissues generally expressed more *RASSF1A* protein than the unmethylated tumors. The thyroid cell lines, which were methylated, did not express *RASSF1A* protein (Figure 1b). Hypermethylation of *RASSF1A* in primary tumors was variable with PTC (32%), FTC (100%), MTC (40%), FA (33%) and HTT (25%) (Table 4 and Figure 2). Non-neoplastic thyroid tissues were all unmethylated (Table 4). Among the subtypes of PTC, a percentage of classical PTC smaller than the follicular variant patterned tumors was methylated (Table 5). The methylation frequencies of the *RASSF1A* gene were 25% (7/28 cases) in classic PTC and 60% (3/5 cases) in PTC follicular variant. The one case of PTC tall-cell variant was also methylated (Table 5).

NORE1A Methylation

The NPA cell line was completely hypermethylated in the promoter region of *NORE1A*, while the TPC1 cell line was heterozygous, with a methylated and an unmethylated band for *NORE1A* (Table 3 and Figure 3). The TT1 cell line was unmethylated for *NORE1A* (Table 3 and Figure 3). All normal and neoplastic human thyroid tissues were unmethylated in the promoter region of *NORE1A* (Table 4 and Figure 3).

BRAF Mutation

Analysis of the three thyroid cell lines showed that the NPA cells had $BRAF^{V600E}$ mutation, but PTC-1 and TT1 cells were negative for $BRAF^{V600E}$ mutations (Table 3). *BRAF* mutations were identified in 16 cases of PTC at the V600E site. All mutations involved the same change at nucleotide position 1799, where thymine was replaced by adenine, resulting in the substitution of valine at position 600 for glutamic acid (Figure 4). One case of ATC had a *BRAF* mutation (10%) (Table 4). None of the other tumor types had a $BRAF^{V600E}$ mutation, including 23 cases of HTT (Figure 5 and Table 4). $BRAF^{V600E}$ mutation was not detected in the 42 cases of matching non-neoplastic thyroid tissues from the PTC (Table 4). Analysis of the subtypes of PTC with *BRAF* mutation showed that 11 cases were classic PTC (35%), while only one of five follicular variant carcinomas (20%) had a $BRAF^{V600E}$ mutation. Most (four of six) tall-cell variant carcinomas (67%) had $BRAF^{V600E}$ mutations (Table 5). The same sequencing

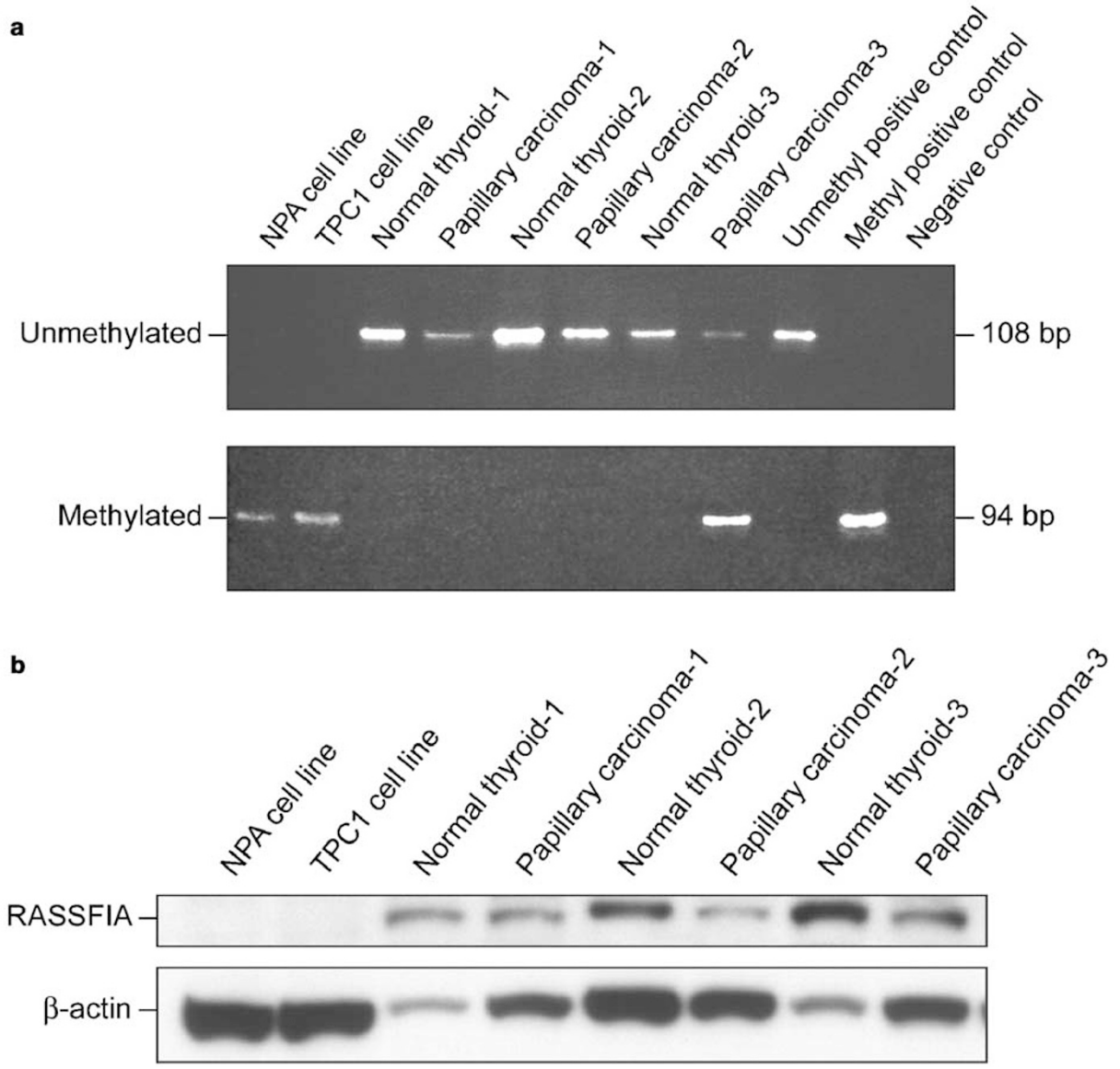

Figure 1 (**a**) Methylation-specific PCR of *RASSF1A*. All non-neoplastic thyroid tissue and some benign and malignant thyroid tumors were unmethylated. NPA and TPC1 cell lines and PTC-3 were methylated in the promoter region of *RASSF1A*. HeLa-DNA and the CpGenome Universal Methylated DNA were used as controls for MS-PCR. Water was used as negative control. (**b**) Western blotting showing *RASSF1A* protein expression. The same samples as in (**a**) were used to show the relationship between unmethylated samples and *RASSF1A* protein expression. Strong expression of *RASSF1A* protein was observed in all non-neoplastic thyroid tissues. *β*-Actin was used as to check for equal loading.

results for *BRAF* mutations were obtained by using two different sets of *BRAF* primers (Table 2).

Western Blot Analysis of MAPK and MEK

Analysis of the NPA and TPC1 cell lines showed equal-size bands for MAPK and phospho-MAPK (Figure 6a). The NPA cell line, which harbored a $BRAF^{V600E}$ mutation, showed increased expression of phospho-MEK (Figure 6b). The TPC1 cell line, which did not have a $BRAF^{V600E}$ mutation, had a weaker band for phospho-MEK compared to MEK.

Discussion

Analysis of *RASSF1A* in normal and neoplastic thyroid tissues showed that all non-neoplastic thyroid tissue was unmethylated, while *RASSF1A* was hypermethylated in both benign and malignant tumors. Methylation studies of *RASSF1A* have shown that this gene is one of the most commonly methylated tumor suppressor genes,[3,4] and the few studies of *RASSF1A* in thyroid tumors[15–17] have not examined the wide spectrum of benign tumors and more aggressive well-differentiated tumors reported in this study. Schagdarsurengin *et al*[15] detected

Table 4 Analysis of *RASSF1A* and *NORE1A* methylation-specific PCR and *BRAF* mutation in thyroid tumors

Diagnosis	*Total no. of cases*	RASSF1A		NOREF1A		BRAF	
		No. of methylated cases/total	*%*	*No. of methylated cases/total*	*%*	*No. of mutated cases/total*	*%*
Non-neoplastic tissue	42	0/27	0	0/11	0	0/42	0
PTC	42	11/34	32	0/32	0	16/42	38
FTC	4	4/4	100	0/3	0	0/4	0
MTC	5	2/5	40	0/5	0	0/5	0
ATC	12	4/12	33	0/4	0	1/10	10
FA	3	1/3	33	0/2	0	0/3	0
HTT	23	5/20	25	0/4	0	0/23	0

PTC: papillary thyroid carcinoma, FTC: follicular thyroid carcinoma, MTC: medullary thyroid carcinoma, ATC: anaplastic thyroid carcinoma, FA: follicular adenoma, HTT: hyalinizing trabecular tumor.

Table 5 Analysis of *RASSF1A* and *NORE1A* by methylation-specific PCR and *BRAF* mutation in papillary thyroid carcinomas

Diagnosis	*Total no. of cases*	RASSF1A		NORE1A		BRAF	
		No. of methylated cases/total	*%*	*No. of methylated cases/total*	*%*	*No. of mutated cases/total*	*%*
Classic PTC	31	7/28	25	0/27	0	11/31	35
Follicular variant	5	3/5	60	0/5	0	1/5	20
Tall cell variant	6	1/1	—	0/1	—	4/6	67

PTC: papillary thyroid carcinoma.

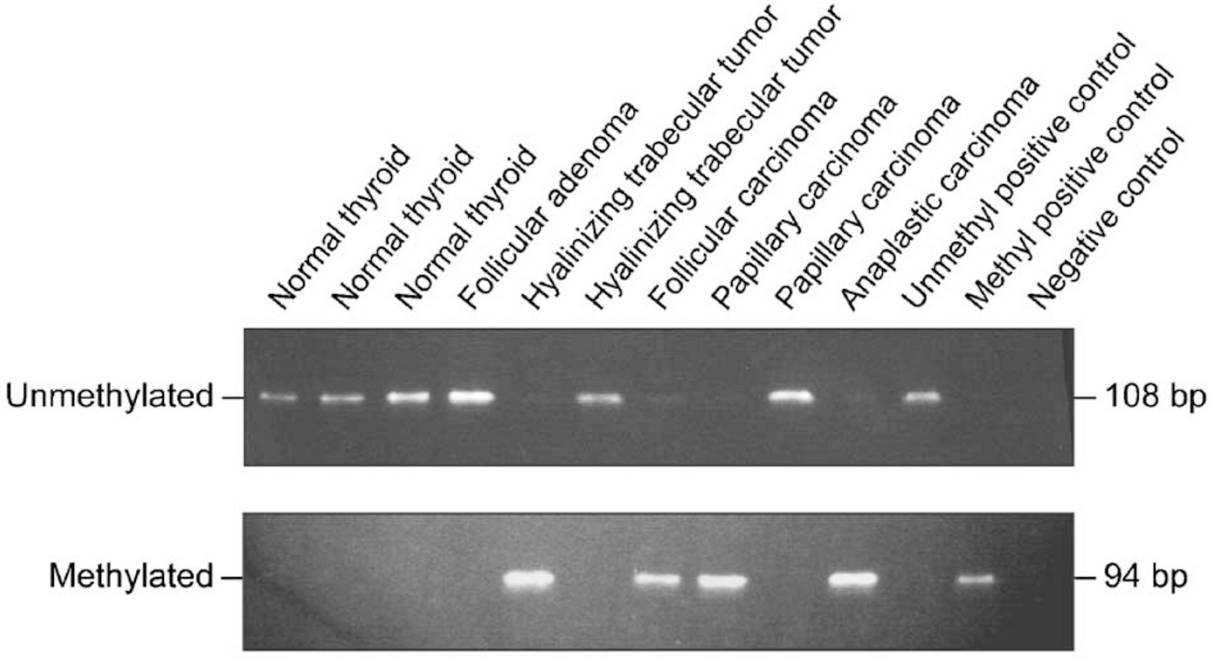

Figure 2 Methylation-specific PCR analysis of *RASSF1A* methylation in different types of thyroid tumors. Benign and malignant thyroid tumors were methylated. Non-neoplastic thyroid tissues were all unmethylated.

RASSF1A methylation in 71% (27/38 cases) of primary thyroid carcinomas, while Wong *et al*[16] reported *RASSF1A* hypermethylation in the two thyroid tumors examined. Xing *et al*[17] reported *RASSF1A* in benign adenomas (44%), FTC tumors (75%) and PTC tumors (20%).

In our study, the finding of methylated *RASSF1A* in both benign and malignant thyroid tumors

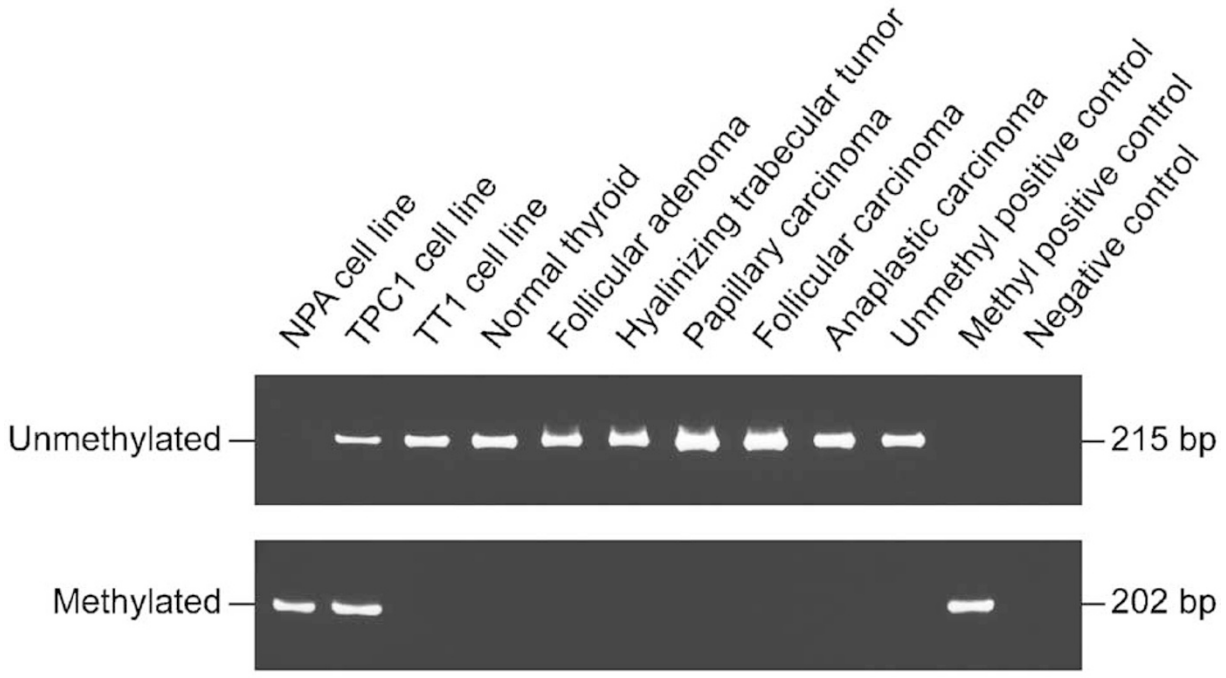

Figure 3 Methylation-specific PCR analysis of *NORE1A*. NPA and TPC1 cell lines were methylated at the promoter region of *NORE1A*. All primary thyroid tumors were unmethylated of the promoter region of *NORE1A*. HeLa-DNA (unmethylated) and the methylated CpGenome Universal DNA were used as controls for MS-PCR. Water was used as negative control.

suggests that it is an early event in thyroid tumorigenesis. This is supported by our finding of unmethylated *RASSF1A* only in the non-neoplastic thyroid tissues and methylated *RASSF1A* in the benign tumors. *RASSF1A* inactivation has been associated with increased tumor cell proliferation via Cyclin D1 and increased apoptosis via MST1, so these mechanisms may also be important in thyroid tumor progression.[7] We show for the first time that in thyroid tumors there is generally good agreement between unmethylated *RASSF1A* and protein expression and that thyroid tumors that were methylated usually did not express *RASSF1A* protein, while most of the unmethylated tumors expressed *RASSF1A* proteins. These observations confirm the functional role of *RASSF1A* protein in signal transduction and cell proliferation in thyroid neoplasms.

Our study of *NORE1A* methylation in thyroid tissues is the first reported analysis of *NORE1A* in thyroid tumors. The results showed that two of three thyroid cell lines were methylated in the *NORE1A* promoter region. However, all of the thyroid tumors analyzed as well as the 42 non-neoplastic thyroids were unmethylated. The presence of *NORE1A* methylation in thyroid cell lines and not in the primary tumors is similar to earlier reports in gliomas, nasopharyngeal carcinomas and small-cell carcinomas of the lungs in which cell lines from these tumors were methylated, while all of the primary tumors were unmethylated.[20,46,41] These findings may be analogous to the finding of a higher rate of methylation in metastatic compared to primary breast cancers including the *RASSF1A* gene, and the possibility that promoter methylation may confer survival advantages as with cells grown *in vitro*.[47] It is also possible that the cell culture conditions may change the promoter methylation status of the cultured cells. Silencing of *NORE1A* by methylation is probably related to *RASSF1A* silencing, since both of these genes are members of the Ras effector group.[18,20]

$BRAF^{V600E}$ mutation was detected in 38% of PTC and in 10% of ATC, but not in other tumor types in this study. This mutation has previously been detected in 29–69% of PTC and in some of the ATC.[17,36–38,48–61] Reports of $BRAF^{V600E}$ mutation in ATC have been quite variable.[37,50,51,53–56,61] These results may vary in part with the methods used to detect the mutation. Nikiforova *et al*[51] reported that there was no difference in various techniques in the detection of $BRAF^{V600E}$ mutations, using LightCycler PCR fluorescence melting curve analysis, single-strand conformational polymorphism and direct sequencing. $BRAF^{V600E}$ mutation is highly specific for PTC. However, variation in the incidence of mutations was dependent on the type of PTC. $BRAF^{V600E}$ mutation was detected in 67% of tall-cell PTC and in 20% of follicular variant of PTC in this study in agreement with earlier reports,[52,55,56,60] indicating that $BRAF^{V600E}$ mutation is more common in more aggressive thyroid tumors.

Although an inverse relationship between $BRAF^{V600E}$ mutation and hypermethylation of *RASS-*

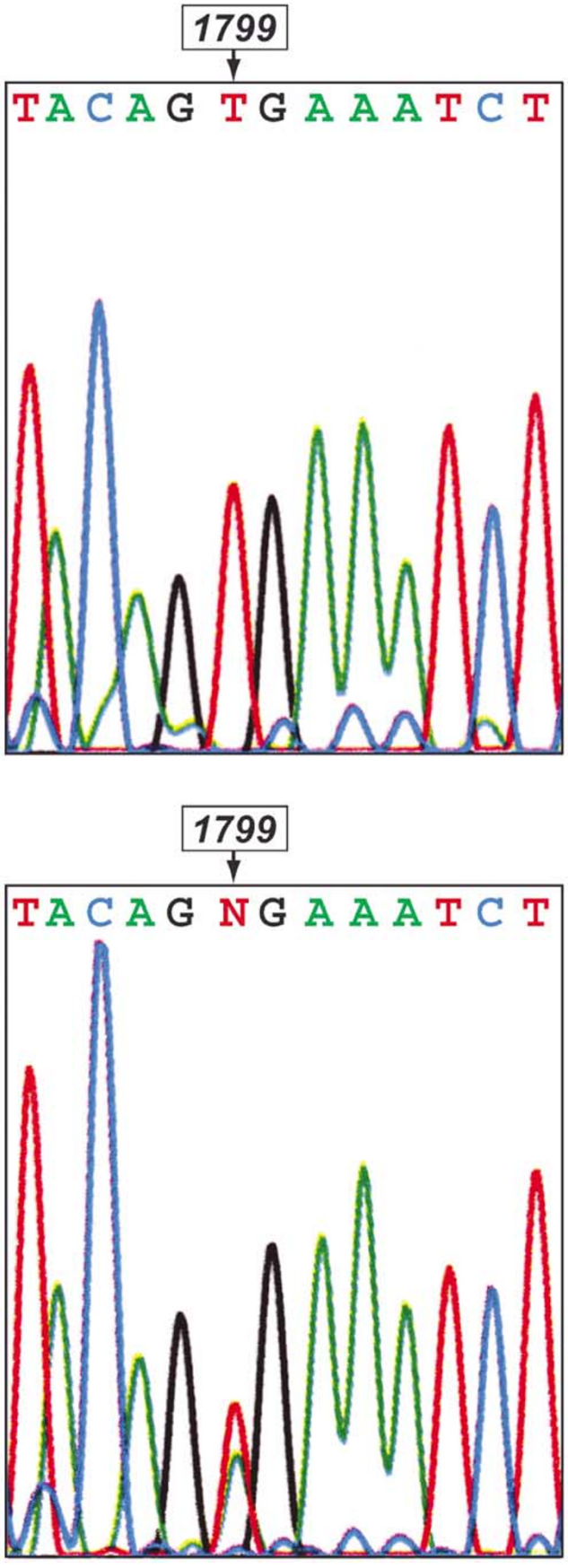

Figure 4 PCR amplification followed by nucleotide sequencing of the PCR products to detect *BRAF*^{V600E} mutations. The matching non-neoplastic thyroid tissue showed a wild-type peak (top), while a PTC from the same case showed a *BRAF*^{V600E} mutation. T→A transversion at nucleotide 1799 (bottom).

F1A was suggested by Xing *et al*,[17] we did not observe this relationship in our study. The relationship between *BRAF*^{V600E} mutation and other mutations in PTC has also been examined. Xu *et al*[38] reported that 38% of their cases had both of *BRAF* mutations and *RET/PTC* rearrangements. Nikiforova *et al*[57] observed that 4% of their cases had *BRAF* mutation and *RET/PTC* rearrangement. Understanding the interaction of these various genes during thyroid tumor progression will require further studies. Our observations of increased expression of phosphorylated MEK in the NPA cell line that has *BRAF* mutations supports the hypothesis that this pathway is important for PTC pathogenesis.

An important practical implication of our study is that HTT ($n=23$) did not have *BRAF* mutations. Trovisco *et al* also observed the absence of *BRAF* mutation in a smaller series ($n=5$) of HTT.[52] HTTs

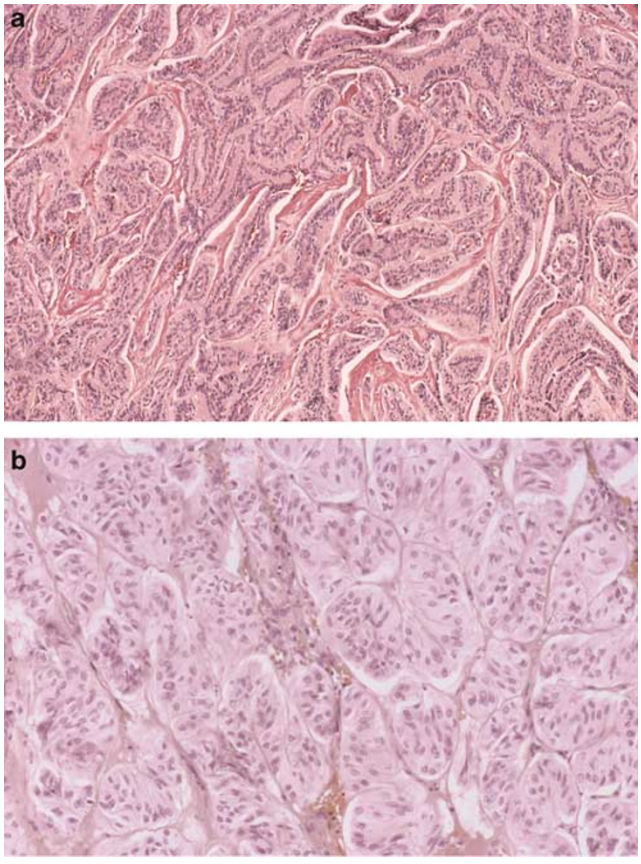

Figure 5 Hematoxylin-and-eosin-stained case of a PTC (**a**) and an HTT (**b**). Although these HTT share some cytologic features with PTC, they were all negative for *BRAF*^{V600E} mutations.

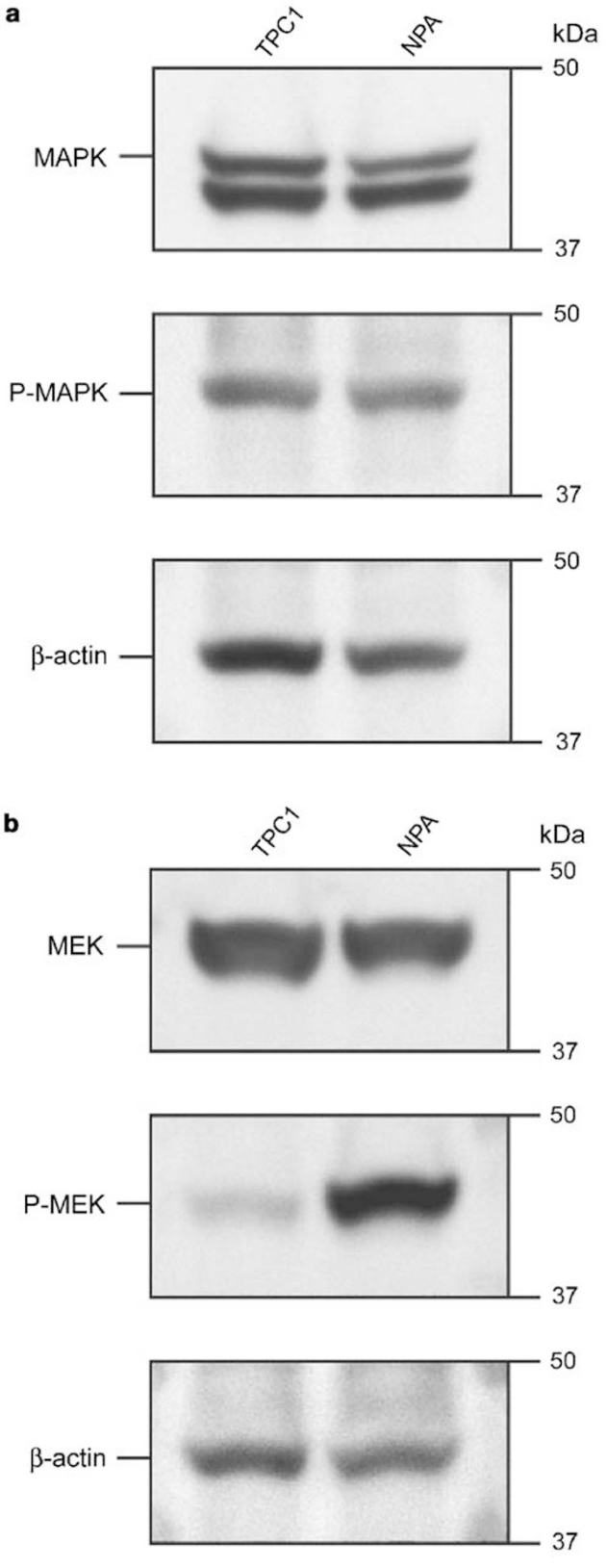

Figure 6 Western blotting of MAPK and phospho-MAPK (**a**), MEK and phospho-MEK (**b**) in NPA and TPC1 thyroid cell lines. Phospho-MEK 1/2 expression was increased in the NPA cell line, which had a *BRAF* mutation supporting the importance of the *BRAF* pathway in the development of PTC.

have many nuclear features in common with PTC and the distinction between HTT and PTC may be difficult in cytological and tissue specimens. Our findings suggest that molecular analysis for *BRAF* in cases in which it is not possible to distinguish between a HTT and PTC in cytological or biopsy specimens will be a very useful molecular assay.

In summary, analysis of the epigenetic changes of *RASSF1A* and *NORE1A* in thyroid tumor indicates that methylation of *RASSF1A* is an early event in thyroid tumor pathogenesis and that *NORE1A* methylation is uncommon in primary thyroid tumors. *BRAF* point mutation occurs later in thyroid tumor progression and is restricted to PTC and some ATCs.

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References

1 Soares P, Maximo V, Sobrinho-Simoes M. Molecular pathology of papillary, follicular and Hurthle cell carcinomas of the thyroid. Arkh Patol 2003;65:45–47.
2 Segev DL, Umbricht C, Zeiger MA. Molecular pathogenesis of thyroid cancer. Surg Oncol 2003;12:69–90.
3 Dammann R, Li C, Yoon JH, *et al.* Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. Nat Genet 2000;25:315–319.
4 Burbee DG, Forgacs E, Zochbauer-Muller S, *et al.* Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. J Natl Cancer Inst 2001;93:691–699.
5 Dreijerink K, Braga E, Kuzmin I, *et al.* The candidate tumor suppressor gene, RASSF1A, from human chromosome 3p21.3 is involved in kidney tumorigenesis. Proc Natl Acad Sci USA 2001;98:7504–7509.
6 Kuzmin I, Gillespie JW, Protopopov A, *et al.* The RASSF1A tumor suppressor gene is inactivated in prostate tumors and suppresses growth of prostate carcinoma cells. Cancer Res 2002;62:3498–3502.
7 Shivakumar L, Minna J, Sakamaki T, *et al.* The RASSF1A tumor suppressor blocks cell cycle progression and inhibits cyclin D1 accumulation. Mol Cell Biol 2002;22:4309–4318.
8 Dammann R, Yang G, Pfeifer GP. 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 2001;61:3105–3109.
9 Dammann R, Takahashi T, Pfeifer GP. The CpG island of the novel tumor suppressor gene RASSF1A is

intensely methylated in primary small cell lung carcinomas. Oncogene 2001;20:3563–3567.
10 Yoon JH, Dammann R, Pfeifer GP. Hypermethylation of the CpG island of the RASSF1A gene in ovarian and renal cell carcinomas. Int J Cancer 2001;94:212–217.
11 van Engeland M, Roemen GM, Brink M, *et al.* K-ras mutations and RASSF1A promoter methylation in colorectal cancer. Oncogene 2002;21:3792–3795.
12 Hasegawa M, Nelson HH, Peters E, *et al.* Patterns of gene promoter methylation in squamous cell cancer of the head and neck. Oncogene 2002;21:4231–4236.
13 Toyooka S, Carbone M, Toyooka KO, *et al.* Progressive aberrant methylation of the RASSF1A gene in simian virus 40 infected human mesothelial cells. Oncogene 2002;21:4340–4344.
14 Harada K, Toyooka S, Maitra A, *et al.* Aberrant promoter methylation and silencing of the RASSF1A gene in pediatric tumors and cell lines. Oncogene 2002;21:4345–4349.
15 Schagdarsurengin U, Gimm O, Hoang-Vu C, *et al.* Frequent epigenetic silencing of the CpG island promoter of RASSF1A in thyroid carcinoma. Cancer Res 2002;62:3698–3701.
16 Wong IH, Chan J, Wong J, *et al.* Ubiquitous aberrant RASSF1A promoter methylation in childhood neoplasia. Clin Cancer Res 2004;10:994–1002.
17 Xing M, Cohen Y, Mambo E, *et al.* Early occurrence of RASSF1A hypermethylation and its mutual exclusion with BRAF mutation in thyroid tumorigenesis. Cancer Res 2004;64:1664–1668.
18 Vavvas D, Li X, Avruch J, *et al.* Identification of Nore1 as a potential Ras effector. J Biol Chem 1998;273: 5439–5442.
19 Tommasi S, Dammann R, Jin SG, *et al.* RASSF3 and NORE1: identification and cloning of two human homologues of the putative tumor suppressor gene RASSF1. Oncogene 2002;21:2713–2720.
20 Hesson L, Dallol A, Minna JD, *et al.* NORE1A, a homologue of RASSF1A tumour suppressor gene is inactivated in human cancers. Oncogene 2003;22: 947–954.
21 Bonner TI, Kerby SB, Sutrave P, *et al.* Structure and biological activity of human homologs of the raf/mil oncogene. Mol Cell Biol 1985;5:1400–1407.
22 Bonner TI, Oppermann H, Seeburg P, *et al.* The complete coding sequence of the human raf oncogene and the corresponding structure of the c-raf-1 gene. Nucleic Acids Res 1986;14:1009–1015.
23 Huebner K, ar-Rushdi A, Griffin CA, *et al.* Actively transcribed genes in the raf oncogene group, located on the X chromosome in mouse and human. Proc Natl Acad Sci USA 1986;83:3934–3938.
24 Huleihel M, Goldsborough M, Cleveland J, *et al.* Characterization of murine A-raf, a new oncogene related to the v-raf oncogene. Mol Cell Biol 1986;6: 2655–2662.
25 Beck TW, Huleihel M, Gunnell M, *et al.* The complete coding sequence of the human A-raf-1 oncogene and transforming activity of a human A-raf carrying retrovirus. Nucleic Acids Res 1987;15:595–609.
26 Ikawa S, Fukui M, Ueyama Y, *et al.* B-raf, a new member of the raf family, is activated by DNA rearrangement. Mol Cell Biol 1988;8:2651–2654.
27 Sithanandam G, Kolch W, Duh FM, *et al.* Complete coding sequence of a human B-raf cDNA and detection of B-raf protein kinase with isozyme specific antibodies. Oncogene 1990;5:1775–1780.
28 Eychene A, Barnier JV, Apiou F, *et al.* Chromosomal assignment of two human B-raf(Rmil) proto-oncogene loci: B-raf-1 encoding the p94Braf/Rmil and B-raf-2, a processed pseudogene. Oncogene 1992;7:1657–1660.
29 Stephens RM, Sithanandam G, Copeland TD, *et al.* 95-kilodalton B-Raf serine/threonine kinase: identification of the protein and its major autophosphorylation site. Mol Cell Biol 1992;12:3733–3742.
30 Hillier LW, Fulton RS, Fulton LA, *et al.* The DNA sequence of human chromosome 7. Nature 2003;424: 157–164.
31 Peyssonnaux C, Eychene A. The Raf/MEK/ERK pathway: new concepts of activation. Biol Cell 2001;93: 53–62.
32 Davies H, Bignell GR, Cox C, *et al.* Mutations of the BRAF gene in human cancer. Nature 2002;417: 949–954.
33 Kumar R, Angelini S, Czene K, *et al.* BRAF mutations in metastatic melanoma: a possible association with clinical outcome. Clin Cancer Res 2003;9:3362–3368.
34 Pollock PM, Harper UL, Hansen KS, *et al.* High frequency of BRAF mutations in nevi. Nat Genet 2003;33:19–20.
35 Domingo E, Espin E, Armengol M, *et al.* Activated BRAF targets proximal colon tumors with mismatch repair deficiency and MLH1 inactivation. Genes Chromosomes Cancer 2004;39:138–142.
36 Kimura ET, Nikiforova MN, Zhu Z, *et al.* 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 2003;63:1454–1457.
37 Namba H, Nakashima M, Hayashi T, *et al.* Clinical implication of hot spot BRAF mutation, V599E, in papillary thyroid cancers. J Clin Endocrinol Metab 2003;88:4393–4397.
38 Xu X, Quiros RM, Gattuso P, *et al.* High prevalence of BRAF gene mutation in papillary thyroid carcinomas and thyroid tumor cell lines. Cancer Res 2003;63: 4561–4567.
39 Frommer M, McDonald LE, Millar DS, *et al.* A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci USA 1992;89:1827–1831.
40 Irimia M, Fraga MF, Sanchez-Cespedes M, *et al.* CpG island promoter hypermethylation of the Ras-effector gene RASSF1A occurs in the context of a wild-type K-ras in lung cancer. Oncogene 2004;23: 8695–8699.
41 Hesson L, Bieche I, Krex D, *et al.* Frequent epigenetic inactivation of RASSF1A and BLU genes located within the critical 3p21.3 region in gliomas. Oncogene 2004;23:2408–2419.
42 Lae ME, Roche PC, Jin L, *et al.* Desmoplastic small round cell tumor: a clinicopathologic, immunohistochemical, and molecular study of 32 tumors. Am J Surg Pathol 2002;26:823–835.
43 Ruebel KH, Jin L, Zhang S, *et al.* Inactivation of the p16 gene in human pituitary nonfunctioning tumors by hypermethylation is more common in null cell adenomas. Endocr Pathol 2001;12:281–289.
44 Riss D, Jin L, Qian X, *et al.* Differential expression of galectin-3 in pituitary tumors. Cancer Res 2003;63: 2251–2255.
45 Lloyd RV, Ruebel KH, Zhang S, *et al.* Pituitary hyperplasia in glycoprotein hormone alpha subunit-, p18(INK4C)-, and p27(kip-1)-null mice: analysis of

proteins influencing p27(kip-1) ubiquitin degradation. Am J Pathol 2002;160:1171–1179.
46 Chow LS, Lo KW, Kwong J, *et al.* Aberrant methylation of RASSF4/AD037 in nasopharyngeal carcinoma. Oncol Rep 2004;12:781–787.
47 Mehrotra J, Vali M, McVeigh M, *et al.* Very high frequency of hypermethylated genes in breast cancer metastasis to the bone, brain and lung. Clin Cancer Res 2004;10:3104–3109.
48 Cohen Y, Xing M, Mambo E, *et al.* BRAF mutation in papillary thyroid carcinoma. J Natl Cancer Inst 2003; 95:625–627.
49 Soares P, Trovisco V, Rocha AS, *et al.* BRAF mutations and RET/PTC rearrangements are alternative events in the etiopathogenesis of PTC. Oncogene 2003;22: 4578–4580.
50 Fukushima T, Suzuki S, Mashiko M, *et al.* BRAF mutations in papillary carcinomas of the thyroid. Oncogene 2003;22:6455–6457.
51 Nikiforova MN, Kimura ET, Gandhi M, *et al.* BRAF mutations in thyroid tumors are restricted to papillary carcinomas and anaplastic or poorly differentiated carcinomas arising from papillary carcinomas. J Clin Endocrinol Metab 2003;88:5399–5404.
52 Trovisco V, Vieira de Castro I, Soares P, *et al.* BRAF mutations are associated with some histological types of papillary thyroid carcinoma. J Pathol 2004;202: 247–251.
53 Xing M, Vasko V, Tallini G, *et al.* BRAF T1796A transversion mutation in various thyroid neoplasms. J Clin Endocrinol Metab 2004;89:1365–1368.
54 Soares P, Trovisco V, Rocha AS, *et al.* BRAF mutations typical of papillary thyroid carcinoma are more frequently detected in undifferentiated than in insular and insular-like poorly differentiated carcinomas. Virchows Arch 2004;444:572–576.
55 Cohen Y, Rosenbaum E, Clark DP, *et al.* Mutational analysis of BRAF in fine needle aspiration biopsies of the thyroid: a potential application for the preoperative assessment of thyroid nodules. Clin Cancer Res 2004;10:2761–2765.
56 Puxeddu E, Moretti S, Elisei R, *et al.* BRAF(V599E) mutation is the leading genetic event in adult sporadic papillary thyroid carcinomas. J Clin Endocrinol Metab 2004;89:2414–2420.
57 Nikiforova MN, Ciampi R, Salvatore G, *et al.* Low prevalence of BRAF mutations in radiation-induced thyroid tumors in contrast to sporadic papillary carcinomas. Cancer Lett 2004;209:1–6.
58 Xing M, Tufano RP, Tufaro AP, *et al.* Detection of BRAF mutation on fine needle aspiration biopsy specimens: a new diagnostic tool for papillary thyroid cancer. J Clin Endocrinol Metab 2004;89: 2867–2872.
59 Fugazzola L, Mannavola D, Cirello V, *et al.* BRAF mutations in an Italian cohort of thyroid cancers. Clin Endocrinol (Oxf) 2004;61:239–243.
60 Frattini M, Ferrario C, Bressan P, *et al.* Alternative mutations of BRAF, RET and NTRK1 are associated with similar but distinct gene expression patterns in papillary thyroid cancer. Oncogene 2004;23: 7436–7440.
61 Begum S, Rosenbaum E, Henrique R, *et al.* BRAF mutations in anaplastic thyroid carcinoma: implications for tumor origin, diagnosis and treatment. Mod Pathol 2004;17:1359–1363.