

## Dual Role of RASSF1 as a Tumor Suppressor and an Oncogene in Neuroendocrine Tumors of the Lung

GIUSEPPE PELOSI<sup>1,2</sup>, CATERINA FUMAGALLI<sup>1</sup>, MAURIZIO TRUBIA<sup>3</sup>, ANGELICA SONZOGNI<sup>1</sup>, NATASHA REKHTMAN<sup>4</sup>, PATRICK MAISONNEUVE<sup>5</sup>, DOMENICO GALETTA<sup>6</sup>, LORENZO SPAGGIARI<sup>2,6</sup>, GIULIA VERONESI<sup>6</sup>, ALDO SCARPA<sup>7</sup>, GIORGIO MALPELI<sup>7</sup> and GIUSEPPE VIALE<sup>1,2</sup>

<sup>1</sup>Division of Pathology and Laboratory Medicine,

European Institute of Oncology and National Cancer Institute, Milan, Italy;

<sup>2</sup>Department of Medicine, Surgery and Dentistry, University of Milan School of Medicine, Milan, Italy;

<sup>3</sup>Department of Biotechnology and Biosciences, Milano-Bicocca University, Milan, Italy;

<sup>4</sup>Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY, USA;

<sup>5</sup>Division of Epidemiology and Biostatistics, European Institute of Oncology, Milan, Italy;

<sup>6</sup>Division of Thoracic Surgery, European Institute of Oncology, Milan, Italy;

<sup>7</sup>Institute of Pathology, University of Verona School of Medicine, Verona, Italy

**Abstract.** Background: Little is known about the dual role of RAS-association domain family 1 (RASSF1) gene at 3p21.3 in neuroendocrine tumors (NET) of the lung. Materials and Methods: Twenty typical carcinoids (TC), 11 atypical carcinoids (ATC), 11 large cell neuroendocrine carcinomas (LCNEC) and 16 small cell lung carcinomas (SCLC) were analyzed for RASSF1 promoter methylation, mRNA and protein expression, and loss of 3p21.3 locus. Results: Promoter 1 was hypermethylated in NET but not in paired non-neoplastic lung tissues nor in 20 control NSCLC, with the degree of hypermethylation paralleling tumor grade. RASSF1 A/E isoform mRNA but not protein expression was lost in most NET compared to NSCLC or non-neoplastic tissues. The relationship between methylation level and mRNA or protein loss varied by NET type, with significant correlation for decreasing RASSF1 A protein in ACT, and marginal correlation for down-regulated RASSF1 A/E mRNA in TC, this suggesting a non linear regulation by methylation in NET. No promoter 2 methylation was detected in NET; however, up-regulation of its RASSF1 C transcript emerged as an adverse prognostic factor in the LCNEC/SCLC group. A correlation was found between 3p21.3 allelic loss and decrease of RASSF1 A/E mRNA

( $p=0.023$ ) and protein ( $p=0.043$ ) expression in ATC, suggesting that 3p21.3 allelic loss contributed to the loss of gene expression. Conclusion: RASSF1 A/E is likely to act as a tumor suppressor gene in most pulmonary NET, and RASSF1 C as an oncogene in high-grade tumors.

RAS GTPases make up a superfamily of molecular switches, which regulate diverse and complex cellular functions, including proliferation, differentiation, motility and apoptosis in response to various extracellular signals. Beside well-known RAS effectors, such as RAF and PIK-3, a new eight exon-spanning gene member has recently been described, the RAS-association domain family 1 (RASSF1) (also known as RASSF1A, NORE2A, 123F2, RDA32 and REH3P21), which maps to chromosome 3p21.3 (1, 2) and encodes at least eight different transcripts (RASSF1 A-H) under control of two promoters (promoter 1 and promoter 2), and alternative splicing modalities (3, 4). Both promoter 1 and promoter 2 contain CpG-rich areas, and methylation of these regions has been implicated as important for regulating these promoters (3, 4).

RASSF1A and RASSF1C are the two most common isoforms expressed in several normal tissues (5-7). Expression of RASSF1A is regulated by promoter 1, while expression of RASSF1C is under control of promoter 2. Isoforms RASSF1B, RASSF1D and RASSF1E have been found predominantly in hematopoietic, cardiac and pancreatic cells, respectively (8), under the same promoter as the RASSF1A isoform (4). Data on the prevalence and biological significance of RASSF1F, RASSF1G and RASSF1H isoforms are still lacking.

In human cancer, regulatory mechanisms of RASSF1A expression, the best known member of this protein family

Correspondence to: Giuseppe Pelosi, MD, MIAC, Dipartimento di Patologia Diagnostica e Laboratorio, Istituto Nazionale dei Tumori, Via G. Venezian, 1, 20133 Milano, Italy. Tel: +39 0223902260, Fax: +39 0223902877, e-mail: giuseppe.pelosi@istitutotumori.mi.it

Key Words: Carcinoma, carcinoid, small cell carcinoma, large cell neuroendocrine carcinoma, methylation, immunofluorescence, survival.

acting as a tumor suppressor, most often include gene promoter methylation and loss of heterozygosity (9, 10), leading to impaired apoptosis (4), increased cell migration (11, 12) and proliferation (13-15). In turn, the functions of RASSF1C isoform are still debated, inasmuch as its role as either tumor suppressor (16) or direct stimulator of cell proliferation (7, 17, 18) has been reported in both normal and several tumor tissues.

Neuroendocrine tumors (NET) of the lung constitute a heterogeneous group of neoplasms including relatively indolent lesions with longer life expectation (typical [TC] and atypical [ATC] carcinoids) and very aggressive tumors with dismal prognosis (large cell neuroendocrine carcinoma [LCNEC] and small cell lung cancer [SCLC]) (19). Several molecular prognostic factors have been proposed (20-24), but histological typing still remains the most powerful predictor of clinical course (25, 26). The greatest challenge for treating these tumors, however, still remains the separation of patient subsets with different tumor biological behavior within each diagnostic category. Therefore, investigations on factors implicated in the development and progression of these tumors, as well as those affecting survival rates, are clearly warranted, inasmuch as they could provide helpful prognostic and predictive information.

Prior studies have shown that the RASSF1A isoform is down-regulated by methylation in most SCLC and pulmonary ATC in comparison with TC (1, 27-29). Epigenetic silencing of *RASSF1A* promoter by methylation and allelic loss of its locus at 3p21.3 are also thought to be relevant to the development of NET derived from the foregut (30-33), abdominal paraganglioma (34) and adrenal glands (34, 35), as well as in the growth of several types of pediatric malignant tumors (36, 37). Furthermore, the presence of both *RASSF1A* methylation and 3p21.3 allelic loss has been associated with malignant behavior of foregut NET (30). In contrast, no SCLC thus far examined has shown evidence for CpG island methylation of *RASSF1C* (1, 5, 27), but a comprehensive analysis of the methylation status, the prevalence and the clinical implications of diverse *RASSF1* isoforms across the whole spectrum of pulmonary NET is still lacking.

In this study, we analyzed *RASSF1* promoter methylation and 3p21.3 allelic loss in relation to *RASSF1A* and *RASSF1C* mRNA and protein expression in a series of 58 pulmonary NET including TC and ATC, LCNEC and SCLC, as well as 20 NSCLC (10 squamous cell carcinomas and 10 adenocarcinomas) and non-tumor samples used as controls. In addition, we studied the clinicopathological correlates of these various parameters.

## Materials and Methods

*Strategy of study, selection of patients and adequacy of samples.* The *RASSF1* gene generates at least five different transcripts (isoforms A to E) under two promoters and alternative splicing modalities. Since epigenetic silencing by methylation seems to play

a role in regulating this expression, we designed and conducted a quantitative methylation analysis of both gene promoters by means of a twofold approach including pyrosequencing technology (38-43) and a customer-designed real-time methylation-specific polymerase chain reaction (PCR) (QMSP) assay.

Using island size >100 bp, GC percentage >50, and observed/expected ratio >0.6 as selection criteria and MethPrimer software (44), two CG dinucleotide-rich regions were confirmed in the promoter 1 sequence (GenBank accession number DQ444319; island 1: 133 bp, start 424, end 556; island 2: 636 bp, start 564, end 1199) and one CG dinucleotide-rich region in the promoter 2 sequence (GenBank accession number NC\_000003 from 3031 to 3770; 637 bp, start 48, end 684) (Figure 1), as previously reported (9). Beta-actin (*ACTB*) was used as the internal reference housekeeping gene in all experiments, inasmuch as it showed the lowest standard deviation in nonneoplastic lung tissue samples after comparison with other housekeeping genes (*GAPDH*, 18S, and beta-2-microglobulin).

Representative samples of both tumor tissue and paired nonneoplastic lung parenchyma were included from 58 neuroendocrine tumor patients, comprising 20 TC, 11 ATC, 11 LCNEC and 16 SCLC, which were collected at the European Institute of Oncology between 1997 and 2006. Ten adenocarcinoma and ten squamous cell carcinoma samples collected at the same Institution in the same time frame were used as a control group of non-NET. All tumor patients entering the study had been exhaustively studied with clinical history, physical examination, respiratory function tests, radiological imaging and routine laboratory profile prior to surgery, and all but three patients (2 bearing TC and 1 SCLC) underwent radical surgery inclusive of extended mediastinal lymph node dissection (median value: 17 excised lymph nodes). There were 31 females and 27 males in the patient cohort under investigation, with ages ranging from 30 to 80 years in the former (mean±SD: 59.6±11.8, median: 62 years) and from 22 to 82 years in the latter (mean±SD: 60.1±12.6, median: 62 years). Complete follow-up information on relapse and survival was available for all but two SCLC patients. Representative data on the entire patient cohort according to the tumor histology is reported in Table I. All patients gave informed consent to be enrolled into the study that had been approved by the Institutional Review Board.

All nonneoplastic and tumor tissues had been immediately removed at the time of surgery, and in part snap-frozen in liquid nitrogen for DNA or RNA extraction within 10 min after excision, in part fixed in formalin and embedded in paraffin for 6 to 8 hours for routine histopathological examination. Diagnostic assessment was carried out according to the criteria of the 2004 WHO classification (19), and only tumor samples containing at least 80% tumor cellularity were included in the study.

*DNA and RNA extraction.* Ten-µm-thick frozen sections were cut from each tissue sample, either tumoral (containing at least 80% of neoplastic cells) or nonneoplastic lung, and immediately processed for DNA (NucleoSpin Tissue Kit; Machery-Nagel, Germany) or RNA (RNeasy Mini Kit; Qiagen, Milan, Italy) extraction according to the manufacturer's instructions. RNase digestion was also included into the DNA extraction protocol to obtain RNA-free samples (Qiagen). Samples were then eluted in 100 µl of sterile filtered water for DNA and in 50 µl of RNase-free water for RNA, both being stored at -80°C until use. The final yield of extracted DNA and RNA was measured spectrophotometrically.

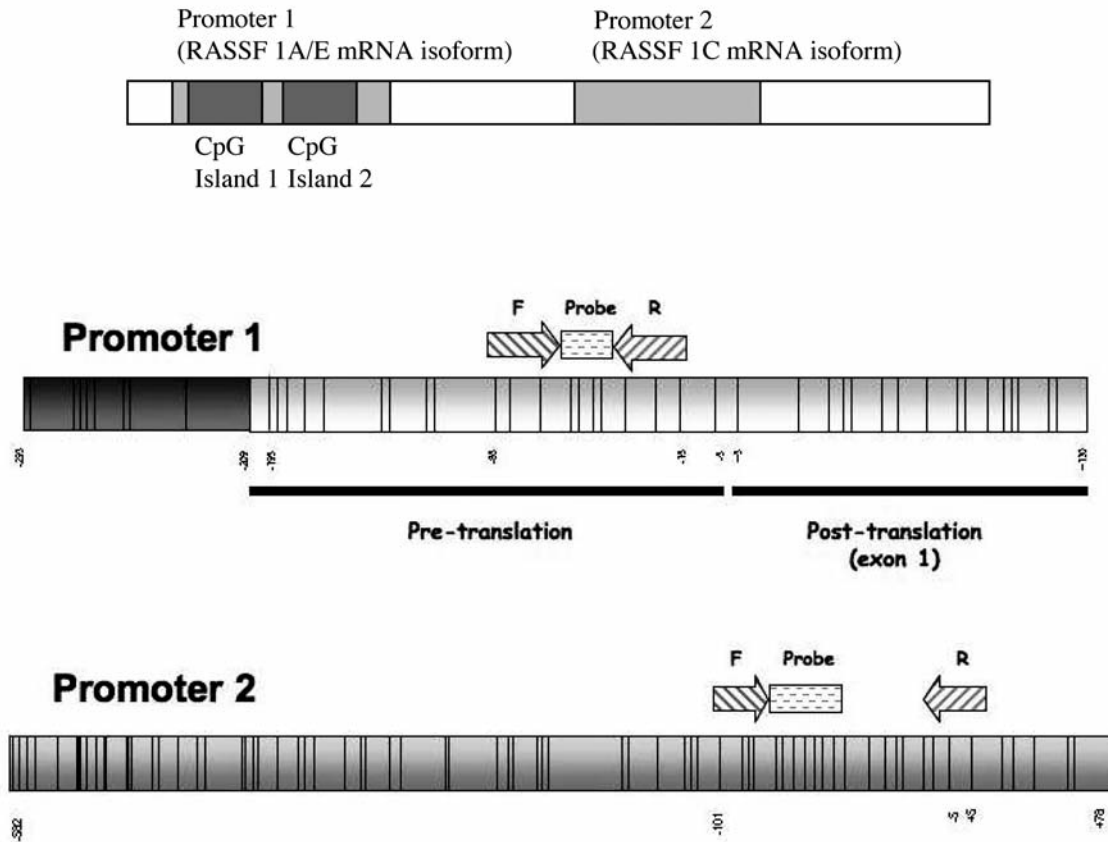


Figure 1. General diagram of RASSF1 promoter and the strategy used in the study for highlighting the methylation status in both promoter 1 and promoter 2. As island 1 of the promoter 1 partially spans encoding exon 1 of the relevant gene, the CpG island has been arbitrarily divided into pre-translational and post-translational sequences.

**Pyrosequencing evaluation.** Pyrosequencing analysis was carried out in the current investigation (38-43) to thoroughly assess the methylation status of both promoter 1 (island 1 and 2) and promoter 2. Island 2 of promoter 1 was arbitrarily split into pre-transcriptional and post-transcriptional regions, as the latter spans encoding exon 1 of the relevant gene (Figure 1). In every tumor case, the results were expressed as the median value of the methylation levels of all CpG dinucleotides present in the relevant regions.

**Quantitative (real-time) methylation-specific PCR (QMSP) assay.** A QMSP assay approach was carried out in the current investigation to analyze island 2 methylation of the pre-translational sequence according to many studies thus far published, which have pointed to this region as being important for affecting protein expression (45-47).

Briefly, sodium-bisulfite treatment for DNA modification was performed with EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. Bisulfite-modified DNA was then used as template for QMSP, designing appropriate primers and probes for promoters 1 and 2 of RASSF1 gene as indicated in Table II. QMSP was carried out in a reaction volume of 25  $\mu$ l, each reaction mixture consisting of 600 nM of each primer, 200 nM probe, TaqMan<sup>®</sup> universal PCR Master Mix NO Amperase UNG 1x and 30 ng of modified DNA. Amplification was performed in an ABI PRISM 7700 Sequence Detector (Applied

Biosystems, Foster City, CA, USA) using as thermal cycling conditions denaturation steps at 95°C for 10 min followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. All samples were run in triplicate and repeated again if they did not match with each other. Each run of amplification included commercially available, CpG dinucleotide-completely methylated DNA (CpGenome Universal Methylated DNA, Millipore at <http://www.millipore.com/antibodies/ab/abhome>) as a positive control, and three different negative controls, either omitting DNA or using leukocyte DNA from healthy volunteers, the latter either sodium bisulfite-unmodified or sodium bisulfite-modified. Five serial dilutions from 50 to 1.563 ng/ml of the positive control were used for constructing standard curves for each amplification run. Linear amplification (with correlation coefficient, 0.999 to 0.995; slope, 3.25 to 3.35) was obtained in each experiment and the 'input amount' values of each sample were calculated by plotting  $C_t$  values on the corresponding standard curve. To determine the relative levels of methylated promoter DNA, the amount of the methylated gene of interest was compared with the amount of DNA modified, based on the internal reference gene, to obtain a ratio that was then multiplied by 100 to give a percentage value (target gene/ACTB  $\times$ 100).

**Quantitative (real-time) PCR assay for RASSF1 isoforms.** After retro-transcription of RNA with MULV Reverse Transcriptase

Table I. Clinicopathological characteristics of 58 patients with neuroendocrine tumors.

Variable	Category	TC (n=20)	ATC (n=11)	p-Value	LCNEC (n=11)	SCLC (n=16)	p-Value
Age (years)	≤49	2	5		1	0	
	50-59	7	1		6	3	
	60-69	6	3		1	11	
	≥70	5	2	<0.001	3	2	0.024
Gender	Male	3	5		3	5	
	Female	17	6	<0.001	8	11	0.203
Surgery	Lobectomy	13	9		8	15	
	Pneumectomy	0	2		3	1	
	Other	7	0	0.021	0	0	0.026
Smoker	Never	14	6		0	1	
	Former	1	3		7	9	
	Current	5	2	<0.001	4	6	0.348
Pack-years	Median	8	11	0.345	45	49	0.567
Metastasis	Yes	17	3		3	4	
	No	3	8	0.005	8	12	0.208
Positive LN %	Median	0	6	0.001	7	12	0.278
Therapy pre-surgery	Yes	0	1		5	5	
	No	20	10	0.001	6	11	0.132
Stage	I	16	5		3	4	
	II-IV	4	6	<0.001	8	12	0.208
pT	1	15	2		3	3	
	2	3	9		4	12	
	3-4	2	0	0.001	4	1	0.001
pN*	N0	17	4		3	3	
	N1	0	5		7	5	
	N2	1	2	0.002	1	7	0.051
Size	≤2 cm	12	2		1	4	
	2-4 cm	8	4		5	6	
	>4 cm	0	5	0.003	5	6	0.275
Vital status*	Alive	20	10		8	10	
	Dead	0	1	0.001	3	4	0.420
Recurrence*	Yes	2	2		5	5	
	No	18	9	0.003	6	9	0.317
Ki-67 index	Median	3	11	<0.001	58	83	<0.001

\*Presence of cases with incomplete information about pN status, vital status and recurrences; TC: typical carcinoids; ATC: atypical carcinoids; LCNEC: large cell neuroendocrine carcinoma; SCLC: small cell lung carcinoma.

(Applied Biosystems) according to the manufacturer's protocol to obtain a final cDNA concentration of 30 ng/μl (thermal conditions included 10-min random hexamer incubation step at 25°C, followed by a 48°C extension for 30 min and 95°C denaturation for 5 min), real-time PCR evaluation was carried out with an ABI Prism 7700 Sequence Detector (Applied Biosystems) for both tumor and paired nonneoplastic tissue samples. The amplification was achieved using commercially available, intron-spanning TaqMan® Gene Expression Assays (Applied Biosystems) for the diverse *RASSF1* gene transcripts (Hs00945257\_m1 specific for isoform A and isoform E, without distinguishing from each other [hence indicated as isoforms A/E]; Hs00945679\_m1 for isoform C; Hs00945252\_m1 for isoform D; Hs00945680\_m1 for isoform F; and Hs00945677\_m1 for isoform G), as well as for β2-microglobulin (Hs99999907\_m1) as housekeeping target gene. No specific assay was available for *RASSF1B* and therefore this transcript was not addressed in our study. Thermal cycling conditions for all assays included 2 min at

50°C, 10 min at 95°C to activate the TaqMan®, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min according to the manufacturer's instructions. All assays were repeated in triplicate, averaging the results for each sample. The specificity of all PCR reactions was checked by omitting cDNA from every assay as negative controls and using commercially available normal lung tissue RNA as positive controls (Human Lung Total RNA from Applied Biosystems and MPV Total RNA Human Adult Lung from Stratagene [Agilent, Santa Clara, CA, USA]).

The relative quantification of the target gene expression was calculated by means of the comparative C<sub>T</sub> method (ΔΔC<sub>T</sub>) (Applied Biosystems), using as endogenous reference β2microglobulin (C<sub>T</sub> REFERENCE) and as calibrator the mean of C<sub>T</sub> values of the two commercial RNA samples (Human Lung Total RNA and MPV Total RNA Human Adult Lung). Briefly, the ΔC<sub>T</sub> value (ΔC<sub>T</sub>=C<sub>T</sub> RASSF1-C<sub>T</sub> REFERENCE) was calculated for each sample under investigation, then compared with the calibrator



sample ( $\Delta\Delta C_T = \Delta C_{T \text{ test sample}} - \Delta C_{T \text{ calibrator sample}}$ ) to obtain a  $2^{-\Delta\Delta C_T}$  threshold corresponding to the amounts of the relevant mRNA normalized to an endogenous reference and relative to a calibrator.

**Immunofluorescence and fluorescence in situ hybridization (FISH) assays.** Frozen samples of both tumor and paired nonneoplastic lung tissue were only immunostained for RASSF1A only because of the lack of commercially available antibodies to other isoforms at the time of the current investigation. Four- $\mu\text{m}$ -thick air-dried sections were fixed in acetone for 5 minutes, reacted with the mouse monoclonal antibody 3F3 (IgG1) specifically recognizing the C1 domain of RASSF1A molecule (Abcam, Cambridge, UK), and then incubated with a fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch, West Grove, PA; USA), the former at a dilution of 10  $\mu\text{g}/\text{ml}$  for 60 minutes, the latter of 40  $\mu\text{g}/\text{ml}$  for 30 min, both at room temperature. The specificity of all immunoreactions was double-checked by substituting the primary antibody with a non-related isotypic mouse immunoglobulin at a comparable dilution, and with normal serum alone. Tumors were considered immunoreactive for RASSF1A if cell membrane, cytoplasmic or nuclear immunofluorescence was observed. Immunoreactivity of nonneoplastic bronchial and alveolar epithelia was used as internal positive controls in all cases. Immunoreactivity on either tumor or nonneoplastic cells was evaluated semiquantitatively and arbitrarily on a scale from negative to 3+. Briefly, samples were considered negative if staining was either completely absent or observed in fewer than 5% cells; 1+ cases showed fluorescence in 5-25% cells; 2+ cases in 26-50% cells and 3+ cases exhibited immunoreactivity in the majority of cells (51 to 100%) (Figure 2).

FISH assay for *RASSF1* gene was performed on 4- $\mu\text{m}$ -thick, consecutive paraffin-embedded sections using a home-made SpectrumOrange-labeled DNA probe for the relevant gene or a commercially available conjugated Cy3, SpectrumOrange-labeled, centromeric enumeration probe (Abbott-Vysis, Downer Grove, IL, USA). Briefly, BAC clones specific for RASSF1 (RP11-894C9), belonging to the Roswell Park Cancer Institute libraries (Pieter J. de Jong at <http://bacpac.chori.org/>), were selected according to previous results from BAC library screening (data not shown). Clones were validated by FISH analysis on normal fibroblasts to confirm their expected chromosomal localization. Paraffin sections were hybridized with the relevant probe labeled by nick translation (48), using 500 ng of probe labeled with either Fluorolink Cy3-dUTP or Fluor-X-dCTP (Amersham, Buckinghamshire, UK). At least 60 nuclei were counted for *RASSF1* signals and alpha-satellite sequences of chromosome 3 centromere (CEP3) on consecutive tissue sections. Results were expressed as the mean value of either gene copy number (GCN) or *RASSF1*:CEP3 signal ratio per cell. Monosomy for 3p21.3 locus containing *RASSF1* gene was defined by a mean of <1.5 signals per cell (49).

**Statistics.** Qualitative data are presented as frequencies and percentages and compared using chi-square test, Fisher's exact *t*-test or Mantel-Haenszel test for trend as appropriate. Continuous data were expressed using median values and contrasted employing the Wilcoxon signed-rank test for pairs or the Kruskal-Wallis test if medians were analyzed between two or more groups, respectively. All correlation tests were performed using Spearman's rank test (*r*). Overall survival was defined as the time between surgery and the

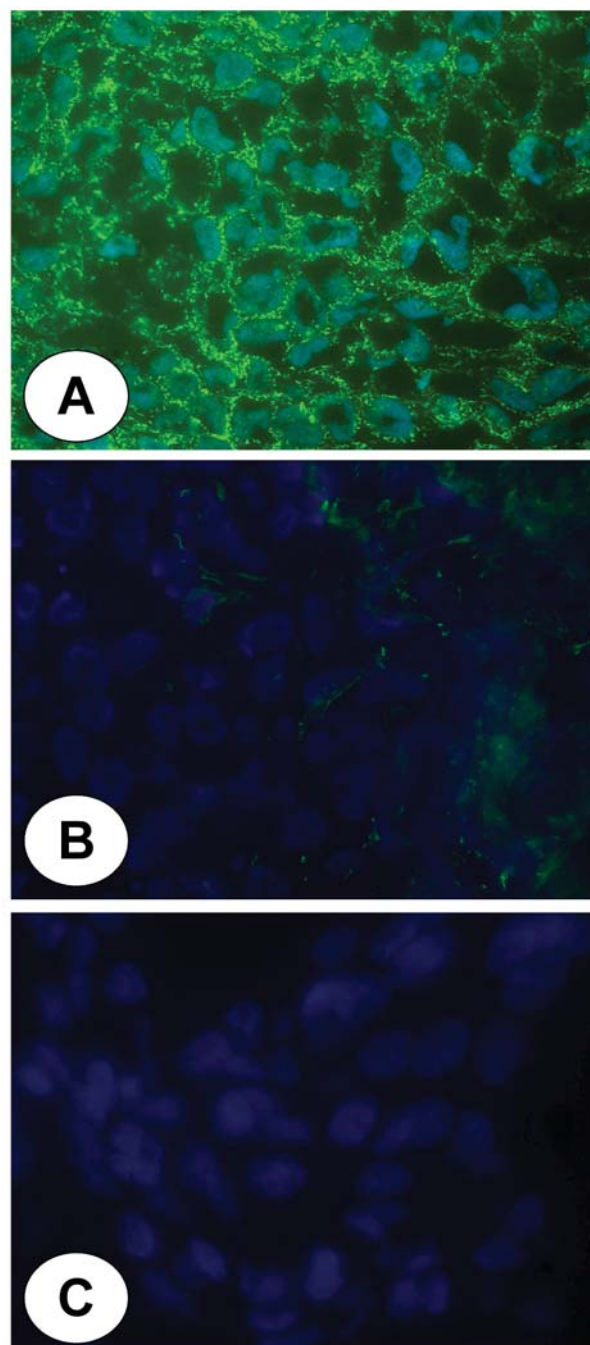


Figure 2. Immunofluorescence results for RASSF1A antibody in tumor samples showing 3+ staining in the majority of tumor cells (A), 1+ staining in 5-25% tumor cells (B), and negative staining, i.e. lack of immunoreactive cells (C).

last follow-up or cancer-related death. If a patient died without cancer recurrence, the patient's survival time was censored at the time of death. Only lung cancer-related deaths or recurrences were considered as events. Disease-free survival was calculated from the date of surgery to the date of progression or the date of last follow-

Table II. Primers used in the study for quantitative (real-time) methylation-specific PCR (QMSP) assay.

Gene		Sequence (5'-3')	Length	No. of examined CpG	GenBank accession number	Amplicon position	Amplicon length
ACTB (β-actin)	F	TGG TGA TGG AGG AGG TTT AGT AAG T	25	-	Y00474	390-522	133 bp
	R	AAC CAA TAA AAC CTA CTC CTC CCT TAA	27	-			
	Probe	6FAM ACC ACC ACC CAA CAC ACA ATA ACA AAC ACA TAMRA	30	-			
RASSF1 PROMOTER 1 (island 2)	F	GCG TTG AAG TCG GGG TTC	18	3	DQ444319	674-748	75 bp
	R	CCC GTA CTT CGC TAA CTT TAA ACG	24	3			
	Probe	6FAM ACA AAC GCG AAC CGA ACG AAA CCA TAMRA	24	4			
RASSF1 PROMOTER 2	F	GTT CGG GCG TAC GCG TAT A	19	4	NC000003	3514-3624	111 bp
	R	CGC CCA TAA CCG TAC CCG	18	3			
	Probe	6FAM TAC GCG TAT ACG TAC GTA CGC GAT CG TAMRA	26	7			

ACTB primers and probe were located in areas without CpG nucleotides, thus amplifying the modified DNA independently of the methylation status of CpG nucleotides; F: forward; R: reverse.

Table III. Methylation of RASSF1 promoter 1 and promoter 2 in the tumor series under evaluation.

Variable	Category	TC (n=20)	ATC (n=11)	p-Value (TC vs. ATC)	LCNEC (n=11)	SCLC (n=16)	p-Value (LCNEC vs. SCLC)	All NET	NSCLC samples	p-Value (All NET vs. NSCLC)	Non- neoplastic lung tissue
Promoter 1:											
Island 1 methylation	Pyrosequencing	35.1	47.5	0.045	40.2	63.5	0.061	46.2	13.0	<0.001	0.0
Promoter 1:											
Island 2 methylation											
Pre-translation	Pyrosequencing	20.0	44.6	0.024	50.0	72.4	0.132	46.1	0.0	<0.001	0.0
Pre-translation	QMSP	0.0	0.6	0.044	38.6	64.0	0.046	1.8	1.2	0.463	0.0
Post-translation	Pyrosequencing	12.9	25.6	0.030	48.8	69.6	0.064	31.4	13.7	0.028	0.0
Promoter 2	QMSP	0.0	0.0	-	0.0	0.0	-	0.0	0.0	-	0.0

TC: Typical carcinoid; ATC: atypical carcinoid; LCNEC: large cell neuroendocrine carcinoma; SCLC: small cell lung carcinoma. QMSP: quantitative methylation-specific protocol; NSCLC: non-small cell lung carcinoma samples. In the table, median values of percentage of promoter methylation are shown.

up. Survival estimates were calculated with Kaplan-Meier's method (50) and compared by the log rank test (51). Any statistical test was considered significant if the corresponding p-value was ≤0.05.

## Results

**Hypermethylation: Promoter 1 hypermethylation is specific to NET and inversely correlates with tumor grade.** Methylation of RASSF1 promoter 1, either CpG island 1 or island 2, was specific to NET, inasmuch as it was consistently lacking in nonneoplastic lung tissue samples and NSCLC. The level of promoter 1 methylation varied according to tumor grade, with TC and ATC exhibiting lower levels of methylation than LCNEC and SCLC, in both island 1 (p=0.032) and island 2 (pre-translation region, p=0.001; post-translation region p<0.001) sequences, when analyzing the four groups of NET together. In particular, higher levels of promoter 1 methylation

were seen in ATC as compared to TC, and SCLC as compared to LCNEC using pyrosequencing and QMSP (Table III). When considered as a whole and compared with NSCLC or nonneoplastic lung tissue, NET showed higher levels of methylation for both island 1 and island 2 of the promoter 1, whereas no methylation was detected in the promoter 2 in any NET, NSCLC or nonneoplastic tissue sample tested (Table III). In all NET types there was a close correlation between island 1 and island 2 methylation levels, independently of the type of assay under evaluation (Table IV).

**RASSF1 mRNA content: NET of the lung show decreased levels of RASSF1 A/E mRNA and increased levels of RASSF1 C mRNA compared to non-tumor tissue and NSCLC.** We found that NET as a group showed down-regulation of RASSF1A/E mRNA (under promoter 1) as compared to nonneoplastic lung tissue samples, with 32 of

58 (55%) NET showing lower levels of methylation than paired nonneoplastic controls. In contrast, *RASSF1C* mRNA (under promoter 2) was overexpressed in 52 out of 58 (90%) NET in comparison with paired nonneoplastic lung tissue samples (*RASSF1A/E* vs. *RASSF1C*,  $p < 0.001$ ). Among NET, *RASSF1A/E* mRNA was more conserved in carcinoids than LCNEC/SCLC, whereas five cases of SCLC exhibited lower levels of *RASSF1C* (Table V). No differences in the level of *RASSF1A/E* and *RASSF1C* mRNA were found comparing TC to ATC, and LCNEC to SCLC, apart from a significant down-regulation of *RASSF1A/E* isoform in SCLC than LCNEC ( $p = 0.041$ ) (Table VI).

When considering NET cases as a whole, fewer expressed *RASSF1A/E* mRNA (55% of cases,  $p = 0.0415$ ) and more *RASSF1C* mRNA (90% of cases,  $p < 0.001$ ) in comparison with NSCLC, whether taking into account the tumor to nonneoplastic lung tissue ratio (Table V) or the median mRNA expression (Table VI). Differences in *RASSF1A/E* mRNA levels were significantly different for each NET tumor type (except LCNEC) compared to NSCLC.

No appreciable levels of RASSF1D, G and F isoforms (all under promoter 1) were found in any NET, NSCLC or nonneoplastic tissue sample under evaluation (data not shown).

*Relationship between methylation status, RASSF1A/E mRNA content and RASSF1 A protein expression.* Correlation of methylation levels with mRNA or protein expression yielded variable results in the different tumor types under evaluation. In NET, association of methylation (island 2, post-translational region) and reduced *RASSF1A/E* mRNA content was detected marginally in TC ( $r = -0.361$ ,  $p = 0.118$ ) only, whereas no correlation of methylation and reduced mRNA level was identified in ATC, LCNEC, and SCLC (data not shown). Neither was there any significant relationship found in NSCLC between methylation and *RASSF1A/E* mRNA content.

Significant association for island 1 ( $r = -0.641$ ,  $p = 0.0337$ ) or marginal for island 2 ( $r = -0.479$ ,  $p = 0.136$ ) methylation and loss of RASSF1A protein expression was observed in ATC but not other in types of NET. In NSCLC, there was a marginal relationship between methylation and down-regulated RASSF1A protein expression for both island 1 ( $r = -0.403$ ,  $p = 0.078$ ) and island 2 ( $r = -0.409$ ,  $p = 0.073$ ).

The proliferation index of NET did not correlate with methylation status, *RASSF1C* or A/E mRNA content or RASSF1A protein expression. No correlation was identified between *RASSF1A/E* mRNA content and RASSF1A protein expression in any tumor tissue, whereas an inverse relationship was found in nonneoplastic lung tissue ( $r = -0.583$ ,  $p = 0.0601$ ).

*Clinical implications: Increased RASSF1C mRNA level predicts recurrences and shorter survival in high-grade NRET, independently of tumor stage.* As LCNEC and SCLC did not differ as to survival, we considered high-grade NET

as a single category for analysis (Figure 3). Carcinoid tumors, either typical or atypical, cannot be separately analyzed for survival or recurrence because of the low number of events in this group.

We found that increased methylation of island 1 or island 2 was marginally associated with better overall survival in the group of high-grade neuroendocrine tumors, with a hazard ratio (HR) of 1.036 (95% CI: 0.995-1.079,  $p = 0.088$ ) for island 1, and HR 1.040 (95% CI: 0.995-1.087,  $p = 0.084$ ) for island 2 hypermethylation. Moreover, an increased content of *RASSF1C* mRNA (median value 1.5) emerged as dismal prognostic factor for overall survival ( $p = 0.036$ ) and, marginally, for disease-free survival ( $p = 0.112$ ). (Figure 4) Likewise, survival of these patient was strictly associated with increased levels of *RASSF1C* mRNA, inasmuch as 6 out of 7 patients who died of disease had tumor mRNA levels  $> 1.5$  in comparison with 7 out of 18 of the surviving patients ( $p = 0.0392$ , test for trend). This association with poorer prognosis was also maintained when the results were stratified for regional lymph node-positive patients (pN1: stage II; pN2: stage III) (Figure 5).

Univariate Cox's survival analysis for continuous variables confirmed the significant association between *RASSF1C* mRNA content and overall (HR: 2.271; 95% CI: 1.281-4.027,  $p = 0.005$ ) and disease-free survival (HR: 1.763; 95% CI: 1.107-2.807,  $p = 0.0169$ ). Methylation status, mRNA content of isoforms other than *RASSF1C*, protein expression, Ki-67 labeling index and the other clinicopathological variables listed in Table I were not associated with patients' survival.

*FISH analysis of RASSF1 gene.* The Distribution of FISH alterations for *RASSF1* gene is presented in Table VII. Monosomy was found in 57% of NET and 50% of NSCLC, but in none of the nonneoplastic lung tissues. Likewise, there were no significant differences in GCN among different types of NET (data not shown).

A positive correlation was found between increased GCN (FISH signals per cell) and *RASSF1A/E* mRNA ( $r = 0.667$ ;  $p = 0.023$ ) or protein ( $r = 0.721$ ;  $p = 0.043$ ) expression in ATC, suggesting a regulation by 3p21.3 loss in this tumor type.

## Discussion

Although similar results were found to some extent when using pyrosequencing and QMSP (Table IV), functional implications (see below) emerged only when exploiting the former, probably because of the lower amounts of CpG dinucleotides highlighted by QMSP in contrast with the pyrosequencing strategy, which allows all known CpG islands to be evaluated in a given genetic region. A key result of our investigation is that that promoter 1 hypermethylation of the *RASSF1* gene controlling the expression of A, B, D and E isoforms was limited to NET of the lung. Methylation

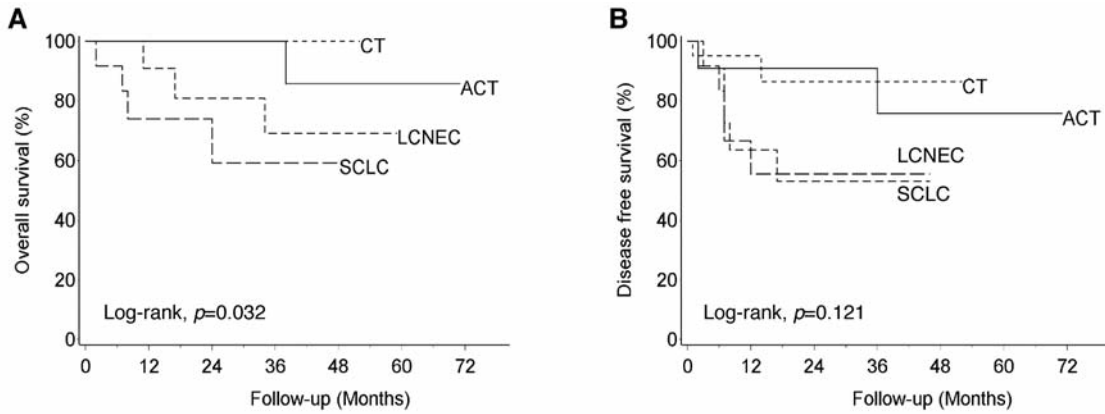


Figure 3. Overall (A) and disease-free (B) survival curves according to tumor histological subtype.

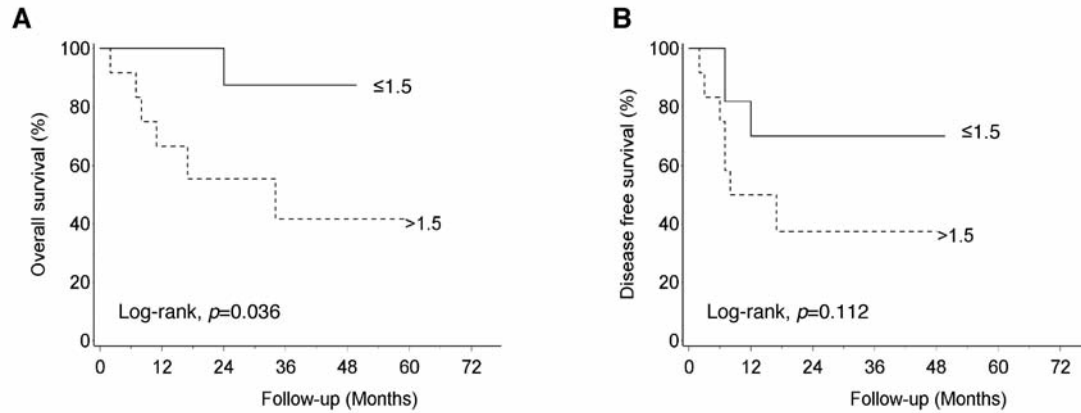


Figure 4. Overall (A) and disease-free (B) survival curves of patients with large cell neuroendocrine carcinoma and those with small cell lung cancer according to RASSF1C mRNA expression.

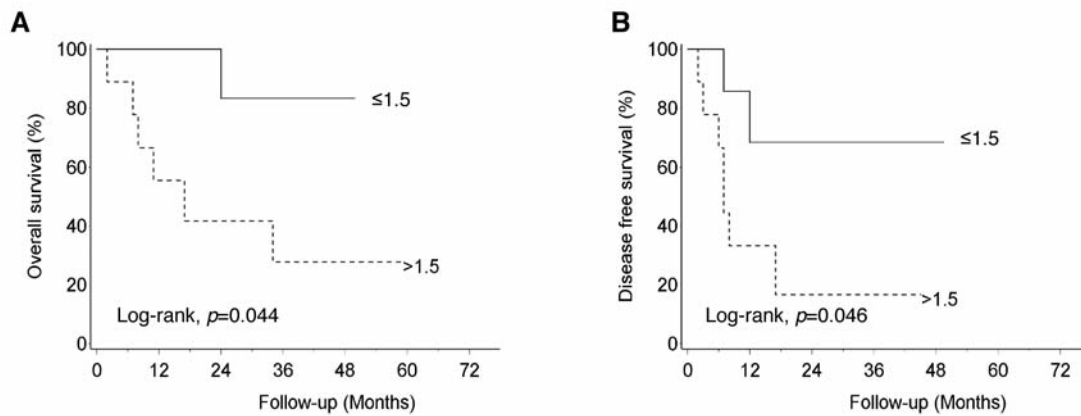


Figure 5. Overall (A) and disease-free (B) survival curves of patients with large cell neuroendocrine carcinoma/small cell lung cancer and node-positive disease according to RASSF1C mRNA expression.



Table IV. Relationship of RASSF1 promoter 1 methylation in neuroendocrine lung tumors.

Tumor type	Number of cases	Island 2					
		Pre-translation (pyrosequencing)		Post-translation (pyrosequencing)		QPCR	
		r	p-Value	r	p-Value	r	p-Value
TC	N=20	0.802	<0.0001	0.770	<0.0001	0.512	0.0209
ATC	N=11	0.836	0.0013	0.909	0.0001	0.815	0.0022
LCNEC	N=11	0.743	0.0088	0.818	0.0021	0.772	0.0053
SCLC	N=16	0.556	0.0254	0.853	<0.0001	0.688	0.0032

TC: Typical carcinoid; ATC: atypical carcinoid; LCNEC: large cell neuroendocrine carcinoma; SCLC: small cell lung carcinoma; Island 1 pyrosequencing is used as reference for correlation tests; r value indicates correlation index according to the Spearman rank method.

of this region was not seen in any samples of adenocarcinoma or squamous cell carcinoma, and all the paired nonneoplastic lung tissue samples exhibited lower levels of methylation or even no methylation. This striking difference supports the nonrandom role for this epigenetic change in the development of pulmonary NET.

We found that the extent of methylation paralleled the increasing grade of clinical aggressiveness of NET from TC to ATC to LCNEC and SCLC, with lowest level of methylation seen in TC and highest levels in SCLC. The methylation levels of low/intermediate grade tumors (TC, ATC) were significantly lower than those in high-grade tumors (LCNEC, SCLC). These observations are in keeping with previous studies on *RASSF1* gene silencing in NET of the lung, documenting increased levels of promoter 1 hypermethylation in most SCLC and ATC when compared to pulmonary TC (1, 27-29).

We also found that the levels of *RASSF1A/E* mRNA, which are under the control of promoter 1, were globally down-regulated in the diverse categories of pulmonary NET when compared with the corresponding samples of paired nonneoplastic lung tissues or NSCLC, thereby establishing a close relationship between gene promoter hypermethylation and *RASSF1* gene silencing, and confirming once again a general function of the tumor suppressor gene for A/E isoform in the development of NET of the lung. The level of *RASSF1A/E* mRNA was more conserved in carcinoids than LCNEC/SCLC and particularly low in SCLC, where it was significantly lower than in LCNEC ( $p=0.041$ ), further supporting the functional role of tumor suppressor for *RASSF1A/E* isoform in NET with a fine modulation paralleling tumor grade.

Despite the striking hypermethylation of *RASSF1* promoter 1 and the global decrease of *RASSF1A/E* mRNA levels in NET, we found no correlation between methylation and mRNA expression levels in individual NET (except for a

marginal association in TC only). A possible explanation for this is that the quantitative extent of promoter methylation (as reflected by the median value of methylation used arbitrarily in our analysis) is not a significant determinant of gene silencing, and even small levels of methylation occurring in critical CpG islands may be sufficient to cause loss of mRNA expression. In this context, if the relationship of methylation and mRNA level is non-linear, it may be interpreted as insignificant. Similar results were also found in our series of NSCLC, thereby suggesting a non-linear relationship of promoter methylation and relevant mRNA expression when using a quantitative approach. Further investigations on a larger series of NET and NSCLC and different cut-off criteria are currently in progress in our laboratory to address this important issue.

Similarly, *RASSF1A* protein level showed variable correlation with methylation: Promoter 1 methylation was associated with *RASSF1A* protein loss in ATC and to some extent NSCLC, but not in any other NET. Furthermore, we did not identify an association between *RASSF1A* mRNA and protein level in any tumor type under evaluation. However, an inverse correlation of mRNA and *RASSF1A* protein was found in nonneoplastic lung tissue, supporting the belief that post-translational modifications may contribute to the *RASSF1A* protein level regulation (37, 52). However, we cannot completely exclude the possibility that our analysis of *RASSF1A* protein levels by immunofluorescence may have some technical limitations in terms of quantitative resolution, and also, this reagent is relatively new and has not been extensively thus far studied in lung cancer samples (53-56). Despite these limitations, the association between methylation and *RASSF1A* protein loss in ATC points to a function of the recessive oncogene for this isoform and a role for island 1 that has not previously been reported in pulmonary NET. Studies with human normal placenta reporting *RASSF1A* protein loss in association with hypermethylation have

Table V. Distribution of *RASSF1* isoform based on tumor to normal ratio according to the histological subtyping.

Tumor type	No. of cases	Isoforms A/E		<i>p</i> -Value	Isoform C		<i>p</i> -Value
		T:N ratio ≤1	T:N ratio >1		T:N ratio ≤1	T:N ratio >1	
TC	20	11	9		1	19	
ATC	11	3	8		0	11	
LCNEC	11	6	5		0	11	
SCLC	16	12	4	<0.001	5	11	<0.001
All NET	58	32	26		6	52	
NSCLC	20	7	13	0.0415	6	14	<0.001

TC: Typical carcinoid; ACT: atypical carcinoid; LCNEC: large cell neuroendocrine carcinoma; SCLC: small cell lung carcinoma; NET: neuroendocrine tumors; NSCLC: non-small cell lung carcinoma.

Table VI. Analysis of *RASSF1* mRNA in neuroendocrine lung tumors, NSCLC and nonneoplastic lung tissue.

Variable	TC (n=20)	ATC (n=11)	<i>p</i> -Value (CT vs. ATC)	LCNEC (n=11)	SCLC (n=16)	<i>p</i> -Value (LCNEC vs. SCLC)	All NET (n=58)	NSCLC (n=20)	<i>p</i> -Value (All NET vs. NSCLC)	Nonneoplastic lung tissue
RASSF1 A/E	1.2	0.9	0.344	1.0	0.8	0.687	0.9	2.5	0.001	1.1
RASSF1 A/E, ratio T:N	0.8	1.2	0.563	1.0	0.5	0.041	0.8	1.8	-	-
RASSF1 C	3.5	3.2	0.234	1.9	1.3	0.223	2.1	1.5	0.198	0.6
RASSF1 C, ratio T:N	4.7	6.1	0.190	3.0	2.6	0.355	3.9	2.2	-	-

All data are expressed as  $2^{-\Delta\Delta C_T}$  threshold; median values were taken into account for the analysis; TC: typical carcinoid; ATC: atypical carcinoid; LCNEC: large cell neuroendocrine carcinoma; SCLC: small cell lung carcinoma; NSCLC: non-small cell lung carcinoma.

Table VII. Comparison of *RASSF1* FISH analysis of neuroendocrine tumors (NET), NSCLC and nonneoplastic lung tissue.

Variable	GCN <1.5 (%)	GCN >1.5 (%)	<i>p</i> -Value	Median GCN (range)	RASSF1:CEP3 ratio (range)
NET (n=58)	33 (57%)	25 (43%)		1.49 (0.93-1.9)	0.78 (0.35-1.0)
NSCLC (n=20)	10 (50%)	10 (50%)		1.51 (0.62-1.75)	0.54 (0.39-0.96)
Nonneoplastic lung tissue (n=58)	0 (0%)	58 (100%)	<0.001	1.9 (1.6-2.33)	0.94 (0.78-1.2)

GCN: Gene copy number; NSCLC: non-small cell lung carcinoma.

explored island 2 (54), as have most of the published studies on NET at different anatomical sites, with similar findings in terms of hypermethylation across the diverse histologies (1, 5, 9, 27-37, 57). An inverse functional correlation with corresponding mRNA isoform levels has been found for island 2 hypermethylation in SCLC (1) and corresponding tumor cell lines (1, 5), in human normal placenta (54), in several malignant pediatric tumors and derived tumor cell lines (36) and in pancreatic cancer cell lines (58), but not in esophageal carcinoma (59). As discussed earlier, we did not find a relationship between hypermethylation of either island 1 or island 2 and decrease in *RASSF1A/E* mRNA content in the NET, aside from a marginal association in TC for island 2 ( $r=-0.361, p=0.118$ ). These partially discrepant results could be due to the different methods used for the assays in different studies (1, 5).

In NET at different anatomical sites (30-33, 35) and in many pediatric malignant tumors with neuroendocrine differentiation (36, 37), epigenetic silencing of *RASSF1* gene may also be caused by allelic loss of its locus at 3p21.3, a chromosomal region harboring several tumor suppressor genes (52, 60). In our study, 57% of NET exhibited fewer than 1.5 GCN per cell, indicative of monosomy, whereas the remaining 43% comprised values between 1.5 and 1.9 indicative of wider dispersion of losses along chromosome 3p that variably includes *RASSF1* gene. These alterations were found to be independent of tumor type, whether neuroendocrine or common lung carcinoma, confirming the close relationship between 3p21.3 alterations and lung epithelial cell carcinogenesis (52, 60). Interestingly, increased FISH signals per cell correlated positively with *RASSF1A/E* mRNA and protein expression

in ATC, suggesting that in addition to regulation by methylation, *RASS1A/E* mRNA and RASSF1 A protein expression may also be regulated by 3p21.3 allelic loss in ATC. This is in accordance with a two-hit mechanism of *RASSF1* inactivation whereby either methylation or 3p21.3 loss are involved in silencing of *RASSF1* gene expression in NET of the lung. A role for *RASSF1A* isoform silencing by gene promoter hypermethylation and/or allelic loss of its locus at 3p21.3 (9, 10) has also been invoked for the development (30-33) and progression (30, 31, 33) of most foregut-derived NET, sporadic and MEN2-associated pheochromocytoma and abdominal paraganglioma (34, 35), and several types of pediatric malignant neoplasm, including rhabdomyosarcoma, medulloblastoma, retinoblastoma and neuroblastoma (36, 37). In all these tumor types, *RASSF1A* isoform is likely to serve as a tumor suppressor gene, leading to impaired apoptosis (4), increased cell migration (11, 12) and increased proliferation rate (13-15). This observation is not surprising because 3p21.3 is known to harbor a tumor suppressor gene cluster whose alterations have been found in most types of human cancer (52, 60).

The differential distribution of *RASSF1* gene promoter 1 hypermethylation across the whole spectrum of pulmonary NET we documented in our investigation probably reflects a specific change contributing to the development of these tumors rather than a simple epiphenomenon associated with pulmonary epithelial cell carcinogenesis, inasmuch as it was less demonstrable in common types of lung carcinomas. A practical application of this finding could be a diagnostic one, for example when experiencing difficulties in differentiating low- to intermediate-grade carcinoids from high-grade neuroendocrine carcinomas, which require completely different therapeutic approaches, in the event of severe crush artifacts in small biopsy samples (61). In addition, distinction of basaloid squamous cell carcinoma from SCLC can present a diagnostic challenge with standard morphological approaches. Analysis of *RASSF1* promoter methylation could service as a useful ancillary tool in this differential diagnosis.

Our investigation showed that promoter 2 was never hypermethylated in NET, NSCLC and paired nonneoplastic lung tissue samples, in accordance with previously reported data indicating that none of the SCLC samples thus far examined showed evidence of CpG island methylation of *RASSF1C* (1, 5, 27). Accordingly, increased levels of *RASSF1C* isoform mRNA were found in all types of lung carcinomas we examined in comparison with nonneoplastic tissue samples, in keeping with the hypothesis that *RASSF1C* isoform could affect lung carcinoma development according to a function of the dominant oncogene, independently of the occurrence of a neuroendocrine differentiation. Interestingly, this dual functions have been described in other genes too, such as retinoblastoma – a prototypical tumor suppressor

gene, which in some settings can act as an oncogene by blocking cell differentiation, as has been described for erythroid differentiation in cultured mouse erythroleukemia cell lines (62).

Another original aspect of our investigation regards the up-regulation of *RASSF1C* transcript as an adverse prognostic factor in the group of high-grade NET, including LCNEC and SCLC, for both overall (HR: 2.271;  $p=0.005$ ) and disease-free survival (HR: 1.763;  $p=0.0169$ ), independently of tumor stage. These findings are in accordance with a function of the dominant oncogene for *RASSF1C* favoring the progression and impairing the survival of patients with pulmonary high-grade neuroendocrine carcinomas as previously reported in other tumor tissues (7, 17, 18). Increased hypermethylation of the island 1 or island 2 was also marginally associated with overall survival in the group of patients with high-grade NET, with HR 1.036 (95% CI: 0.995-1.079,  $p=0.088$ ) for the former and HR 1.040 (95% CI: 0.995-1.087,  $p=0.084$ ) for the latter, suggesting a role of a tumor suppressor gene for *RASSF1A/E* isoforms as previously indicated for SCLC (5).

In conclusion, our study supports the view that the *RASSF1* gene plays an important role in the development and progression of pulmonary NET, with dual function as tumor suppressor gene in all types of NET for *RASSF1A/E* isoform and as oncogene impairing patients' survival for *RASSF1C* isoform in high-grade NET.

## Acknowledgements

This work was supported by Associazione Italiana per la Ricerca sul Cancro and is dedicated to the memory of Carlotta, an extraordinarily lively girl who untimely died of cancer in the prime of life.

## References

- 1 Dammann R, Li C, Yoon JH, Chin PL, Bates S and Pfeifer GP: Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet* 25: 315-319, 2000.
- 2 Lerman MI and Minna JD: 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, 2000.
- 3 Donninger H, Vos MD and Clark GJ: The *RASSF1A* tumor suppressor. *J Cell Sci* 120: 3163-3172, 2007.
- 4 van der Weyden L and Adams D: The Ras-association domain family (RASSF) members and their role in human tumorigenesis. *Biochim Biophys Acta* 1776: 58-85, 2007.
- 5 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, Zbarovsky E, White M and Minna JD: Epigenetic inactivation of *RASSF1A* in lung and breast cancers and malignant phenotype suppression. *J Natl Cancer Inst* 93: 691-699, 2001.

- 6 Dammann R, Strunnikova M, Schagdarsurengin U, Rastetter M, Papritz M, Hattenhorst UE, Hofmann HS, Silber RE, Burdach S and Hansen G: CpG island methylation and expression of tumour-associated genes in lung carcinoma. *Eur J Cancer* 41: 1223-1236, 2005.
- 7 Amaar YG, Baylink DJ and Mohan S: Ras-association domain family 1 protein, RASSF1C, is an IGFBP-5 binding partner and a potential regulator of osteoblast cell proliferation. *J Bone Miner Res* 20: 1430-1439, 2005.
- 8 Agathangelou A, Cooper WN and Latif F: Role of the Ras-association domain family 1 tumor suppressor gene in human cancers. *Cancer Res* 65: 3497-3508, 2005.
- 9 Agathangelou 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 and Latif F: Methylation associated inactivation of *RASSF1A* from region 3p21.3 in lung, breast and ovarian tumours. *Oncogene* 20: 1509-1518, 2001.
- 10 Pfeifer GP and Dammann R: Methylation of the tumor suppressor gene *RASSF1A* in human tumors. *Biochemistry (Mosc)* 70: 576-583, 2005.
- 11 Dallol A, Agathangelou A, Tommasi S, Pfeifer GP, Maher ER and Latif F: Involvement of the *RASSF1A* tumor suppressor gene in controlling cell migration. *Cancer Res* 65: 7653-7659, 2005.
- 12 Dallol A, Agathangelou A, Fenton SL, Ahmed-Choudhury J, Hesson L, Vos MD, Clark GJ, Downward J, Maher ER and Latif F: RASSF1A interacts with microtubule-associated proteins and modulates microtubule dynamics. *Cancer Res* 64: 4112-4116, 2004.
- 13 Shivakumar L, Minna J, Sakamaki T, Pestell R and White MA: The RASSF1A tumor suppressor blocks cell cycle progression and inhibits cyclin D1 accumulation. *Mol Cell Biol* 22: 4309-4318, 2002.
- 14 Song MS, Song SJ, Ayad NG, Chang JS, Lee JH, Hong HK, Lee H, Choi N, Kim J, Kim H, Kim JW, Choi EJ, Kirschner MW and Lim DS: The tumour suppressor RASSF1A regulates mitosis by inhibiting the APC-Cdc20 complex. *Nat Cell Biol* 6: 129-137, 2004.
- 15 Rong R, Jin W, Zhang J, Sheikh MS and Huang Y: Tumor suppressor RASSF1A is a microtubule-binding protein that stabilizes microtubules and induces G<sub>2</sub>/M arrest. *Oncogene* 23: 8216-8230, 2004.
- 16 Li J, Wang F, Protopopov A, Malyukova A, Kashuba V, Minna JD, Lerman MI, Klein G and Zbarovsky E: Inactivation of *RASSF1C* during *in vivo* tumor growth identifies it as a tumor suppressor gene. *Oncogene* 23: 5941-5949, 2004.
- 17 Amaar YG, Minera MG, Hatran LK, Strong DD, Mohan S and Reeves ME: Ras association domain family 1C protein stimulates human lung cancer cell proliferation. *Am J Physiol Lung Cell Mol Physiol* 291: L1185-1190, 2006.
- 18 Estrabaud E, Lassot I, Blot G, Le Rouzic E, Tanchou V, Quemeneur E, Daviet L, Margottin-Goguet F and Benarous R: RASSF1C, an isoform of the tumor suppressor RASSF1A, promotes the accumulation of beta-catenin by interacting with betaTrCP. *Cancer Res* 67: 1054-1061, 2007.
- 19 Travis W, Brambilla E, Muller-Hermelink H and Harris C: *Tumours of the Lung, Pleura, Thymus and Heart*. Lyon: IARC Press, pp. 344, 2004.
- 20 Onuki N, Wistuba I, Travis W, Virmani A, Yashima K, Brambilla E, Hasleton P and Gazdar A: Genetic changes in the spectrum of neuroendocrine lung tumors. *Cancer* 85: 600-607, 1999.
- 21 Padberg B, Woenckhaus J, Hilger G, Beccu L, Jochum W, Range U, Kastendieck H and Schroder S: DNA cytophotometry and prognosis in typical and atypical bronchopulmonary carcinoids. A clinicomorphologic study of 100 neuroendocrine lung tumors. *Am J Surg Pathol* 20: 815-822, 1996.
- 22 Petzmann S, Ullmann R, Klemen H, Renner H and Popper H: Loss of heterozygosity on chromosome arm 11q in lung carcinoids. *Hum Pathol* 32: 333-338, 2001.
- 23 Przygodzki R, Finkelstein S, Langer J, Swalsky P, Fishback N, Bakker A, Guinee D, Koss M and Travis W: Analysis of p53, K-ras-2, and C-raf-1 in pulmonary neuroendocrine tumors. Correlation with histological subtype and clinical outcome. *Am J Pathol* 148: 1531-1541, 1996.
- 24 Walch A, Zitzelsberger RH, Aubele M, Mattis A, Bauchinger M, Candidus S, Prauer H, Werner M and Hofler H: Typical and atypical carcinoid tumors of the lung are characterized by 11q deletions as detected by comparative genomic hybridization. *Am J Pathol* 153: 1089-1098, 1998.
- 25 Beasley M, Thunnissen F, Brambilla E, Hasleton P, Steele R, Hammar S, Colby T, Sheppard M, Shimosato Y, Koss M, Falk R and Travis W: Pulmonary atypical carcinoid: predictors of survival in 106 cases. *Hum Pathol* 31: 1255-1265, 2000.
- 26 Garcia-Yuste M, Matilla J, Alvarez-Gago T, Duque J, Heras F, Cerezal L and Ramos G: Prognostic factors in neuroendocrine lung tumors: a Spanish Multicenter Study. Spanish Multicenter Study of Neuroendocrine Tumors of the Lung of the Spanish Society of Pneumology and Thoracic Surgery (EMETNE-SEPAR). *Ann Thorac Surg* 70: 258-263, 2000.
- 27 Dammann R, Takahashi T and Pfeifer GP: The CpG island of the novel tumor suppressor gene *RASSF1A* is intensely methylated in primary small cell lung carcinomas. *Oncogene* 20: 3563-3567, 2001.
- 28 Toyooka S, Toyooka KO, Maruyama R, Virmani AK, Girard L, Miyajima K, Harada K, Ariyoshi Y, Takahashi T, Sugio K, Brambilla E, Gilcrease M, Minna JD and Gazdar AF: DNA methylation profiles of lung tumors. *Mol Cancer Ther* 1: 61-67, 2001.
- 29 Leotlela PD, Jauch A, Holtgreve-Grez H and Thakker RV: Genetics of neuroendocrine and carcinoid tumours. *Endocr Relat Cancer* 10: 437-450, 2003.
- 30 Pizzi S, Azzoni C, Bottarelli L, Campanini N, D'Adda T, Pasquali C, Rossi G, Rindi G and Bordi C: *RASSF1A* promoter methylation and 3p21.3 loss of heterozygosity are features of foregut, but not midgut and hindgut, malignant endocrine tumours. *J Pathol* 206: 409-416, 2005.
- 31 Zhang HY, Rumilla KM, Jin L, Nakamura N, Stilling GA, Ruebel KH, Hobday TJ, Erlichman C, Erickson LA and Lloyd RV: Association of DNA methylation and epigenetic inactivation of RASSF1A and beta-catenin with metastasis in small bowel carcinoid tumors. *Endocrine* 30: 299-306, 2006.
- 32 House MG, Herman JG, Guo MZ, Hooker CM, Schulick RD, Lillemo KD, Cameron JL, Hruban RH, Maitra A and Yeo CJ: Aberrant hypermethylation of tumor suppressor genes in pancreatic endocrine neoplasms. *Ann Surg* 238: 423-431; discussion 431-422, 2003.
- 33 Liu L, Broaddus RR, Yao JC, Xie S, White JA, Wu TT, Hamilton SR and Rashid A: Epigenetic alterations in neuroendocrine tumors: methylation of RAS-association domain family 1, isoform A and *p16* genes are associated with metastasis. *Mod Pathol* 18: 1632-1640, 2005.



- 34 Geli J, Kiss N, Lanner F, Foukakis T, Natalishvili N, Larsson O, Kogner P, Hoog A, Clark GJ, Ekstrom TJ, Backdahl M, Farnebo F and Larsson C: The Ras effectors *NORE1A* and *RASSF1A* are frequently inactivated in pheochromocytoma and abdominal paraganglioma. *Endocr Relat Cancer* 14: 125-134, 2007.
- 35 Dammann R, Schagdarsurengin U, Seidel C, Trumpler C, Hoang-Vu C, Gimm O, Dralle H, Pfeifer GP and Brauckhoff M: Frequent promoter methylation of tumor-related genes in sporadic and MEN2-associated pheochromocytomas. *Exp Clin Endocrinol Diabetes* 113: 1-7, 2005.
- 36 Harada K, Toyooka S, Maitra A, Maruyama R, Toyooka KO, Timmons CF, Tomlinson GE, Mastrangelo D, Hay RJ, Minna JD and Gazdar AF: Aberrant promoter methylation and silencing of the *RASSF1A* gene in pediatric tumors and cell lines. *Oncogene* 21: 4345-4349, 2002.
- 37 Harada K, Toyooka S, Shivapurkar N, Maitra A, Reddy JL, Matta H, Miyajima K, Timmons CF, Tomlinson GE, Mastrangelo D, Hay RJ, Chaudhary PM and Gazdar AF: Deregulation of caspase 8 and 10 expression in pediatric tumors and cell lines. *Cancer Res* 62: 5897-5901, 2002.
- 38 Uhlmann K, Brinckmann A, Toliat MR, Ritter H and Nurnberg P: Evaluation of a potential epigenetic biomarker by quantitative methyl-single nucleotide polymorphism analysis. *Electrophoresis* 23: 4072-4079, 2002.
- 39 Colella S, Shen L, Baggerly KA, Issa JP and Krahe R: Sensitive and quantitative universal pyrosequencing methylation analysis of CpG sites. *Biotechniques* 35: 146-150, 2003.
- 40 Dupont JM, Tost J, Jammes H and Gut IG: *De novo* quantitative bisulfite sequencing using the pyrosequencing technology. *Anal Biochem* 333: 119-127, 2004.
- 41 Ronaghi M: Pyrosequencing sheds light on DNA sequencing. *Genome Res* 11: 3-11, 2001.
- 42 Tost J, Dunker J and Gut IG: Analysis and quantification of multiple methylation variable positions in CpG islands by pyrosequencing. *Biotechniques* 35: 152-156, 2003.
- 43 Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH and Issa JP: A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Res* 32: e38, 2004.
- 44 Li LC and Dahiya R: MethPrimer: designing primers for methylation PCRs. *Bioinformatics* 18: 1427-1431, 2002.
- 45 Cohen Y, Singer G, Lavie O, Dong SM, Beller U and Sidransky D: The *RASSF1A* tumor suppressor gene is commonly inactivated in adenocarcinoma of the uterine cervix. *Clin Cancer Res* 9: 2981-2984, 2003.
- 46 Topaloglu O, Hoque MO, Tokumaru Y, Lee J, Ratovitski E, Sidransky D and Moon CS: Detection of promoter hypermethylation of multiple genes in the tumor and bronchoalveolar lavage of patients with lung cancer. *Clin Cancer Res* 10: 2284-2288, 2004.
- 47 Xing M, Cohen Y, Mambo E, Tallini G, Udelsman R, Ladenson PW and Sidransky D: Early occurrence of *RASSF1A* hypermethylation and its mutual exclusion with *BRAF* mutation in thyroid tumorigenesis. *Cancer Res* 64: 1664-1668, 2004.
- 48 Lichter P, Tang C, Call K, Hermanson G, Evans G, Housman D and Ward D: High-resolution mapping of human chromosome 11 by *in situ* hybridization with cosmid clones. *Science* 247: 64-69, 1990.
- 49 Ma Y, Lespagnard L, Durbecq V, Paesmans M, Desmedt C, Gomez-Galdon M, Veys I, Cardoso F, Sotiriou C, Di Leo A, Piccart M and Larsimont D: Polysomy 17 in HER-2/neu status elaboration in breast cancer: effect on daily practice. *Clin Cancer Res* 11: 4393-4399, 2005.
- 50 Kaplan E and Meier P: Nonparametric estimation from incomplete observations. *Am Stat Assoc J* 53: 457-481, 1958.
- 51 Mantel N and Haenszel W: Statistical aspects of the analysis of data from retrospective studies of disease. *J Natl Cancer Inst* 22: 719-748, 1959.
- 52 Ji L, Minna JD and Roth JA: 3p21.3 Tumor suppressor cluster: prospects for translational applications. *Future Oncol* 1: 79-92, 2005.
- 53 Bennett KL, Romigh T and Eng C: AP-2alpha induces epigenetic silencing of tumor suppressive genes and microsatellite instability in head and neck squamous cell carcinoma. *PLoS One* 4: e6931, 2009.
- 54 Chiu RW, Chim SS, Wong IH, Wong CS, Lee WS, To KF, Tong JH, Yuen RK, Shum AS, Chan JK, Chan LY, Yuen JW, Tong YK, Weier JF, Ferlatte C, Leung TN, Lau TK, Lo KW and Lo YM: Hypermethylation of *RASSF1A* in human and rhesus placentas. *Am J Pathol* 170: 941-950, 2007.
- 55 Thaler S, Hahnel PS, Schad A, Dammann R and Schuler M: *RASSF1A* mediates p21/Cip1/Waf1-dependent cell cycle arrest and senescence through modulation of the Raf-MEK-ERK pathway and inhibition of Akt. *Cancer Res* 69: 1748-1757, 2009.
- 56 Wang X, Di K, Zhang X, Han H, Wong Y, Leung S and Ling M: Id-1 promotes chromosomal instability through modification of APC/C activity during mitosis in response to microtubule disruption. *Oncogene* 27: 4456-4466, 2008.
- 57 Zochbauer-Muller S, Fong K, Maitra A, Lam S, Geradts J, Ashfaq R, Virmani A, Milchgrub S, Gazdar A and Minna J: 5' CpG island methylation of the *FHIT* gene is correlated with loss of gene expression in lung and breast cancer. *Cancer Res* 61: 3581-3585, 2001.
- 58 Dammann R, Schagdarsurengin U, Liu L, Otto N, Gimm O, Dralle H, Boehm BO, Pfeifer GP and Hoang-Vu C: Frequent *RASSF1A* promoter hypermethylation and *KRAS* mutations in pancreatic carcinoma. *Oncogene* 22: 3806-3812, 2003.
- 59 Kuroki T, Trapasso F, Yendamuri S, Matsuyama A, Alder H, Mori M and Croce CM: Allele loss and promoter hypermethylation of *VHL*, *RAR-beta*, *RASSF1A*, and *FHIT* tumor suppressor genes on chromosome 3p in esophageal squamous cell carcinoma. *Cancer Res* 63: 3724-3728, 2003.
- 60 Hesson LB, Cooper WN and Latif F: Evaluation of the 3p21.3 tumour-suppressor gene cluster. *Oncogene* 26: 7283-7301, 2007.
- 61 Pelosi G, Rodriguez J, Viale G and Rosai J: Typical and atypical pulmonary carcinoid tumor overdiagnosed as small cell carcinoma on biopsy specimens: a major pitfall in the management of lung cancer patients. *Am J Surg Pathol* 29: 179-187, 2005.
- 62 Rekhtman N, Choe KS, Matushansky I, Murray S, Stopka T and Skoultchi AI: PU.1 and pRB interact and cooperate to repress GATA-1 and block erythroid differentiation. *Mol Cell Biol* 23: 7460-7474, 2003.

Received July 4, 2010  
Revised September 1, 2010  
Accepted September 7, 2010