Endocrine (2025) 87:1246–1256
https://doi.org/10.1007/s12020-024-04113-7

ORIGINAL ARTICLE

Aberrant promoter methylation, expression and function of *RASSF1A* gene in a series of Italian parathyroid tumors

Chiara Verdelli[1] · Federico Pio Fabrizio[2,3] · Paola Maroni[1] · Annamaria Morotti[4,5] · Giulia Stefania Tavanti[1,6] · Silvia Carrara[7] · Vito Guarnieri[8] · Filomena Cetani[9] · Alfredo Scillitani[10] · Riccardo Maggiore[11] · Francesca Perticone[12] · Valentina Vaira[4,5] · Lucia Anna Muscarella[2] · Sabrina Corbetta[6,13]

Received: 13 October 2024 / Accepted: 13 November 2024 / Published online: 28 November 2024

Abstract

Purpose Aberrant epigenetic features are key events involved in parathyroid tumorigenesis, including DNA methylation, histone methylation, and non-coding RNAs. Ras Association Domain Family Protein1 Isoform A (RASSF1A) and Adenomatous Polyposis of Colon (APC) are frequently downregulated in human cancers. Here, we investigated their deregulated expression and the potential role in parathyroid neoplasms.

Methods methylation of *RASSF1A* and *APC* promoters was analyzed in a series of parathyroid adenomas (PAds, n = 80) and parathyroid carcinomas (PCas, n = 9) from Italian patients with primary hyperparathyroidism,

Results *RASSF1A* and *APC* promoter methylation occurred in about 90% of PAds samples. PCas displayed *RASSF1A* promoter methylation, while *APC* promoter was methylated only in 2 samples. Of note, *RASSF1A* promoter methylation negatively correlated with PAds tumor size. However, *RASSF1A* transcript and protein levels were reduced in PAds and PCas compared with parathyroid normal glands. Investigating the potential mechanism involved in *RASSF1A* promoter methylation, we found that DNA methyltransferases (DNMTs) activity was variable in PAds and inversely correlated with RASSF1A protein levels. In addition, the *RASSF1A* promoter methylation negatively correlated with long-non-coding Antisense Intronic Noncoding RASSF1A (*ANRASSF1A*) mRNA levels, excluding the involvement of *ANRASSF1* in RASSF1A regulation. In HEK293A cells transfected with the calcium sensing receptor (CASR), loss of RASSF1A increased basal phosphorylated Extracellular signal-regulated kinase (pERK/ERK) levels blunting the CASR-induced increases.

Conclusion *RASSF1A* and *APC* promoter methylation is a hallmark of parathyroid tumors; deregulation of DNMTs activity contributes to modulation of *RASSF1A* expression. Loss of RASSF1A may be involved in the tuning of ERK pathway in parathyroid tumors.

Keywords parathyroid tumors · APC · RASSF1 · PTH · DNMTs · DNA Methylation

Introduction

Parathyroid tumors are the second most common endocrine neoplasia following thyroid tumors. They are mostly benign, and while malignancy is rare, can be fatal in at least half of cases. Epigenetic aberrations are frequent in parathyroid tumors, some of which are shared with the most common human cancers, though the effects in parathyroid tumorigenesis are not defined. Global methylation was similar in parathyroid tumors and normal parathyroid glands, while global hypermethylation has been reported in type 1 multiple endocrine neoplasia (MEN1)-related parathyroid tumors [1]. Moreover, a 5-hydroxymethylcytosine (5mC), marker of DNA demethylation by the ten-eleven translocation (TET) family of methylcytosine hydroxylases, was reduced in parathyroid adenomas (PAds) and absent in parathyroid carcinomas (PCas) when compared with normal parathyroid glands (PaNs) [2]. Methylation of cytosine to 5mC is a central epigenetic modification that feeds back on

These authors contributed equally: Chiara Verdelli, Federico Pio Fabrizio

These authors jointly supervised this work: Lucia Anna Muscarella, Sabrina Corbetta

✉ Sabrina Corbetta
sabrina.corbetta@unimi.it

Extended author information available on the last page of the article

Table 1 Clinical and hormonal characteristics of the first series of parathyroid tumors investigated for the *RASSF1A* and *APC* promoter methylation

Histotype	n	Sex F/M	Age Years	*CDC73* mutations	Parafibromin Loss IHC	S Ca mg/dl	PTH pg/ml	Tumor size cm
PCas	9	4/5	44.9 ± 3.7	2/7	6	12.6 ± 0.4	369.5 ± 61.5	2.7 ± 0.3
PAts	3	3/0	50.9 ± 5.4	0/3	-	11.5 ± 0.2	280.1 ± 75.5	2.5 ± 0.4
PAds	80	62/18	57.6 ± 2.3	-	-	11.7 ± 0.2	335.9 ± 52.0	2.7 ± 0.3*
PaNs	3	3/0	-	-	-	-	-	-

cellular processes including genome regulation, organism development, and disease [3]. Alterations in the methylation of the promoters of some genes have been reported in parathyroid tumors; in particular, *RASSF1A* and *APC* promoters, which are unmethylated in normal parathyroid tissue, have been found variably methylated [4]. However, the promoter methylation of specific parathyroid genes such as those encoding for parathormone (*PTH*), calcium-sensing receptor (*CASR*), tumor oncosuppressors *MEN1* and *CDC73* in parathyroid tumors has been found similar to that detected in normal parathyroid tissue [4].

The human genome has ten genes belonging to the Ras Association domain Family (RASSF). RASSF is made up of two subclasses, C-RASSF and N-RASSF, coding both for proteins containing the Ras association binding domain and frequently suppressed by DNA hypermethylation in human cancers [3]. The tumor suppressor gene *RASSF1A* encodes a microtubule-associated and multitasking scaffold protein communicating with the RAS pathway, estrogen receptor signaling, and Hippo pathway [3]. In addition, RASSF1A controls cell cycle and cell migration by interacting with APC, an inhibitory component of the WNT/β-catenin pathway [5], whose inactivating mutations characterize colon cancer. Loss of either RASSF1A or APC is associated with the activation of the WNT/β-catenin pathway [6, 7]. Promoter methylation of *RASSF1A* and/or *APC* genes is a hallmark of human neoplasia, and it has also been described in parathyroid tumors, though its role in parathyroid tumorigenesis has never been investigated.

In the present study, methylation of the promoter of *RASSF1A* and *APC* genes was investigated in an Italian series of parathyroid tumors compared with normal parathyroid glands. Moreover, we tested the hypothesis that the DNA methyltransferases (DNMTs) and /or the long non-coding RNA *ANRASSF1A* are involved in the *RASSF1A* promoter methylation in parathyroid tumors. Finally, we provided evidence suggesting that *RASSF1A* may modulate CASR-stimulated intracellular ERK levels in parathyroid tumor cells.

Materials and methods

Parathyroid tumor samples

The DNA obtained from a series of 3 PaNs incidentally removed from normocalcemic patients that had undergone surgery for thyroid disease, 9 parathyroid carcinomas (PCas), 3 atypical parathyroid adenomas (PAts), 80 parathyroid adenomas (PAds) samples were analyzed by quantitative methylation-specific PCR (Table 1). Histological diagnosis of PCas and aPAds was established according to WHO guideline [8].

Data are expressed as mean ± SEM. n, sample size; F, females; M, males; age, age at diagnosis; *CDC73* mutations, number of patients harboring inactivating mutation of *CDC73* gene/number of patients harboring wildtype allele; Parafibromin IHC negativity, number of FFPE samples with negative immunostaining for Parafibromin; S Ca, serum albumin-corrected calcium levels at diagnosis; PTH, plasma PTH levels at diagnosis were measured by different assay (reference range: 10–65 pg/ml for each specific assay); Tumor size, maximum diameter of fresh surgically removed parathyroid tumors recorded by pathologists; PCas, parathyroid carcinomas; PAts, atypical parathyroid adenomas; PAds, sporadic parathyroid adenomas; PaNs, normal parathyroid glands incidentally removed from normocalcemic patients during thyroid surgery. *, tumor size was available only for 20 PAds.

A second independent series of 3 PaNs, 7 PCas, 6 PAts, and 35 PAds, was analyzed for gene expression, whose clinical and hormonal features were similar to those of the first series and previously published in Verdelli et al. [9]. Both tumor sample series were collected from patients affected with primary hyperparathyroidism (PHPT) referred to the Endocrine Units of the third level centers IRCCS Casa Sollievo della Sofferenza in San Giovanni Rotondo, University Hospital in Pisa, and IRCCS Ospedale Galeazzi-Sant'Ambrogio/IRCCS Ospedale San Raffaele in Milan. The diagnosis of PHPT was based on increased ionized or albumin-corrected serum calcium and increased or inappropriately normal intact PTH levels [10]. Fasting serum total and ionized calcium were measured by a multichannel autoanalyzer. Plasma PTH was measured by second-generation assays among the different centers (reference range: 10–65 pg/ml for each specific assay).

This research was performed in accordance with the World Medical Association Declaration of Helsinki. The study was approved by an Institutional Ethical Committee (Ospedale San Raffaele Ethical Committee, protocol no. GPRC6A PARA, 07/03/2019; CE40/2019), and informed consent was obtained from all patients.

DNA extraction and quantification

DNA was extracted from peripheral whole blood lymphocytes by automated EZ1 Bio-Robot (Qiagen) and quantified at the Nanodrop (Eppendorf).

Parathyroid tumor specimens obtained from the first series of PHPT patients were cut into 3-µm-thick Formalin-Fixed Paraffin-Embedded (FFPE) sections, which were previously fixed in neutral-buffer and successively stained with Hematoxilyn and Eosin (H&E) in order to establish tumor cellularity. DNA was enriched with manual microdissection from the corresponding unstained 12-µm-thick section and was isolated by using GeneRead FFPE Kit (Qiagen, Germantown, MD, USA) following the manufacturer's instructions [11]. For DNA extracted from peripheral blood withdrawal, the classic salting in-out protocol was applied. DNA quantification and purity was analyzed by NanoDrop™ 1000 Spectrophotometer (Thermo Scientific).

DNA bisulfite treatment and quantitative methylation-specific PCR (QMSP) analysis

DNA was preliminarily subjected to bisulfite conversion and purification by using Epitect Bisulfite kit (QiagenSci, MD, USA) according to manufacturer's instruction. Bisulfite-converted genomic DNA was then amplified using QMSP. Primers/probe sets used to quantify methylation of *APC* and *RASSF1A* promoter regions were previously reported [12]: *APC* forward 5′-GAACCAAAACGCTC CCCAT-3′, *APC* reverse 5′-TTATATGTGGTTAGGTGCG TTTATAT-3′ and *APC* probe FAM-CCCGTCGAAAA CCCGCCGATTA-TAMRA; *RASSF1A* forward 5′-GCG TTGAAGTCGGGGTTC-3′, *RASSF1A* reverse 5′-CCC GTACTTCGCTAACTTTAAACG-3′ and *RASSF1A* probe FAM-ACAAACGCGAACCGAACGAAACCA-TAMRA. As reference, a primer/probe set was specifically designed to cover the unmethylated promoter region of the β-Actin (*ACTB*) gene: forward 5′-TGGTGATGGAGGAGGTT-TAGTAAGT-3′, reverse 5′-AACCAATAAAACCTAC TCCTCCCTTAA-3′ and probe FAM-ACCACCACCCAA CACACAATAACAAACACA-TAMRA. *APC* and *RASS F1A* methylation levels were assessed by using a relative quantification method with a standard curve. Each calibration curve was obtained from ten-fold dilutions (50–0.05 ng) of commercially available fully methylated DNA (CpGenome Universal Methylated DNA, Millipore, Bedford, MA, USA). Reactions were made in triplicate using 50 ng of bisulfite-modified DNA aliquoted in 384-well plates and were run on ABI PRISM 7900 Sequence detection system, using SDS 2.4.1 as analysis software (Thermo Fisher Inc., Applied Biosystems Division). The relative level of methylated DNA was finally calculated as target gene/ACTBx1000 [13] and used as log2 transformed. The cut-off level for hypermethylation was arbitrary set at ≥5.0.

Gene expression

Total RNA was isolated using TRIzol Reagent (Ambion, Thermo Fisher Scientific), and 1 µg of RNA was digested with DNase I (Thermo Fisher Scientific). 300 ng of DNA-free RNA were reverse transcribed to cDNA using iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Real-time PCR (qRT-PCR) was performed using TaqMan gene expression assay and StepOne Plus PCR System with the following assays: *RASSF1A* Hs00200394_m1, *AN-RASSF1A* Hs04402917_s1, Hydroxymethylbilane synthase (*HMBS*) Hs00609297_m1 and Beta-2-Microglobulin (*B2M*) Hs99999907_m1. The reference genes *HMBS* and *B2M* were used as an internal control for relative quantification using the comparative Ct method. Then, raw data were median-normalized and log2 transformed.

Immunohistochemistry

Samples were collected from 6 PAds, 3 PCas, and 3 PaNs incidentally removed from normocalcemic patients treated with thyroid surgery. Diagnosis of PCas was performed according to World Health Organization guidelines [8]. After antigen retrieval, FFPE parathyroid sections were incubated overnight at 4 °C with a rabbit monoclonal antibody specific for RASSF1 (ab126764, Abcam). Immunostaining was performed with a streptavidin–biotin system (ABC kit, Santa-Cruz Biotechnology) and detected by diaminobenzidine (Novolink Polymer Detection System, Novocastra Laboratories, Leica Microsystems). Counterstaining was performed with Mayer's hematoxylin solution. Negative-control sections were subjected to the same staining procedure without the primary antibody. Immunoreactivity was checked by light microscopy (CKX41 Olympus, Olypus Co., Tokyo, Japan).

Cell cultures and transfections

The human embryonic kidney HEK293A cell line (Invitrogen, catalog n.R705- 07) was cultured in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, and 100 U/ml penicillin-streptomycin. For transfection experiments, cells were seeded in 6-well plates at 1.2×10^5 cells/well density without antibiotics. The day after, cells were transfected with a custom *RASSF1A*-directed siRNA (RASSF1A-1, GACCUCUGUGGCGACUUCAdTdT) or a control siRNA (ON-TARGET Plus Control Non-Targeting pool, D-001810-10-05) in Opti-MEM media (Gibco, ThermoFisher Scientific) using Dharmafect1 (T-2001-02) as a

transfection reagent. All reagents used for *RASSF1A* silencing were purchased from Dharmacon. After 24 hours, cells were transiently transfected with a plasmid encoding for CASR, obtained by site-directed mutagenesis, as previously described [14]. Four micrograms of CASR plasmid were transfected using TurboFect Transfection Reagent (R0533, ThermoFisher) in DMEM serum-free medium, according to the manufacturer 's instructions and as previously described [15]. Preliminary experiments were performed to determine the optimal concentration of siRNA and to define the best timing for carrying out the co-transfection. After 48 hours, *CASR* and *RASSF1* expression levels were analyzed by qRT-PCR and western blot to verify the up-regulation and the downregulation of CASR and RASSF1 expression levels, respectively.

Treatments of CASR-HEK293A silenced for *RASSF1A* with R568

Forty-eight hours after CASR transfection and 72 hours after *RASSF1A* silencing, co-transfected HEK293A cells were treated with an increasing concentration of R568 (Cayman Chemical Company), a CASR agonist, for 10 minutes. Treatments were carried out in a physiological saline solution (PSS) (NaCl 125 mM, KCl 4 mM, HEPES 20 mM, D-Glucose 0.1%, NaH_2PO_4 0.8 mM, $MgCl_2$ 1 mM, pH 7.45), supplemented with 0.1% Bovine serum albumin (BSA) and in the presence of 1.5 mM extracellular calcium ($[Ca^{2+}]_o$). Before treatments, cells were serum starved for 24 hours, using a serum-free medium supplemented with 0.2% BSA and 1% L-Glutamine, and pre-treated for 30 minutes with physiological saline solution (PSS) added with Ca^{2+}. Cells were then harvested and lysed to perform analysis of total proteins or fractioned protein lysates. Untreated cells (NT) were used as controls.

Protein extraction and western blot analysis

Cells were homogenized using NP40 buffer (FNN0021, ThermoFisher Scientific) containing protease and phosphatase inhibitors to obtain total protein extracts. Nuclear extracts from cells were obtained using the Subcellular Protein Fractionation Kit (78840, ThermoFisher Scientific), while nuclear extracts from snap-frozen PAds tissues (n = 16) were obtained by using a Dounce homogenizer and the Subcellular Protein Fractionation Kit for Tissues (87790, ThermoFisher Scientific) following the manufacturer's instruction. Protein concentration was determined using the Pierce BCA (bicinchoninic acid) Protein assay kit (ThermoFisher Scientific). After separation by SDS-PAGE, polypeptides were electrophoretically transferred to nitrocellulose membranes (Bio-Rad), and membranes were incubated using the following primary antibodies: anti-RASSF1 (ab126764, Abcam), anti-CASR (ab19347, Abcam), phosphorylated ERK and total ERK (#4370S and #9107S, respectively, Cell Signaling). Anti-Vinculin (ab129002, Abcam) was used as a loading control for whole lysates. After the incubation with the appropriate horseradish-peroxidase (HRP)-conjugated secondary antibody-specific bands were visualized using Clarity Western Blot ECL with a ChemiDoc Imaging system (Bio-Rad). Densitometry was performed using ImageLab software (Bio-Rad).

DNA methyltransferases (DNMT) activity/inhibition assay

The DNMTs Activity/Inhibition Assay is a non-radioactive assay to measure the activity or inhibition of DNA methyltransferases 1, 3a, 3b (DNMT1, DNMT3a and DNMT3b; Catalog No. V13-55006, Vinci Biochem). The sensitive ELISA-based method utilizes the high-affinity binding of methyl CpG binding domain (MBD) protein towards methylated DNA in order to detect DNA methyltransferase activity on the provided CpG-enriched DNA substrate. The standard curve was prepared using the CpG methyltransferase enzyme provided by the kit as a positive control. The assay is quantified by spectrophotometry at 450 nm. For this experiment, nuclear extracts (10 µg) from PAds (n = 16) were prepared using the Subcellular Protein Fractionation Kit for Tissues (87790, ThermoFisher Scientific).

Statistical analysis

Data are presented as mean±standard error media (SEM). All data were checked for normality by D'Agostino and Pearson omnibus normality test. Data failing the test were normalized by log2 transformation. Comparisons among multiple parameters were analyzed by ordinary one-way ANOVA with Holm-Sidak correction for multiple comparisons. For each comparison the multiplicity adjusted P value was reported. Correlations between parameters were tested by Pearson correlation coefficients. Statistical analysis was performed by GraphPad Prism version 6.0 (GraphPad Software, La Jolla, California, USA).

Results

DNA promoter methylation of the *RASSF1A* gene in parathyroid tumors

The DNA promoter region of *RASSF1A* gene was unmethylated in PaNs (n = 3). In PCas (n = 9), *RASSF1A*

promoter region was variably methylated in all samples (100%) with methylation levels ranging 40.6 to 385.9. In PAds (n = 80), the methylation of *RASSF1A* promoter ranged 0.0–6384.0. PAts also showed *RASSF1A* promoter methylation in all 3 samples ranging 1807.0 to 3243.0 (Fig. 1a). The levels of DNA methylation were significantly higher in parathyroid tumors compared with normal samples, while mean *RASSF1A* promoter methylation level was lower in PCas compared with those detected in PAds and PAts.

DNA promoter methylation of the *APC* gene in parathyroid tumors

The DNA promoter region of *APC* gene was unmethylated in PaNs (n = 3). In PCas (n = 9), the *APC* promoter was demethylated in all samples but one (89%). By contrast, the *APC* promoter was variably methylated in most PAds (68 out of 80, 85%) ranging 0.0–17234.0. PAts showed a similar pattern of methylation as *APC* promoter was methylated in 2 out of 3 samples (Fig. 1b). Similar to what observed for the methylation status of the *RASSF1A* promoter, the mean level of DNA methylation was significantly higher in PAds compared with both PaNs and PCas samples.

Considering the PAds harboring the methylation of both *RASSF1A* and *APC* promoters (73 out of 80, 91%), a significant positive correlation between the DNA methylation levels of *RASSF1A* and those of *APC* gene promoters was detected ($r^2 = 0.287$, $P < 0.0001$)(Fig. 1c).

Correlations between the degrees of *RASSF1A* and *APC* promoters methylation and clinical and biochemical parameters

The DNA methylation levels of both *RASSF1A* and *APC* promoters did not show any significant correlation with the circulating albumin-corrected calcium and PTH levels (data not shown). Indeed, in a subset of 20 PAds, whose major diameters were available, *RASSF1A* promoter methylation levels negatively correlated with the tumor size ($r = -0.512$, $P = 0.021$)(Fig. 1d).

RASSF1A mRNA and protein expression in parathyroid tumors

We focused our attention on the oncosuppressor *RASSF1A*. *RASSF1A* mRNAs could be analyzed in a subset of 35 PAds included in the first tumor samples series. *RASSF1A* transcripts were significantly reduced in PAds compared with PaNs (n = 3)(Fig. 1e). Nonetheless, any significant correlation between *RASSF1A* mRNA levels and levels of *RASSF1A* promoter methylation could be detected (Fig. 1f).

RASSF1A protein was detectable by immunohistochemistry in the cytoplasm of cells in normal parathyroid glands (n = 3) and the rim of normal glands surrounding parathyroid adenomas (Fig. 1g–j), while the cytoplasm of adenomatous (n = 6, Fig. 1k, l) and cancerous parathyroid cells (n = 3, Fig. 1m) were weakly positive or negative.

Potential molecular mechanisms involved in *RASSF1A* promoter methylation in parathyroid tumors

RASSF1A promoter methylation emerges as a hallmark of PAds. We investigated two potential molecular mechanisms, whose alterations may promote *RASSF1A* promoter methylation.

1. *DNMTs activity*
   DNMT1 methylates both *RASSF1A* and *APC* gene promoters [16]. To test whether *RASSF1A* and *APC* gene promoters hypermethylation may be induced by deregulated DNMTs activity, nuclear extracts from 16 PAds were analyzed. The assay showed that DNMTs activity was differentially modulated in the different samples (Fig. 2a). Of note, DNMTs activity inversely correlated with RASSF1A protein levels ($r^2 = 0.400$, $P = 0.0086$ by linear regression analysis; Fig. 2b). These data suggest a potential role of DNMTs in RASSF1A regulation.
2. *ANRASSF1 long non coding RNA expression*

The antisense long non-coding RNA *RASSF1-AS1*, also termed Antisense Intronic Noncoding RASSF1 *ANRASSF1*, was implicated in a locus-specific mechanism for the *RASSF1A* epigenetic repression mediated by the Polycomb Repressive Complex 2 (PRC2) [17]. *ANRASSF1* has a *cis* function in the epigenetic silencing of *RASSF1A*, through the recruitment of the PRC2 components Enhancer of zeste homolog 2 (EZH2) and Polycomb Repressive Complex 2 Subunit (SUZ12) on the *RASSF1A* promoter and the subsequent trimethylation of the lysine 27 of H3 histone (H3K27me3). EZH2, the catalytic subunit of PRC2 complex showing histone methyltransferase activity [18], is also able to interact with DNMTs [19]. Thus, the cis-acting function of *ANRASSF1* mediated by PRC2 provides a possible link between histone modifications (H3K27me3) and de novo locus-specific methylation. Moreover, *ANRASSF1* could indirectly reinforce *RASSF1A* long-term epigenetic silencing via DNA methylation [20].

Given this background, we correlated *ANRASSF1* and *RASSF1A* mRNA levels, in 35 PAds, finding a positive correlation ($r = 0.788$, $P = 0.0001$, by Pearson coefficient correlation)(Fig. 2c). Similarly, *RASSF1A* promoter methylation negatively correlated with *ANRASSF1A* mRNA levels

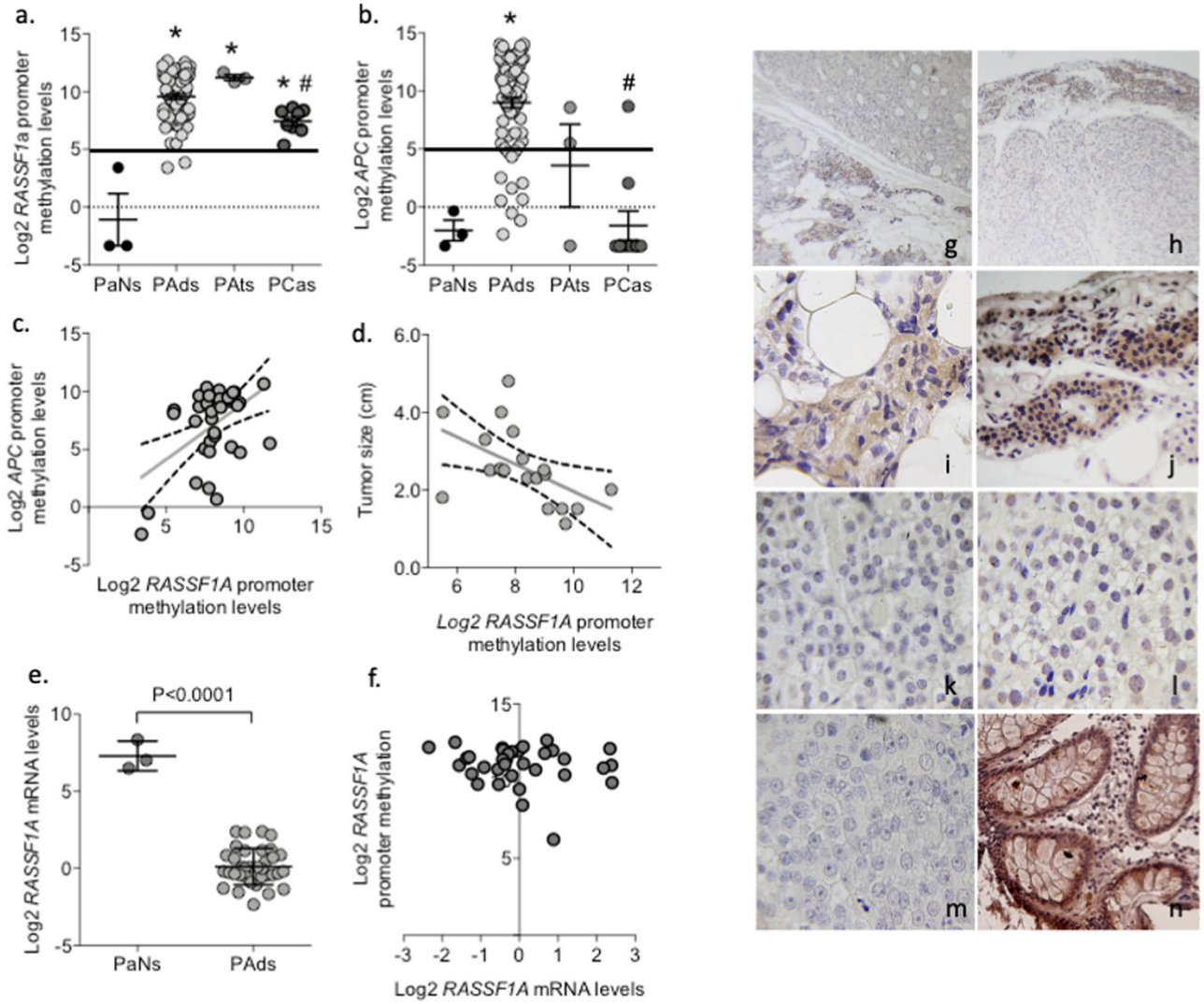

Fig. 1 Methylation levels of the promoters of the *RASSF1A* and *APC* genes and RASSF1A expression levels in human parathyroid tissues. **a** Mean promoter methylation levels of the *RASSF1A* gene in normal parathyroid glands (PaNs), parathyroid adenomas (PAds; *$p < 0.0001$ *vs* PaNs), atypical adenomas (PAts; *$p < 0.0001$ *vs* PaNs) and carcinomas (PCas; *$p < 0.0001$ *vs* PaNs; #$p = 0.029$ and 0.035 *vs* PAds and PAts, respectively). Comparisons were tested by ordinary one-way ANOVA corrected for multiple comparisons. Promoter methylation levels were presented as log2 transformed. The black thick line represents the cut-off level for hypermethylation, arbitrary set at ≥5.0. **b** Mean promoter methylation levels of the *APC* gene in PaNs, PAds (*$p < 0.0001$ *vs* PaNs), PAts, PCas (#$p < 0.0001$ *vs* PAds). Comparisons were tested by ordinary one-way ANOVA corrected for multiple comparisons. Promoter methylation levels were presented as log2 transformed. The black thick line represents the cut-off level for hypermethylation, arbitrary set at ≥5.0. **c** Correlation between *RASSF1A* promoter methylation and *APC* promoter methylation levels in PAds ($r^2 = 0.250$, $p = 0.0016$, by linear regression analysis). **d** Correlation between *RASSF1A* promoter methylation levels and PAds tumor size ($r = -0.512$, $P = 0.021$, by Pearson correlation coefficient). **e** *RASSF1A* gene expression levels in PAds compared with PaNs ($p < 0.0001$). **f** Correlation between *RASSF1A* gene expression and promoter methylation levels. Expression of RASSF1A protein in parathyroid tissues. Representative images showed immunostaining in PAds (**g–l**), and in parathyroid carcinoma (**m**). **n** Sections from human colon were used as positive control

($r = 0.366$, $P = 0.031$, by Pearson coefficient correlation) (Fig. 2d). These findings exclude the role of the lncRNA *ANRASSF1* in *RASSF1A* promoter methylation in PAds.

Effects of *RASSF1A* silencing on CASR-stimulated ERK intracellular signaling in CASR-HEK293A cells

Using HEK293A cells transiently transfected with human *CASR* as an experimental model (CASR-HEK293A), we investigated the effects of *RASSF1A* silencing on phosphorylated ERK/total ERK (pERK/ERK) ratios stimulated by the CASR positive allosteric modulator R568. *RASSF1A* silencing was tested at three different siRNA concentrations (5 nM, 25 nM, 50 nM); a maximum silencing efficiency was obtained with the low concentration of 5 nM (Fig. 3a, b). Moreover, it has been tested that *RASSF1A* silencing did not affect the expression levels of CASR protein (Fig. 3b). *RASSF1A* silencing increased basal pERK/ERK levels and

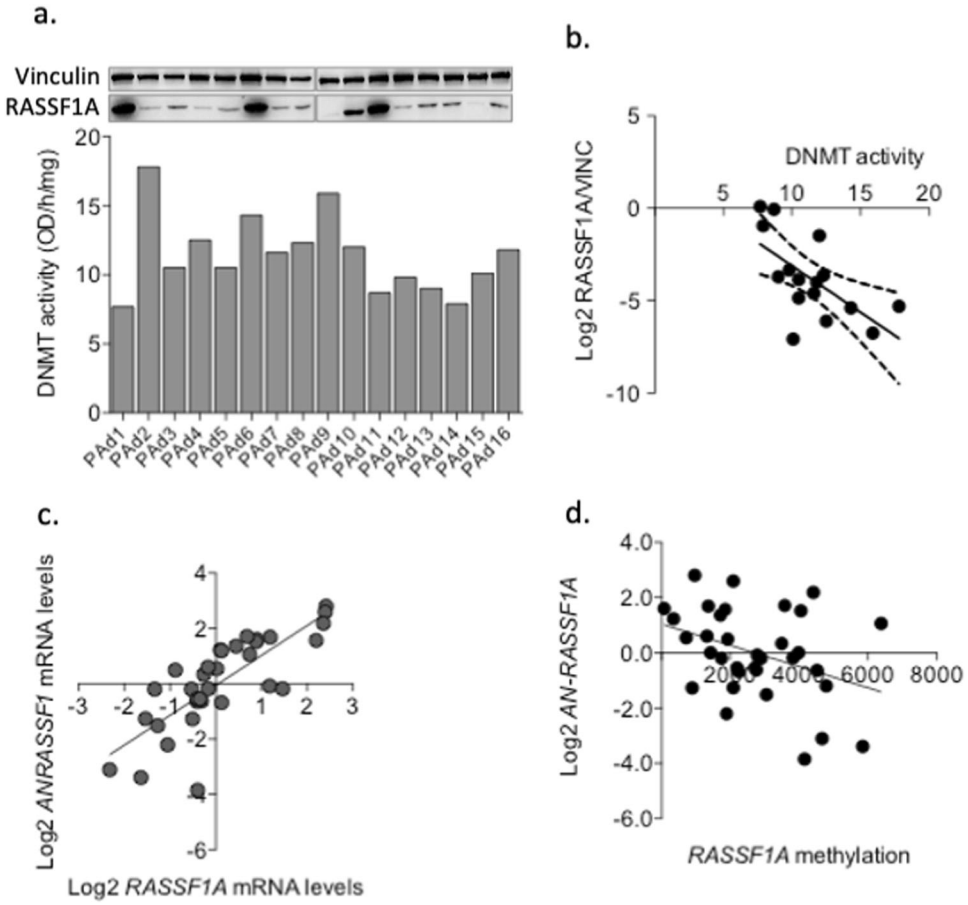

Fig. 2 Regulation of RASSF1A expression. **a** DNMT activity in a series of 16 PAds characterized for the RASSF1A protein expression levels is shown in the upper panel. **b** Correlation between DNMT activity and RASSF1A protein expression levels in PAds ($r^2 = 0.400$, $p = 0.0086$, by linear regression analysis). **c** Correlation between *RASSF1A* gene expression levels and the long non-coding RNA *ANRASSF1* expression levels ($r = 0.7884$, $p < 0.0001$, by Pearson correlation coefficient). **d** the *ANRASSF1* expression levels negatively correlated with the *RASSF1A* promoter methylation ($r = -0.3657$, $p = 0.0308$, by Pearson correlation coefficient)

blunted the pERK/ERK increases induced by R568-mediated CASR activation (Fig. 3c), suggesting that loss of RASSF1A may contribute to the parathyroid cell desensitization towards extracellular calcium concentrations observed in parathyroid tumors.

Discussion

The role of the gene promoter methylation in parathyroid tumors is controversial. Global hypermethylation has been described in tumors with loss of the oncosuppressor *MEN1* gene [1], while sporadic parathyroid tumors, both adenomas, and carcinomas, showed global promoter methylation density by Long Interspersed Elements 1 (LINE-1) similar to that in normal parathyroid glands (mean 70%) [4]. Indeed, a gradient of CpG hypermethylation from normal tissues to adenomas and carcinomas was identified in a subset of genes involved into key pathways, including *APC* and *RASSF1A* [21]. In the present study, most PAds, PAts and PCas displayed variable methylation of the *RASSF1* promoter, while *APC* promoter methylation was detected in most PAds only. This finding was in line with previous studies, reporting methylation of *APC* promoter in 56% [22] and 71% of PAds [23] and of *RASSF1A* promoter in 71% [20], 90% [19] and 98% [23] of PAds. Normal parathyroid tissue was invariably unmethylated.

Considering PCas, previous reports investigated *APC* promoter methylation in a very small number of samples yielding controversial results: in a series of 5 PCas, *APC* promoter methylation of 10 CpG islands has been detected in all samples by pyrosequencing [24], while in a series of 3 PCas only one sample harbored *APC* methylation at low level [22]. We had the opportunity to analyze 9 PCa samples, providing the most consistent series investigated so far for *APC* promoter methylation, and in all samples, except two, we failed in detecting a significant methylation of the *APC* promoter. Besides, *RASSF1A* promoter was methylated in most samples, though with lower levels than those detected in PAds.

In PAds harboring both gene promoters methylation, the methylation levels of *RASSF1A* promoter positively correlated with those of the *APC* promoter, suggesting the existence of a molecular mechanism common to the methylation of both gene promoters. Besides, in contrast with the known oncosuppressor role of RASSF1A reported in most common cancers, *RASSF1A* promoter methylation

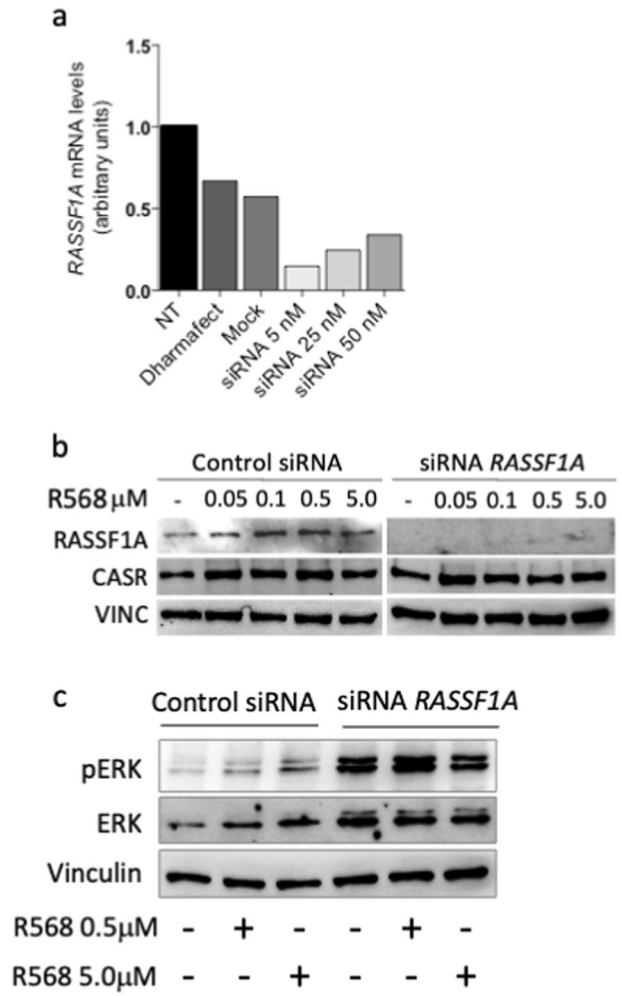

Fig. 3 Effects of *RASSF1A* silencing on the CASR-stimulated intracellular signaling pathways in CASR-HEK293A cells. Transient downregulation of *RASSF1A* by siRNA in HEK293A cells (n = 3) was verified by RT-qPCR (**a**) and western blot analysis (**b**), relative to cells treated with a negative control siRNA (Mock in panel a, control siRNA in panel **b**. In panel **b**, RASSF1A and CASR protein expression levels in HEK293A cells co-transfected with CASR and negative control or *RASSF1A* siRNA were shown. CASR-HEK293A cells transfected with control siRNA or with RASSF1A siRNA were treated with increasing concentrations of the CASR positive allosteric modulator R568. **c** pERK/ERK expression levels in CASR-HEK293A cells transfected with control siRNA or *RASSF1A* siRNA and stimulated with R568 for 10 minutes (n = 3)

inversely correlated with the size of parathyroid adenomas, suggesting that RASSF1A may not play a role in regulating cell proliferation in parathyroid tumorigenesis.

Aberrant hypermethylation of the *RASSF1A* promoter, one of the most common events in human cancers, is caused by DNA methyltransferases deregulation and it is associated with loss of *RASSF1A* expression [25]. *APC* expression has been reported to be lost in most parathyroid cancers and in a subset of PAds [18], while no data are available about RASSF1A in human parathyroid tissues. Here, we firstly reported consistent reduction or absence of RASSF1A protein by immunohistochemistry and western blot in the cytoplasm of cells in both PAds and PCas compared with cells of the normal parathyroid glands in the peripheral rim of PAds.

RASSF1 promoter, as well as *APC* promoter, was methylated by the DNA methyltransferase DNMT1 in different tumors [16, 26–29], which is overexpressed in most common human cancers and also in aggressive pituitary tumors [30]. DNMT1 expression levels have been reported to be similar in parathyroid normal glands and in parathyroid tumors [1]. However, investigating DNMTs activity in a series of PAds, we found that parathyroid tumors with higher DNMTs activity showed reduced RASSF1A cytoplasmic expression, suggesting that deregulation of DNMTs activity is involved in RASSF1A promoter methylation. Besides, though long-non-coding RNAs have been demonstrated to be deregulated in parathyroid tumors [31], in the present study the long-non-coding RNA *ANRASSF1A* was unlikely involved in the modulation of the expression of *RASSF1A* in human PAds.

Finally, the present data suggested that the oncosuppressor RASSF1A may not be involved in cell proliferation in parathyroid tumors; therefore, we investigated the role of RASSF1A in modulating sensitivity to extracellular calcium. Using HEK293A cells transfected with functional human CASR, the effect of loss of RASSF1A on intracellular signaling coupled to CASR activation has been analyzed. HEK293A cells can be a suitable cell model to investigate RASSF1A, as previously reported [5]. Loss of RASSF1A increased basal pERK/ERK levels blunting the CASR-induced increases, a feature resembling what observed in PAds-derived parathyroid cells with reduced sensitivity to extracellular calcium [32, 33]. In line with our data, it has been demonstrated that RASSF1A expression suppresses ERK1 activation in epithelial cells [34, 35].

Admittedly, the present study suffers from some limits: (1) it was focused on RASSF1A and APC genes, therefore the interaction with the methylation of other genes was not considered; (2) the methylation analysis could not discriminate among the different CpG islands in the RASSF1A promoter; (3) the sample size is limited, though the analyzed PCas case series is the most consistent up to now; (4) the role of DNMTs and ANRASSF1 deregulation has been analyzed based on correlations, and it could not investigated with silencing/overexpressing experiments; (5) the effect of loss of RASSF1A in parathyroid cell proliferation could not be investigated, due to lack of a commercially available cell line; (6) similarly, the effect of loss of RASSF1A on sensitivity to extracellular calcium suggested by the experiments in CASR-HEK293 could not be confirmed in parathyroid cells.

Conclusions

RASSF1A and *APC* promoter methylation is a hallmark of sporadic parathyroid adenomas; *RASSF1A* promoter methylation was confirmed in most parathyroid cancers, while methylation of the *APC* promoter remains controversial and needs further studies in wider sample series. DNMT activity is deregulated in parathyroid tumors and may contribute to modulation of *RASSF1A* expression. Finally, loss of RASSF1A may be involved in ERK tuning rather than in cell proliferation. We are tempted to speculate that loss of RASSF1A in PAds can be involved in parathyroid tumor cells sensitivity to extracellular calcium.

Data availability

The datasets generated and analyzed during the current study are available at https://doi.org/10.5281/zenodo.13475637.

Author contributions Conceptualization, V.V., L.A.M. and S.C.; methodology, C.V., P.M., A.M. and F.F.P.; software, C.V. and G.S.T.; validation, F.F.P., C.V., L.M. and S.C.; formal analysis, C.V., G.S.T., A.M.; investigation, S.C., V.G., F.C., A.S., R.M, F.P.; resources, F.C., A.S., R.M., F.P.; data curation, C.V. and P.M.; writing—original draft preparation, C.V. and S.C.; writing—review and editing, L.A.M., V.V. and S.C.; visualization, S.C.; supervision, S.C.; project administration, S.C.; funding acquisition, L.A.M., V.V. and S.C. All authors have read and agreed to the published version of the manuscript.

Funding This research was supported by the Italian Ministry of Health - Ricerca Corrente and University of Milan Linea 2 2022 grant.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Institutional Review Board Statement The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Ospedale San Raffaele Ethical Committee in Milan, Italy (protocol code CE40/2019 GPRC6A PARA, approved on 07/03/2019).

Informed Consent Statement Informed consent was obtained from all subjects involved in the study.

References

1. Z. Yuan, C. Sánchez Claros, M. Suzuki, E.C. Maggi, J.D. Kaner, N. Kinstlinger, J. Gorecka, T.J. Quinn, R. Geha, A. Corn, J. Pastoriza, Q. Jing, A. Adem, H. Wu, G. Alemu, Y.-C. Du, D. Zheng, J.M. Greally, S.K. Libutti, Loss of MEN1 activates DNMT1 implicating DNA hypermethylation as a driver of MEN1 tumorigenesis. Oncotarget **7**, 12633–12650 (2016). https://doi.org/10.18632/oncotarget.7279
2. E. Barazeghi, A.J. Gill, S. Sidhu, O. Norlén, R. Dina, F.F. Palazzo, P. Hellman, P. Stålberg, G. Westin, 5-Hydroxymethylcytosine discriminates between parathyroid adenoma and carcinoma. Clin. Epigenet. **8**, 31 (2016). https://doi.org/10.1186/s13148-016-0197-2
3. G. Malpeli, G. Innamorati, I. Decimo, M. Bencivenga, A.H. Nwabo Kamdje, R. Perris, C. Bassi, Methylation dynamics of RASSF1A and its impact on cancer. Cancers **11**, 959 (2019). https://doi.org/10.3390/cancers11070959
4. V. Guarnieri, L.A. Muscarella, C. Verdelli, S. Corbetta, Alterations of DNA methylation in parathyroid tumors. Mol. Cell. Endocrinol. **469**, 60–69 (2018). https://doi.org/10.1016/j.mce.2017.05.010
5. C. Chow, N. Wong, M. Pagano, S.W. Lun, K.I. Nakayama, K. Nakayama, K.W. Lo, Regulation of APC/CCdc20 activity by RASSF1A-APC/CCdc20 circuitry. Oncogene **31**, 1975–1987 (2012). https://doi.org/10.1038/onc.2011.372
6. E. Estrabaud, I. Lassot, G. Blot, E. Le Rouzic, V. Tanchou, E. Quemeneur, L. Daviet, F. Margottin-Goguet, R. Benarous, RASSF1C, an isoform of the tumor suppressor RASSF1A, promotes the accumulation of beta-catenin by interacting with betaTrCP. Cancer. Res. **67**, 1054–1061 (2007). https://doi.org/10.1158/0008-5472.CAN-06-2530
7. W.G. Wang, S.J. Chen, J.S. He, J.S. Li, X.F. Zang, The tumor suppressive role of RASSF1A in osteosarcoma through the Wnt signaling pathway. Tumour. Biol. **37**, 8869–8877 (2016). https://doi.org/10.1007/s13277-015-4660-z
8. WHO Classification of Tumours Editorial Board. Endocrine and neuroendocrine tumours. Lyon, France: International Agency for Research on Cancer, 2022.
9. C. Verdelli, A. Morotti, G.S. Tavanti, R. Silipigni, S. Guerneri, S. Ferrero, L. Vicentini, V. Vaira, S. Corbetta, The core stem genes SOX2, POU5F1/OCT4, and NANOG are expressed in human parathyroid tumors and modulated by MEN1, YAP1, and β-catenin pathways activation. Biomedicines **9**, 637 (2021). https://doi.org/10.3390/biomedicines9060637
10. J.P. Bilezikian, A.A. Khan, S.J. Silverberg, G.E. Fuleihan, C. Marcocci, S. Minisola, N. Perrier, A. Sitges-Serra, R.V. Thakker, G. Guyatt, M. Mannstadt, J.T. Potts, B.L. Clarke, M.L. Brandi, International Workshop on Primary Hyperparathyroidism.: Evaluation and management of primary hyperparathyroidism: summary statement and guidelines from the Fifth International Workshop. J. Bone Miner. Res. **37**, 2293–2314 (2022). https://doi.org/10.1002/jbmr.4677
11. L.A. Muscarella, F.P. Fabrizio, M. De Bonis, M.T. Mancini, T. Balsamo, P. Graziano, F. Centra, A. Sparaneo, D. Trombetta, A. Bonfitto, V. Scagliusi, P. Larizza, E.D. Capoluongo, V.M. Fazio, Automated workflow for somatic and germline next generation sequencing analysis in routine clinical cancer diagnostics. Cancers **11**, 1691 (2019). https://doi.org/10.3390/cancers11111691
12. A. La Torre, L.A. Muscarella, P. Parrella, T. Balsamo, M. Bisceglia, V.M. Valori, A. La Torre, R. Barbano, E. Perrella, M.L. Poeta, G. Melchionda, G. Merla, E. Maiello, R. Pellicano, V.M. Fazio, Aberrant genes promoter methylation in neural crest-derived tumors. Int. J. Biol. Markers. **27**, 389–394 (2012). https://doi.org/10.5301/JBM.2012.9766
13. F.P. Fabrizio, A. Sparaneo, F. Centra, D. Trombetta, C.T. Storlazzi, P. Graziano, E. Maiello, V.M. Fazio, L.A. Muscarella, Methylation density pattern of KEAP1 gene in lung cancer cell lines detected by quantitative methylation specific PCR and pyrosequencing. Int. J. Mol. Sci. **20**, 2697 (2019). https://doi.org/10.3390/ijms20112697
14. A. Mingione, C. Verdelli, S. Ferrero, V. Vaira, V. Guarnieri, A. Scillitani, L. Vicentini, G. Balza, E. Beretta, A. Terranegra, G. Vezzoli, L. Soldati, S. Corbetta, Filamin A is reduced and contributes to the CASR sensitivity in human parathyroid tumors. J. Mol. Endocrinol. **58**, 91–103 (2017). https://doi.org/10.1530/JME-16-0184
15. G.S. Tavanti, C. Verdelli, A. Morotti, P. Maroni, V. Guarnieri, A. Scillitani, R. Silipigni, S. Guerneri, R. Maggiore, G. Mari, L. Vicentini, P. Dalino Ciaramella, V. Vaira, S. Corbetta, Yes-

Associated Protein 1 is a novel Calcium Sensing Receptor target in human parathyroid tumors. Int. J. Mol. Sci. **22**, 2016 (2021). https://doi.org/10.3390/ijms22042016
16. Q. Lai, Y.H. Xu, Q. Chen, L. Tang, A.G. Li, L.F. Zhang, C.F. Zhang, J.F. Song, Z.Z. Du, The loss-of-function of DNA methyltransferase 1 by siRNA impairs the growth of non-small cell lung cancer with alleviated side effects via reactivation of RASSF1A and APC in vitro and vivo. Oncotarget **8**, 59301–59311 (2017). https://doi.org/10.18632/oncotarget.19573
17. F.C. Beckedorff, A.C. Ayupe, R. Crocci-Souza, M.S. Amaral, H.I. Nakaya, D.T. Soltys, C.F. Menck, E.M. Reis, S. Verjovski-Almeida, The intronic long noncoding RNA ANRASSF1 recruits PRC2 to the RASSF1A promoter, reducing the expression of RASSF1A and increasing cell proliferation. PLoS. Genet. **9**, e1003705 (2013). https://doi.org/10.1371/journal.pgen.1003705
18. C.C. Juhlin, I.L. Nilsson, K. Johansson, F. Haglund, A. Villablanca, A. Höög, C. Larsson, Parafibromin and APC as screening markers for malignant potential in atypical parathyroid adenomas. Endocr. Pathol. **21**, 166–177 (2010). https://doi.org/10.1007/s12022-010-9121-z
19. A.K. Arya, S.K. Bhadada, P. Singh, N. Sachdeva, U.N. Saikia, D. Dahiya, A. Behera, A. Bhansali, S.D. Rao, Promoter hypermethylation inactivates CDKN2A, CDKN2B and RASSF1A genes in sporadic parathyroid adenomas. Sci. Rep. **7**, 3123 (2017). https://doi.org/10.1038/s41598-017-03143-8
20. N. Calanca, A.P. Paschoal, É.P. Munhoz, L.T. Galindo, B.M. Barbosa, J.R.F. Caldeira, R.A. Oliveira, L.R. Cavalli, S.R. Rogatto, C.A. Rainho, The long non-coding RNA ANRASSF1 in the regulation of alternative protein-coding transcripts RASSF1A and RASSF1C in human breast cancer cells: implications to epigenetic therapy. Epigenetics **14**, 741–750 (2019). https://doi.org/10.1080/15592294.2019.1615355
21. L.F. Starker, J. Svedlund, R. Udelsman, H. Dralle, G. Akerström, G. Westin, R.P. Lifton, P. Björklund, T. Carling, The DNA methylome of benign and malignant parathyroid tumors. Genes Chromosomes Cancer **50**, 735–745 (2011). https://doi.org/10.1002/gcc.20895
22. L. Sulaiman, C.C. Juhlin, I.L. Nilsson, O. Fotouhi, C. Larsson, J. Hashemi, Global and gene-specific promoter methylation analysis in primary hyperparathyroidism. Epigenetics **8**, 646–655 (2013). https://doi.org/10.4161/epi.24823
23. C.C. Juhlin, N.B. Kiss, A. Villablanca, F. Haglund, J. Nordenström, A. Höög, C. Larsson, Frequent promoter hypermethylation of the APC and RASSF1A tumour suppressors in parathyroid tumours. PLoS One **5**, e9472 (2010). https://doi.org/10.1371/journal.pone.0009472
24. J. Svedlund, M. Aurén, M. Sundström, H. Dralle, G. Akerström, P. Björklund, G. Westin, Aberrant WNT/β-catenin signaling in parathyroid carcinoma. Mol. Cancer. **9**, 294 (2010). https://doi.org/10.1186/1476-4598-9-294
25. L. García-Gutiérrez, S. McKenna, W. Kolch, D. Matallanas, RASSF1A tumour suppressor: target the network for effective cancer therapy. Cancers **12**, 229 (2020). https://doi.org/10.3390/cancers12010229
26. J. Xiang, F. Luo, Y. Chen, F. Zhu, J. Wang, si-DNMT1 restore tumor suppressor genes expression through the reversal of DNA hypermethylation in cholangiocarcinoma. Clin. Res. Hepatol. Gastroenterol. **38**, 181–189 (2014). https://doi.org/10.1016/j.clinre.2013.11.004
27. Z. Du, K. Ma, X. Sun, A. Li, H. Wang, L. Zhang, F. Lin, X. Feng, J. Song, Methylation of RASSF1A gene promoter and the correlation with DNMT1 expression that may contribute to esophageal squamous cell carcinoma. World J. Surg. Oncol. **13**, 141 (2015). https://doi.org/10.1186/s12957-015-0557-y
28. J. Bai, X. Zhang, K. Hu, B. Liu, H. Wang, A. Li, F. Lin, L. Zhang, X. Sun, Z. Du, J. Song, Silencing DNA methyltransferase 1 (DNMT1) inhibits proliferation, metastasis and invasion in ESCC by suppressing methylation of RASSF1A and DAPK. Oncotarget **7**, 44129–44141 (2016). https://doi.org/10.18632/oncotarget.9866
29. F. Khatami, B. Larijani, R. Heshmat, S. Nasiri, H. Saffar, G. Shafiee, A. Mossafa, S.M. Tavangar, Promoter methylation of four tumor suppressor genes in human papillary thyroid carcinoma. Iran. J. Pathol. **14**, 290–298 (2019). https://doi.org/10.30699/ijp.2019.94401.1922
30. H.S. Ma, E.L. Wang, W.F. Xu, S. Yamada, K. Yoshimoto, Z.R. Qian, L. Shi, L.L. Liu, X.H. Li, Overexpression of DNA (cytosine-5)-methyltransferase 1 (DNMT1) and DNA (cytosine-5)-methyltransferase 3A (DNMT3A) is associated with aggressive behavior and hypermethylation of tumor suppressor genes in human pituitary adenomas. Med. Sci. Monit. **24**, 4841–4850 (2018). https://doi.org/10.12659/MSM.910608
31. A. Morotti, I. Forno, C. Verdelli, V. Guarnieri, F. Cetani, A. Terrasi, R. Silipigni, S. Guerneri, V. Andrè, A. Scillitani, L. Vicentini, S. Ferrero, S. Corbetta, V. Vaira, The oncosuppressors MEN1 and CDC73 are involved in lncRNA deregulation in human parathyroid tumors. J. Bone Miner. Res. **35**, 2423–2431 (2020). https://doi.org/10.1002/jbmr.4154
32. S. Corbetta, A. Lania, M. Filopanti, L. Vicentini, E. Ballaré, A. Spada, Mitogen-activated protein kinase cascade in human normal and tumoral parathyroid cells. J. Clin. Endocrinol. Metab. **87**, 2201–2205 (2002). https://doi.org/10.1210/jcem.87.5.8492
33. O. Kifor, I. Kifor, F.D. Moore Jr., R.R. Butters Jr., T. Cantor, P. Gao, E.M. Brown, Decreased expression of caveolin-1 and altered regulation of mitogen-activated protein kinase in cultured bovine parathyroid cells and human parathyroid adenomas. J. Clin. Endocrinol. Metab. **88**, 4455–4464 (2003). https://doi.org/10.1210/jc.2002-021427
34. R.R. Ram, S. Mendiratta, B.O. Bodemann, M.J. Torres, U. Eskiocak, M.A. White, RASSF1A inactivation unleashes a tumor suppressor/oncogene cascade with context-dependent consequences on cell cycle progression. Mol. Cell. Biol. **34**, 2350–2358 (2014). https://doi.org/10.1128/MCB.01506-13
35. F. Nan, S. Wei, D. Guan, L. Zhang, Q. Guo, S. Cao, Y. Liu, Y. Liu, M. Sun, Suppressive efficiency of RASSF1A in endometrial carcinoma via inhabiting estrogen receptor alpha expression and ERK pathway activation. Int. J. Clin. Exp. Pathol. **11**, 577–585 (2018). PMC6958056

Affiliations

Chiara Verdelli[1] · Federico Pio Fabrizio[2,3] · Paola Maroni[1] · Annamaria Morotti[4,5] · Giulia Stefania Tavanti[1,6] · Silvia Carrara[7] · Vito Guarnieri[8] · Filomena Cetani[9] · Alfredo Scillitani[10] · Riccardo Maggiore[11] · Francesca Perticone[12] · Valentina Vaira[4,5] · Lucia Anna Muscarella[2] · Sabrina Corbetta[6,13]

1 Laboratory of Experimental Biochemistry and Molecular Biology, IRCCS Ospedale Galeazzi-Sant'Ambrogio, Milan, Italy

2 Laboratory of Oncology, Fondazione IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, FG, Italy

3 Department of Medicine and Surgery, University of Enna "Kore", 94100, Enna, Italy

4 Division of Pathology, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

5 Department of Pathophysiology and Transplantation, University of Milan, Milan, Italy

6 Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy

7 Department of Medical Biotechnology and Translational Medicine, University of Milan, Milan, Italy

8 Division of Medical Genetics, Fondazione IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, FG, Italy

9 Department of Clinical and Experimental Medicine, Unit of Endocrinology, University of Pisa, Pisa, Italy

10 Endocrine Unit, Fondazione IRCCS Ospedale Casa Sollievo della Sofferenza, San Giovanni Rotondo, FG, Italy

11 Endocrine Surgery, IRCCS Ospedale San Raffaele, Milan, Italy

12 Endocrine Unit, IRCCS Ospedale San Raffaele, Milan, Italy

13 Bone Metabolic Diseases and Diabetes Unit, Department of Endocrine and Metabolic Diseases, IRCCS Istituto Auxologico Italiano, Milan, Italy