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BRIEF REPORT

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

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ABSTRACT

Alternative protein-coding transcripts of the *RASSF1* gene have been associated with dual functions in human cancer: while *RASSF1C* isoform has oncogenic properties, *RASSF1A* is a tumour suppressor frequently silenced by hypermethylation. Recently, the antisense long non-coding RNA RASSF1 (*ANRASSF1)* was implicated in a *locus*-specific mechanism for the *RASSF1A* epigenetic repression mediated by PRC2 (Polycomb Repressive Complex 2). Here, we evaluated the methylation patterns of the promoter regions of *RASSF1A* and *RASSF1C* and the expression levels of these *RASSF1* transcripts in breast cancer and breast cancer cell lines. As expected, *RASSF1C* remained unmethylated and *RASSF1A* was hypermethylated at high frequencies in 75 primary breast cancers, and also in a panel of three mammary epithelial cells (MEC) and 10 breast cancer cell lines (BCC). Although *RASSF1C* was expressed in all cell lines, only two of them expressed the transcript *RASSF1A*. *ANRASSF1* expression levels were increased in six BCCs. *In vitro* induced demethylation with 5-Aza-2′-deoxicytydine (5-Aza-dC) resulted in up-regulation of *RASSF1A* and an inverse correlation with *ANRASSF1* relative abundance in BCCs. However, increased levels of both transcripts were observed in two MECs (184A1 and MCF10A) after treatment with 5-Aza-dC. Overall, these findings indicate that *ANRASSF1* is differentially expressed in MECs and BCCs. The lncRNA *ANRASSF1* provides new perspectives as a therapeutic target for *locus*-specific regulation of *RASSF1A*.

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Introduction

Tumour suppressor function has been demonstrated for several proteins of the Ras-Association Domain (RASSF) family, which comprises 10 members (RASSF1 to RASSF10). These proteins have been implicated in a broad range of cellular processes such as cell cycle control, mitosis, apoptosis, microtubule stabilization, cell adhesion and polarization [1]. *RASSF1* was one of the first genes identified as a member of this family [2]. This gene is located within the 120Kb critical deletion region on 3p21.3, which is now recognized as a *locus* that harbour a tumour suppressor cluster [3–5]. Multiple isoforms termed *RASSF1A* to *RASSF1G* are originated from alternative splicing and by differential usage of two distinct promoters, both associated with specific CpG-islands. The well-studied variants are *RASSF1A* and *RASSF1C*, transcribed from the upstream and downstream promoters, respectively [6].

RASSF1A and *RASSF1C* variants are ubiquitously expressed in non-tumour tissues and are also recognized by their cancer-related functions [7]. Whilst *RASSF1A* can be inactivated by deletion or rarely by point mutations, the transcriptional silencing by promoter hypermethylation is the major mechanism leading to loss or suppression of its function in cancer cells. Aberrant *RASSF1A* methylation is described as an early and frequent event in human cancers [8,9]. In breast cancer, *RASSF1A* methylation is observed at high frequencies, and this

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aberrant epigenetic mark has also been detected in the serum from breast cancer patients in association with metastasis occurrence, large tumour size, and low response to adjuvant therapy [10]. Thus, it has been suggested that hypermethylation of *RASSFIA* could be used as a potential diagnostic and/or prognostic biomarker in breast cancer.

Contrary to *RASSF1A, RASSF1C* has been associated to oncogenic activities [11–13], and no hypermethylation is described in its promoter in human cancer. Given these opposite effects in cancer cells, it has been proposed that *RASSF1* has a dual function. In normal tissues, the RASSF1A protein presents a dominant action; thus, a high expression ratio of RASSF1A/RASSF1C could suppress the oncogenic effects of RASSF1C. In cancer cells, the effects on cell growth and apoptosis mediated by RASSF1C isoform could accompany RASSF1A loss or inactivation [14].

In our previous study, we described that *RASSF1A* isoform is epigenetically silenced in the majority of breast cancer cell lines (15 out of 17), while *RASSF1C* expression was maintained in all of them [15]. Additionally, we also demonstrated that induced demethylation by using 5-Aza-2′-deoxycytidine (5-Aza-dC), isolated or in combination with the histone deacetylase inhibitor Trichostatin A (TSA), simultaneously up-regulated *RASSF1A* and its flanking genes while no significant changes were observed in *RASSF1C* expression levels [15]. These findings suggested the existence of a fine-tuned and *locus*-specific epigenetic control of the *RASSF1* alternative transcripts.

Recently, a new nuclear long non-coding RNA (lncRNA), *RASSF1-AS1* (also termed Antisense Intronic Noncoding *RASSF1* or *ANRASSF1*) was characterized by Beckedorff et al. [16]. The authors proposed a model in which this lncRNA has a *cis* function in the epigenetic silencing of *RASSF1A*. Accordingly, as *ANRASSF1* is transcribed by RNA polymerase II, it remains tethered to its transcriptional site and recruit histone modifying complexes. Functional assays using HeLa cells overexpressing *ANRASSF1* and RNA immunoprecipitation with antibody specific to members of PRC2 complex (i.e. SUZ12 and EZH2) indicated an enrichment of the lncRNA *ANRASSF1*. The occupancy of PRC2 at the *RASSF1A* promoter was associated with the trimethylation of the lysine 27 of H3 histone (H3K27me3). This repressive histone mark was neither observed in the promoter regions of *RASSF1C* nor in the four neighbouring genes [16].

The protein EZH2, catalytic subunit of PRC2 complex showing histone methyltransferase activity [17], is also able to interact with DNA methyltransferases (DNMTs) [18]. Thus, the *cis*-acting function of *ANRASSF1* mediated by PRC2 provides a possible link between histone modifications (H3K27me) and *de novo locus*-specific methylation. *ANRASSF1* could indirectly reinforce *RASSF1A* long-term epigenetic silencing via DNA methylation. In this context, the proposed mechanism of action of *ANRASSF1* would explain why the promoter-associated CpG island of *RASSF1A* is frequently methylated in several human solid tumours while the closest CpG island associated to the *RASSF1C* promoter region remains unmethylated. Most studies have only focused on the DNA methylation analysis of *RASSF1A* variant and few reports described simultaneously the methylation patterns of both CpG islands of the gene *RASSF1* in the same series of tumour tissue samples.

In this study, we first determined the methylation patterns of *RASSF1A* and *RASSF1C* in primary breast tumours and breast cancer cell lines. Then, we correlated the promoter methylation patterns of both variants with the expression levels of the *ANRASSF1* non-coding RNA and the protein-coding *RASSF1A, RASSF1C*, and *EZH2* transcripts in breast cancer cells lines and discussed the potential of *ANRASSF1* as a new breast cancer biomarker and therapeutic target.

Results

RASSF1A and RASSF1C methylation in breast cancer and breast cancer cell lines

Initially, the methylation patterns of both *RASSF1A* and *RASSF1C* promoter regions were evaluated in a set of 75 ductal breast carcinomas by conventional MS-PCR (Methylation-Specific Polymerase Chain Reaction). As expected, a high frequency of *RASSF1A* methylation was observed (85% of the tissue samples), whereas all samples showed exclusively non-methylated alleles at *RASSF1C* promoter-region. Table 1 shows the significant associations between clinical parameters and disease-specific

Table 1. Disease-specific survival analysis in 75 breast cancer patients.

	Univariate analysis (N = 73/75 cases)		Multivariate analysis	
Parameters	HR (CI 95%)	*p*	HR (CI 95%)	*p*
Age (years)				
≤55	1.0	0.1154		
>55	5.658 (0.654–48.949)			
Clinical stage				
I, IIA, IIB	**1.0**	**0.0050**		
IIIA, IIIB	**23.301 (2.589–209.744)**			
Tumour size (mm)				
≤20	1.0	0.9950		
>20	nd			
Lymph nodes involvement				
≤4	**1.0**	**0.0163**		
>4	**14.271 (1.632–124.788)**			
Metastasis				
Presence	**1.0**	**0.0014**	**1.0**	**0.0014**
Absence	**18.792 (3.093–114.186)**		**18.792 (3.093 –114.186)**	
Histological grade				
I, II	1.0	0.8001		
III	1.230 (0.248–6.111)			
ER				
Positive	1.0	0.0971		
Negative	4.288 (0.768–23.939)			
PgR				
Positive	1.0	0.9959		
Negative	nd			
Her-2				
Negative	1.0	0.7151		
Positive	1.506 (0.167–13.590)			
p53				
Negative	1.0	0.6169		
Positive	0.568 (0.062–5.213)			
Proliferative index (Ki-67)				
Low (≤25%)	1.0	0.6069		
High (>25%)	0.640 (0.117–3.508)			

HR: Hazard Ratio; CI: Confidence Interval; N: Number of cases; nd: not determined; in bold: p-value <0,05.

survival rates in this cohort. However, no significant differences were found between clinical and histopathological parameters and *RASSF1A* methylation (Table 2). Also, *RASSF1A* methylation was not associated with disease-free survival (p = 0.1293, Kaplan-Meier estimation).

Table 2. *RASSF1A* methylation according to clinical, histopathological, and immunohistochemical markers in 75 invasive ductal breast carcinomas.

Variables	Methylation status		
	Methylated	Unmethylated	*p-value*
Age (years)			
≤55	31	4	0.5276
>55	33	7	
Clinical stage*			
I, IIA, IIB	51	11	0.1930
IIIA, IIIB	12	0	
Tumour size**			
≤20 mm	14	4	0.4474
>20 mm	48	7	
Histological grade			
I, II	35	6	1.0
III	29	5	
Lymph nodes involvement*			
≤4	49	10	0.4430
>4	14	1	
Distant metastasis			
Presence	8	0	0.5950
Absence	56	11	
p53***			
Negative	43	8	0.999
Positive	16	3	
Her-2			
Negative	56	9	0.6145
Positive	7	2	
ER*			
Negative	19	5	0.3207
Positive	44	6	
PgR*			
Negative	33	6	0.9999
Positive	30	5	
Ki-67			
Low (≤25%)	40	6	0.7405
High (>25%)	24	5	

data available for (*)74, (**) 73, and (***) 70 breast cancer patients; p-value obtained by Fisher's exact test.

The *RASSF1A* and *RASSF1C* methylation patterns were also evaluated in 10 breast cancer cell lines (BCCs: BT-483, BT-549, Hs578T, MCF7, MDA-MB-134, MDA-MB-231, MDA-MB-415, MDA-MB-453, MDA-MB-468, and SK-BR-3) and in three mammary epithelial cell lines (MECs: 184A1 and 184B5 derived from normal breast epithelium and MCF10A from benign fibrocystic disease). Figure 1(a,e) illustrates respectively the physical position of the two CpG islands of the *RASSF1* gene and the MS-PCR results. Only MDA-MB-415 cells showed exclusively unmethylated alleles of *RASSF1A* promoter region. This region was fully methylated in eight breast cancer cell lines. A hemimethylated pattern (presence of both methylated and unmethylated alleles) was detected in Hs578T cancer cells and in three

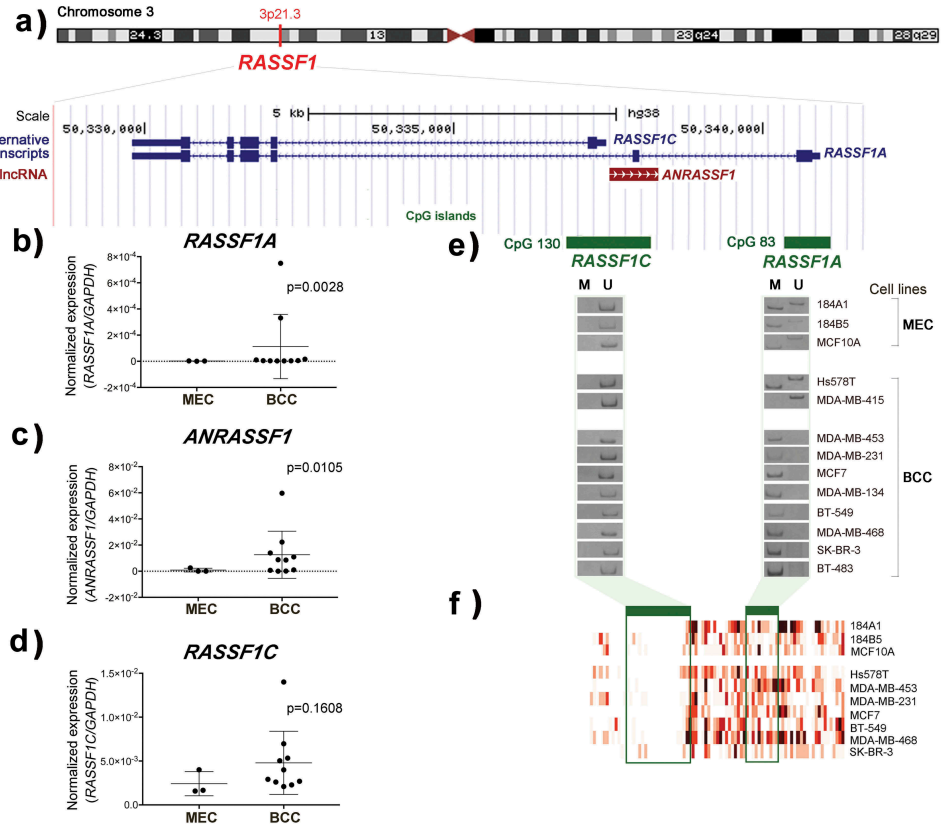

Figure 1. (a) Illustrative physical map of *RASSF1 locus* showing the overlapping of associated coding and non-coding RNAs, as well as the two promoter-associated CpG islands 130 and 83 of the alternative transcripts *RASSF1C* and *RASSF1A*, respectively [modified from UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38) Assembly, http://genome.ucsc.edu)]. (b-d) Expression levels of *RASSF1A, ANRASSF1* and *RASSF1C*, respectively, in three mammary epithelial cells (MECs) and ten breast cancer cell lines (BCCs). The expression of each target gene was normalized with the *GAPDH* gene expression levels. (e) DNA methylation status of *RASSF1A* and *RASSF1C* in MECs and BCCs (M = methylated and U = unmethylated). (f) The colour red represents a gradient heatmap of methylation intensities of the two promoter regions of *RASSF1* gene. These data were retrieved from the public database Cancer Methylome System (http://cbbiweb.uthscsa.edu/KMethylomes/), obtained upon genome-wide MBDCap-sequencing (Methyl-CpG binding domain-based capture and sequencing) (p-value was determined by the Mann–Whitney test).

MECs used as normal references. As observed in the primary breast cancer tissue samples, the *RASSF1A* promoter region was commonly methylated while the promoter-specific CpG island of *RASSF1C* remained unmethylated in all of them.

Expression levels of the lncRNA ANRASSF1 and protein-coding transcripts in breast cancer cell lines

The data of DNA methylation analysis were correlated with the expression of *RASSF1* transcripts by RT-qPCR: *RASSF1C* was unmethylated and expressed in all MEC and BCC cells, while *RASSF1A* was hypermethylated and silenced in most of BCCs (Figure 1(b,d)). The *RASSF1A* transcript was detected only in the MDA-MB-415 and Hs578T cells, which exhibit unmethylated and hemimethylated patterns, respectively. Although expressed at very low levels in one MEC (184B5), the lncRNA *ANRASSF1* was detected in six BCCs (BT-483, MCF7, MDA-MB-134, MDA-MB-415, MDA-MB-453, MDA-MB-468) (Figure 1(c)). Furthermore, the BCCs Hs578T (hemimethylated) and BT-549, MDA-MB-231, and SK-BR-3 (fully methylated) showed results similar to MECs. The gene *EZH2* was equally expressed among MECs and BCCs, with exception of MDA-MB-468 cells which expressed higher levels (Figure S1A).

Effect of 5-Aza-dC treatment in the transcriptional expression levels

Next, we compared the expression levels of the lncRNA *ANRASSF1* and *RASSF1A* transcripts after 5-Aza-dC treatment. Increased *RASSF1A* expression levels ($p = 0.0020$) were observed after induced

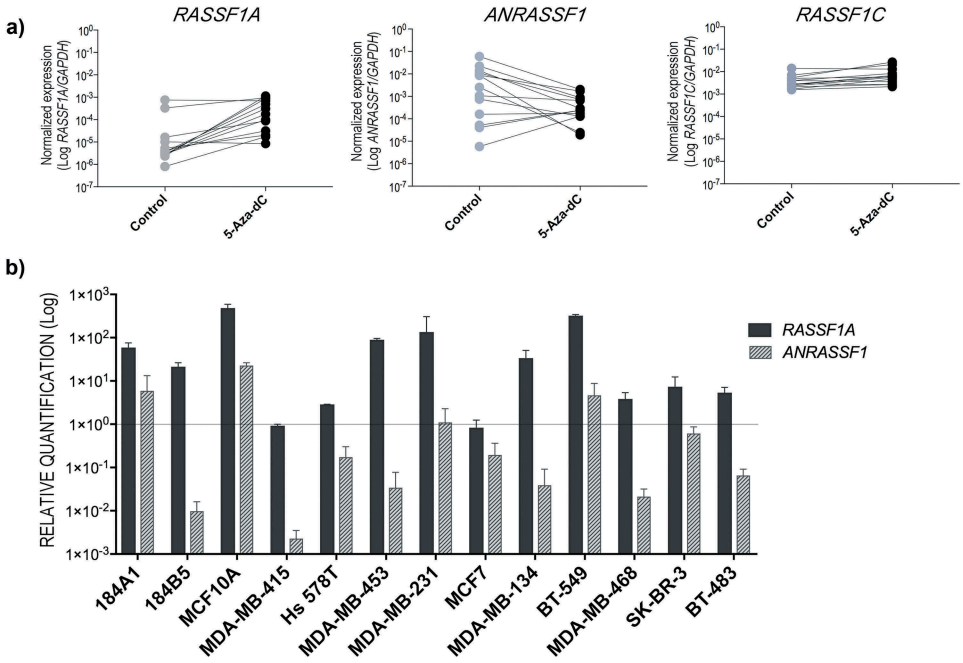

Figure 2. (a) Effect of induced demethylation on the expression levels of *RASSF1* transcripts after *in vitro* treatment with the DNMT inhibitor 5-Aza-2-deoxycytidine (5-Aza-dC) in MECs and BCCs. These data show the average of two independent experiments and technical triplicates for each cell line. (b) Abundance of the *RASSF1A* and *ANRASSF1* transcripts after 5-Aza-dC treatment in each cell line relative to the respective paired untreated control (p-value was determined by the Wilcoxon matched-pairs signed rank test).

demethylation, indicating its re-expression in both MEC and BCC groups. As expected, minor changes were observed for *RASSF1C* expression levels ($p = 0.0081$). Contrarily, we observed a trend to lower levels of *ANRASSF1* expression under this experimental condition ($p = 0.0105$) (Figure 2(a)). Figure 2(b) shows the relative abundance of *RASSF1A* and *ANRASSF1* transcripts in each cell line: 10 of 13 cell lines showed an increase of at least four times in the *RASSF1A* expression levels. In addition, the relative expression levels of *ANRASSF1* were lower compared to untreated cells in 1 MEC and 8 BCCs. Interestingly, *ANRASSF1* levels were increased in two MECs (184A1 and MCF10A). In addition, after the treatment with 5-Aza-dC in MDA-MB-415 cells, although no alteration in the relative expression of *RASSF1A* was found, *ANRASSF1A* showed the most negative effect compared to the other cell lines. No effects in *EZH2* gene expression were observed after 5-Aza-dC treatment (Figure S1B).

Discussion

The results of the present study reinforce that while the promoter-associated CpG island of *RASSF1A* is hypermethylated in high frequency in breast carcinomas, the downstream CpG island associated with the alternative *RASSF1C* promoter remains unmethylated. These DNA methylation data are in accordance with those available in the public database such as Cancer Methylome System [19] (Figure 1(f)) and The Cancer Genome Atlas (TCGA) [20] (Figure S2). *RASSF1A* methylation is a common epigenetic alteration in a broad spectrum of human solid tumours [8–10], commonly associated with poor prognosis [21–23]. Epigenetic inactivation of *RASSF1A* has also been associated with specific molecular subtypes in breast cancer [24], although we did not find significant associations probably due to the small sample size.

A panel of three MECs and 10 BCCs was used to evaluate the effect of DNA methylation of the promoter-associated CpG islands of the *RASSF1* gene in the expression levels of its protein-coding transcripts (*RASSF1A* and *RASSF1C*) and in the recently characterized lncRNA *ANRASSF1*. In 2004, Reis et al. [25] reported the first evidence of an intronic and antisense transcript associated with the *RASSF1* gene which was also correlated with tumour differentiation in prostate cancer. Subsequently, the same research group effectively characterized this new

transcript as *ANRASSF1* [16]. The authors described higher *ANRASSF1* expression levels in breast (MDA-MB-231 and MCF7) and prostate (LNCap and DU145) cancer cell lines when compared with non-tumour cell lines (MCF10A and RWPE-1, respectively). An inverse correlation between *ANRASSF1* and *RASSF1A* expression was also found [16].

Only two other studies have investigated the *ANRASSF1* expression levels in primary human cancers [26,27]. Iranpour et al. [26] reported that, in comparison to matched adjacent noncancerous tissues, *ANRASSF1* is overexpressed in breast cancer especially of the triple-negative subtype [26]. A more recent study showed that *ANRASSF1* was also frequently overexpressed in gastric cancer in comparison with adjacent tissues [27]. These findings suggest a relevant role of *ANRASSF1* in cancer progression.

The importance of non-coding RNA (ncRNA) is increasingly being recognized as key regulators of physiological programs during development and in human diseases, including cancer [28,29]. Few lncRNAs have been completely characterized. To date, *ANRASSF1* is described as an endogenous unspliced lncRNA, which is transcribed through RNA polymerase II, it is capped and polyadenylated, exhibits nuclear localization and ability to bind to PRC2. Despite its shorter half-life compared to other lncRNAs that bind to PRC2, it has been proposed that the interaction *ANRASSF1*/PRC2 could act directly in the epigenetic silencing of the *RASSF1A* isoform [16]. Further, the importance of antisense transcripts at this *locus* was also demonstrated through an analogue system described in plants and based on small interfering RNA (siRNA)-directed CpG methylation. Castanotto et al. [30] demonstrated that short hairpin RNAs (shRNAs) complementary to the *RASSF1A* promoter can direct lower levels of *de novo* DNA methylation and partial gene silencing in HeLa cells.

Herein, we described that *ANRASSF1* abundance is lower in three MECs when compared with a panel of 10 BCCs. In addition, the lncRNA was detected in cell lines methylated as well as in a cell line with exclusively unmethylated *RASSF1A* alleles (MDA-MB -415). These changes in the DNA methylation pattern were not associated with the expression of *EZH2*. Notably, very low abundance of *ANRASSF1* was detected in four hemimethylated cell lines (3 MECs and in Hs578T breast cancer cell line) as well as in three fully methylated BCCs (BT-549, MDA- MB-231, and SK-BR-3). The detection of *ANRASSF1* in partially methylated normal mammary epithelial cells and in a cancer cell line unmethylated suggest that this lncRNA may have other functions than those related with *RASSF1A* epigenetic silencing.

We observed a heterogeneous effect of 5-Aza-dC treatment in the relative abundance of *ANRASSF1* and *RASSF1A* transcripts. While the expression levels of *ANRASSF1* clearly decreased in six BCCs (MDA-MB-415 was unmethylated at *RASSF1A* promoter, and BT-483, MCF7, MDA-MB-134, MDA-MB-453, and MDA-MB-468 were fully methylated), two of three MECs (184A1 and MCF10A) and BT-549 breast cancer cells showed an increase in its relative abundance. Several factors should contribute to this heterogeneous profile, including the differential sensitivity to the DNMT inhibitor and effects of gene dosage.

Mayor et al.[31] showed that although the *in vitro* treatment with 5-Aza-dC was able to restore the expression of hypermethylated genes in HCT116 colon cancer cells, the remethylation of the associated promoter regions occurred 16 days after 5-Aza-dC withdrawal. These authors also described that the retained levels of chromatin marks at these chromosomal regions accompanied the restitution of the DNA methylation. According to the proposed model for the epigenetic silencing of the *RASSF1A* driven by *ANRASSF1* [16], one could speculate that *ANRASSF1* could antagonize the effects of drug-induced demethylation in the promoter region of *RASSF1A*, contributing for its remethylation after a short-term period in the absence of the DNA methylation inhibitors. In this context, *ANRASSF1* could be considered as a promisor molecular target to epigenetic therapy.

The characterization of the molecular mechanisms involved in *RASSF1A* epigenetic silencing could lead to a deeper understanding of tumour development and allow the identification of new treatment strategies. Commonly, the expression levels of lncRNAs are lower and tend to show tissue-specific patterns in non-tumour tissues, but are overexpressed in cancer cells [32,33]. Overall, these biological features support the use of lncRNAs as diagnostic and prognostic markers, as well as therapeutic targets in cancer [34,35].

Currently, approaches to target lncRNAs have been emerging as an innovative therapeutic strategy to induce the up-regulation of *locus*-specific endogenous genes [35]. In this context, *ANRASSF1* is located in the nucleus and has been implicated in the epigenetic repression of the tumour suppressor *RASSF1A*. Therapeutic target of *ANRASSF1* have potential to revert the repressive chromatin modifications introduced by PRC2 at *RASSF1A* promoter, potentially leading to *RASSF1A* up-regulation in breast cancer.

Material and methods

Patients

Breast cancer samples were obtained from Amaral Carvalho Hospital, Jaú, São Paulo, Brazil, under patient-informed consent and Institutional Ethics Committee approval. The DNA methylation patterns of both promoter-associated CpG islands of the *RASSF1* gene (*RASSF1A* and *RASSF1C*) were analysed in a set of 75 invasive ductal breast carcinoma samples from unrelated patients. The samples were obtained from patients naïve of radio and/or chemotherapy prior to surgery. The mean age of the patients was 58.9 ± 15.7 years and the mean follow-up period was 89.2 ± 35.12 months, ranging from 6 to 153 months.

Methylation specific-polymerase chain reaction (MS-PCR)

Genomic DNA was isolated by standard SDS/proteinase K digestion followed by phenol and chloroform extraction and ethanol precipitation. Approximately 1µg of genomic DNA was subjected to bisulfite modification using the EpiTect Bisulfite Modification Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, and eluted in a final volume of 40µL. In order to evaluate the conversion efficiency, 5µL of modified DNA were amplified by PCR using a primer set that recognize the bisulfite-modified template (control region) but do not discriminate between methylated and unmethylated alleles. The DNA methylation patterns of *RASSF1A* and *RASSF1C* promoter regions were determined by conventional MS-PCR. Bisulfite-converted CpG methylated HeLa genomic DNA (New England Biolabs, Ipswich, MA, USA) and the unmethylated EpiTect control DNA (Qiagen, Hilden, Germany) were used to evaluate the specificity of each primer set for methylated and unmethylated alleles, respectively (supplementary material, Table S1). The amplification reactions were performed at a final volume of 25µL containing 0.25mM of each primer, 200mM of each dNTP, 15mM Tris-HCl (pH 8.0), 50mM KCl, 2.5mM $MgCl_2$ and 1U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). The PCR conditions were as follows: 1 cycle at 95°C for 5 minutes; 31 cycles at 95°C for 45 seconds; 56°C for 30 seconds and 72°C for 30 seconds, and one cycle at 72°C for 4 minutes. The amplified products were visualized after electrophoresis in 6% polyacrylamide gels and stained with silver nitrate.

Cell lines and 5-Aza-dC treatment

Three mammary epithelial cell lines (MECs: 184A1 and 184B5, both chemically transformed and MCF10A derived from benign fibrocystic disease) and 10 breast cancer cell lines (BCCs: BT-483, BT-549, Hs578T, MCF7, MDA-MB-134, MDA-MB-231, MDA-MB-415, MDA-MB-453, MDA-MB-468, and SK-BR-3) were obtained from the Tissue Culture Shared Resource (TCSR) at the Lombardi Comprehensive Cancer Center, Georgetown University, Washington DC. All cell lines were fingerprinted using the Cell ID™ System (Promega, Madison, WI, USA) for genomic authentication by the TCSR of Georgetown University, prior to the experiments. The cultures were maintained at 37°C in a humidified environment containing 5% CO_2 and supplemented medium as previously described [15].

Total RNA extraction and mRNA expression analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), purified with DNase I amplification grade (Invitrogen, Carlsbad, CA, USA), and quantified on a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher, Waltham, MA, USA). Total RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The complementary DNA (cDNA) was obtained with the High-Capacity cDNA

Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), using random primers in a final volume of 20μL, according to the manufacturer's instructions. Relative expression levels of the *RASSF1A, RASSF1C*, and *EZH2* alternative transcripts were quantified by RT-qPCR (quantitative real-time polymerase chain reaction) as previously reported [15].

Strand-specific cDNA synthesis and expression analysis of ANRASSF1 by reverse transcriptase-polymerase chain reaction (RT-PCR)

The experimental strategy used for the *ANRASSF1* expression analysis based on qualitative and quantitative RT-PCR is detailed in the supplementary Figure S3. Orientation-specific RT-PCR was performed with 1.5 μg of total RNA, reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). cDNA synthesis used primer complementary to the antisense or sense strand of *ANRASSF1* in a final volume of 20μL. The occurrence of self-priming and DNA contamination was verified in cDNA reactions with no primer or transcriptase reverse, respectively, as additional controls. The *ANRASSF1* was detected as previously described using a primer set that generated an amplicon of 516bp [16]. Direct Sanger sequencing confirmed the identity of this PCR product using the DNA automated sequencer ABI 3500 (Applied Biosystems, Foster City, CA, USA) and the *CLC Main Workbench software* 6.0 (CLC Bio, Aarhus, Denmark) (supplementary material, Table S1 and Figure S1). For the quantitative RT-PCR analysis, the reverse transcription reaction was diluted 1:10 with sterile ultra pure water. Two μL of diluted cDNA were used in a 15μL reaction using the GoTaq qPCR master mix (Promega, Madison, WI, USA) following the conditions: 1 cycle at 95°C for 5 minutes followed by 35 cycles of two steps: denaturation at 95°C for 10 seconds and annealing/extension at 60°C for 30 seconds. The reactions were performed in triplicate using the StepOne Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The results were calculated using the ΔΔCt-method normalized with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression levels, which was experimentally selected in a previous study of our group [15].

Immunohistochemical analysis

Estrogen (ER), Progesterone (PgR), Epidermal Growth Factor II receptor (HER2), p53 and Ki-67 antigen were analysed in formalin-fixed and paraffin-embedded (FFPE) tissues by immunohistochemistry analysis, as previously described by Caldeira et al. [36]. ER and PgR scoring were reviewed according to American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Immunohistochemical Testing of Estrogen and Progesterone Receptors in Breast Cancer [37].

Statistical analysis

Pairwise associations between *RASSF1A* methylation patterns and clinical, histopathological and immunohistological variables were assessed using Fisher's exact test. The survival data, obtained from clinical records, were analysed using the non-parametric Kaplan–Meier method, and the differences were evaluated by log-rank test. Multivariate statistical analysis was performed using the Cox model to evaluate the combined effects of these variables in the risk of death. Comparisons of gene expression levels were based on the average and standard deviation of two independent experiments and three technical replicates. The significance level of 5% was applied for all statistical analysis using the statistical software package SAS/STAT, version 6.0 (SAS Institute, Inc., Cary, NC, USA) or GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

Disclosure statement

No potential conflict of interest was reported by the authors.

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