Promoter methylation of *RASSF1A* modulates the effect of the microtubule-targeting agent docetaxel in breast cancer

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Abstract. Docetaxel is one of the most commonly used chemotherapeutic agents in breast cancer. To avert from significant toxicities with no clinical benefit, identification of predictive markers for response is one of the most important unsolved clinical needs. Therefore, the potential associations of *RASSF1A* hypermethylation and response to docetaxel-based chemotherapy were evaluated, and the underlying mechanism was studied. The expression of RASSF1A in breast cancer cell lines and tissues of normal breast, ductal carcinoma *in situ* (DCIS), and breast cancer (n=45) was analyzed by immunohistochemistry and western blot analysis. Immunohistochemical staining showed that the expression of RASSF1A was frequently lost in primary breast cancers and human breast cancer cell lines, while normal breast tissues or DCIS displayed moderate to strong expression. Furthermore, quantitative methylation analysis of the *RASSF1A* promoter region in 45 primary breast cancers revealed that *RASSF1A* was frequently methylated in primary breast cancers (≥20% methylation in 53% of the patients), and prospective analysis in patients with locally advanced or recurrent breast cancer showed that the mean level of methylation of *RASSF1A* was significantly higher in patients who did not respond to docetaxel-based chemotherapy (30.6±8.5%) than patients with partial or complete response (20.1±11.2%, p=0.042). Finally, *in vitro* studies showed that RASSF1A had cooperative activity in suppression of cancer cell growth and proliferation by enhancing docetaxel-induced cell cycle arrest. Our results suggest that hypermethylated *RASSF1A* is an important modulating factor for the efficacy of docetaxel-based chemotherapy in breast cancer.

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Introduction

Breast cancer is one the most common cancers in women worldwide as well as in Korea (1). Although treatment outcome of breast cancer has been greatly improved due to early diagnosis and development of various targeted agents, prognosis of patients with locally advanced breast cancer still remains to be improved. Recently, neoadjuvant chemotherapy has increasingly been considered, since the chance for breast conservation is significantly increased and the treatment outcome was similar to that in patients who received adjuvant chemotherapy after primary surgery (2,3). Therefore, it is critical to identify biologic markers for response to commonly used chemotherapeutic agents in a neoadjuvant setting. To date, several studies have been conducted for the identification of the predictive markers for response to neoadjuvant chemotherapy, and some biologic characteristics of tumors such as hormone receptor status, HER-2 overexpression, and Ki-67 labeling index have been suggested as potential biomarkers (4,5). However, none of these has been proposed as a marker based on the biologically relevant mechanisms in their cytotoxicity for a specific chemotherapeutic agent. Thus, the multi-factorial molecular mechanisms determining chemotherapy response remain largely unclear.

Taxanes, including docetaxel, in combination with other cyctotoxic agents or targeted agents are the standard treatment for locally advanced or metastatic breast cancers. Many clinical trials have demonstrated the efficacy of the taxanes in this group of patients, however, about 20% of breast cancers do not shrink significantly or progress with neoadjuvant treatment and 30-40% of the patients who had residual disease ultimately get recurrent cancer (2,3). Therefore, identification of promising biomarker which can guide physicians to select best treatment regimen is clearly unmet need in this era of personalized care.

In breast cancers, multitudes of genes are known to be suppressed by epigenetic mechanisms in the promoter region, and *RASSF1A* is one of the most frequently silenced genes in this type of cancer (6). Moreover, inactivation of the RASSF1A

gene by the methylation is known as an early event in the carcinogenesis of breast cancer (7-9), and recent studies have suggested detection of methylated *RASSF1A* in serum or body fluids as a biomarker for early breast cancer development (9-11). Furthermore, there is a growing amount of data, demonstrating that methylation status in the promoter region of *RASSF1A* is associated with poor prognosis in breast cancer patients (12-14) as well as patients with other cancers (15-18). Epigenetic or genetic modification of RASSF1A gene may have effects on key biological processes including apoptosis, cell cycle regulation, mitosis, and microtubule dynamics in cancer cells, which have been shown in numerous studies (19-22). Our recent data showed that RASSF1A protein enhances microtubule-targeted drugs in inducing G2/M arrest in non-small cell lung carcinoma (NSCLC) cells (23), implicating *RASSF1A* in cytotoxicity of anti-mitotic drugs. However, only a few studies have thus far been focused on the biologic consequence of the methylation of *RASSF1A* in various cancer types (24,25), but not in breast cancer. Therefore, we hypothesized that commonly-silenced *RASSF1A* in breast cancer can modulate the response to the frequently used cytotoxic agent, docetaxel.

To this end, we prospectively investigated the relationship between the methylation level of RASSF1A gene and response to docetaxel-based chemotherapy in patients with locally advanced or recurrent breast cancer using methylation-specific pyrosequencing analysis, and found that the mean level of methylation in the promoter region of *RASSF1A* showed a significant association with response to docetaxel-based chemotherapy. Further *in vitro* study showed that *RASSF1A* had cooperative activity in suppression of cancer cell growth and proliferation by enhancing docetaxel-induced cell cycle arrest.

Materials and methods

Cell culture and transfection. Three human breast cancer cell lines, ZR-75-1, MDA-MB-231, and MCF-7 cells, were obtained from the Korean Cell Line Bank (Seoul, Korea). For transfection, cells were transiently transfected with 1 μg of RASSF1A DNA (kindly provided by Dr S. Tommasi of the Beckman Research Institute, Duarte, CA, USA) or the empty pcDNA 3.1 plasmid using the Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). To generate cells stably expressing RASSF1A, cells were transfected and selected in geneticin (G418) (Life Technologies Inc., Grand Island, NY, USA). Only low passage cells (passage <10) were used for experiments.

Patients and tissues. A total of 45 primary breast cancer tissues were obtained from newly diagnosed breast cancer patients who were scheduled to receive docetaxel-based chemotherapy for locally advanced or metastatic disease. Ten non-cancer tissues were obtained from 5 patients with DCIS and 5 normal breast tissues from reduction mammoplasty. All patients gave their informed consent, and the study was approved by the Ethics and Scientific Committees of our Institution (no. 2008-267). Tissues were processed and stored as frozen block at -80˚C or paraffin-embedded block in Korea Lung Tissue Bank (Seoul, Korea) until analysis. Clinicopathological data involving age, TNM stage, tumor grade, ER/PR/HER-2 expression status, and response to docetaxel-based chemotherapy were collected. Responders in this study were defined as the patients who achieved a partial or

complete response to chemotherapy, according to the RECIST criteria version 1.1 (26).

Cell proliferation assays. Cell proliferation was assessed by both colorimetric MTT assay and [^{3}H]-thymidine incorporation. Thus, each breast cancer cell line was incubated with docetaxel for 48 h after 24 h of serum starvation. For the MTT assay, the cells were incubated for 4 h with MTT reagent and then lysed in 50% dimethylformamide (DMSO) and 20% SDS-PAGE at 37˚C. Optical densities (OD) at 550 and 670 nm were measured using a plate reader (BioRad, Hercules, CA, USA), and differential OD between 550 and 670 nm (OD 550-670 nm) was determined. For [^{3}H]-thymidine incorporation assay, 1 μl of [^{3}H]-thymidine (Amersham, Buckinghamshire, UK) was added per well and plates were incubated for 18 h. Then, cells were harvested in a cell harvester, and radioactivity (dpm) was counted using a β-counter. The results were expressed as dpm/mg protein or as percentage of the control (defined as 100%).

Methylation-specific PCR (MSP) analysis. Genomic DNA was extracted from control, 5-aza-deoxycytidine-treated (10 μM for 3 days) cells using QIAamp® DNA Blood Mini Kit (Qiagen, Valencia, CA, USA), by following the manufacturer's instructions. Bisulfite modification of DNA (1 μg) was performed using the EZ DNA Methylation-Gold kit™ (Zymo Research, Orange, CA, USA). Based on the promoter sequence of *RASSF1A*, methylation- and unmethylation-specific primers were designed using Serologicals CpGware software (http://apps.serologicals. com/CPGWARE/dna_form2.html) methylated, 5'-GCTAAC AAACGCGAACCG-3'; 5'-GGGTTTTGCGAGAGCGCG-3' and unmethylated, 5'-CACTAACAAACACAAAC CAAAC-3'; 5'-GGTTTTGTGAGAGTGTGTTTAG-3'; product sizes 169 and 169 bp, respectively (PMID: 11333291). Bisulfite-modified DNA (2 μl out of 10 μl elute) was used for each PCR. All experiments were performed in triplicate and repeated at least three times.

Quantitation of RASSF1A promoter methylation by pyrose-quencing. Bisulfite-treated DNA from tumors was used for PCR amplifications. Pyrosequencing was performed using the PSQ96ID system (Qiagen) including PyroMark Gold Q96 reagents. Primers were designed by the PyroMark Assay design 2.0 (Qiagen). The primers amplify a stretch of the *RASSF1A* exon 1 (Ensemble ID: ENST00000266020). The primers target CpG regions within this stretch. PCR was carried out using 2 μl bisulfite-treated DNA under the following conditions: 94˚C for 5 min, 45x (94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 30 sec), 72˚C for 5 min. Intactness of the PCR product was assessed by electro-phoresis on 2% agarose gel (Seakem® LE Agarose, Lonza) and subsequent ethidium bromide staining. The pyrose-quencing primers target a 46-nt segment, which lies within the previously amplified stretch of RASSF1A exon 1 and contains 7 CpGs(GTCGGGGGTTCGTTTTGTGGTTTCGTTCGG TTCGCGTTTGTTAGCGT).

Immunohistochemistry and immunofluorescence. Expression of RASSF1A protein in tissue samples and breast cancer cell lines were determined with anti-RASSF1A monoclonal antibody (clone eB114, eBioscience, Minneapolis, MI, USA) as previously described (27). The staining results were evaluated according to

the immunodetection of stain intensity and amounts of positive cells by two pathologists (A.K. and H.J.), who discussed each case until they reached a consensus. The degree of staining was subdivided as follows: the stain intensity could be from 0 to 3 (0, no staining; 1, focal or fine granular, weak staining; 2, linear or cluster, strong staining; and 3, diffuse, intense staining); and the positive cells in the observed breast tissue samples ranged from 0 to 3 in percentage (0, no staining; 1, <30%; 2, 30-70%; and 3, >70%). The samples were scored by their summation: 0-1 (-); 2-3 (+); 4 (++); 5-6 (+++). Any staining score 2 or above (+) was considered as positive expression.

For immunofluorescence, cells were plated on 18 mm coverslips and treated on the next day with 10 μM 5-aza-deoxy-cytidine (Sigma Co., St. Louis, MO, USA). After 48-h treatment, cells were fixed with 4% paraformaldehyde, permeabilized in 0.25% Triton X-100 in PBS, and blocked in PBS/5% BSA. RASSF1A was detected using anti-RASSF1A (eBioscience) followed by incubation with fluorescent conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA). After washing, cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma Co.), mounted on glass slides, and examined by a DP40 (Olympus, Tokyo, Japan).

Western blot analysis. The expressions of proteins were detected using relevant antibodies (anti-RASSF1A, Cdk1, Cdk2, Cdk4, cyclin B, Cell Signaling, Boston, MA, USA; anti-cyclin D1, Calbiochem, San Diego, CA, USA; anti-p21, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The collected cells were lysed with 10 μl of dilution buffer containing 20 mM 3-morpholinopropanesulfonic acid (pH 7.2), 25 mM β-glycerophosphate, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, and protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). The total

cell lysates were resolved on 8-12% SDS-PAGE as previously described (23).

Cell cycle analysis. The cell cycle distribution was determined by flow cytometric analysis of propidium iodide (PI) labeled cells. Thus, cells were seeded at 1×10^5 cells/60-mm plate and treated with docetaxel. The cells were harvested, fixed in 70% ethanol, and then stored at -20°C. The cells were then washed twice with ice-cold PBS and incubated with ribonuclease and PI. Cell cycle was analyzed by BD FACScan flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) and CellQuest software.

Statistical analysis. Mean methylation level of RASSF1A according to the clinicopathological parameters were analyzed by Student's t-test. A logistic regression model was applied to determine whether a factor was independent predictor of response to chemotherapy in a multivariate analysis. A two-sided 0.05-level test was determined for statistical significance. All data analyses were conducted with SPSS software (SPSS Inc., Chicago, IL, USA).

Results

RASSF1A protein and mRNA expressions are downregulated in breast cancer cell lines and primary breast cancers. Western blot analysis and RT-PCR revealed the loss of RASSF1A protein and mRNA expression in 3 breast cancer cell lines (ZR-75-1, MDA-MB-231, MCF-7) (Fig. 1A). Next, we evaluated the expression of RASSF1A using immunohistochemical analysis, and Fig. 1B shows that benign breast tissues and DCIS tissues were strongly positive for RASSF1A, whereas invasive ductal carcinoma tissues were negative for RASSF1A staining. Further analysis of primary invasive ductal carcinoma of breast revealed that RASSF1A was lost in 27 (60%) out of 45 primary breast samples (Table I), thus indicating that RASSF1A expression is low or lost in many cases of primary breast cancer specimens as well as selected breast cancer cell lines. These results support earlier hypothesis that the loss of RASSF1A is one of the important events in the breast cancer carcinogenesis (7,8).

RASSF1A promoter is methylated in breast cancer cell lines. Next, we investigated underlying mechanism of downregulation of *RASSF1A*, and tested whether any epigenetic modification regulates RASSF1A gene expression in breast
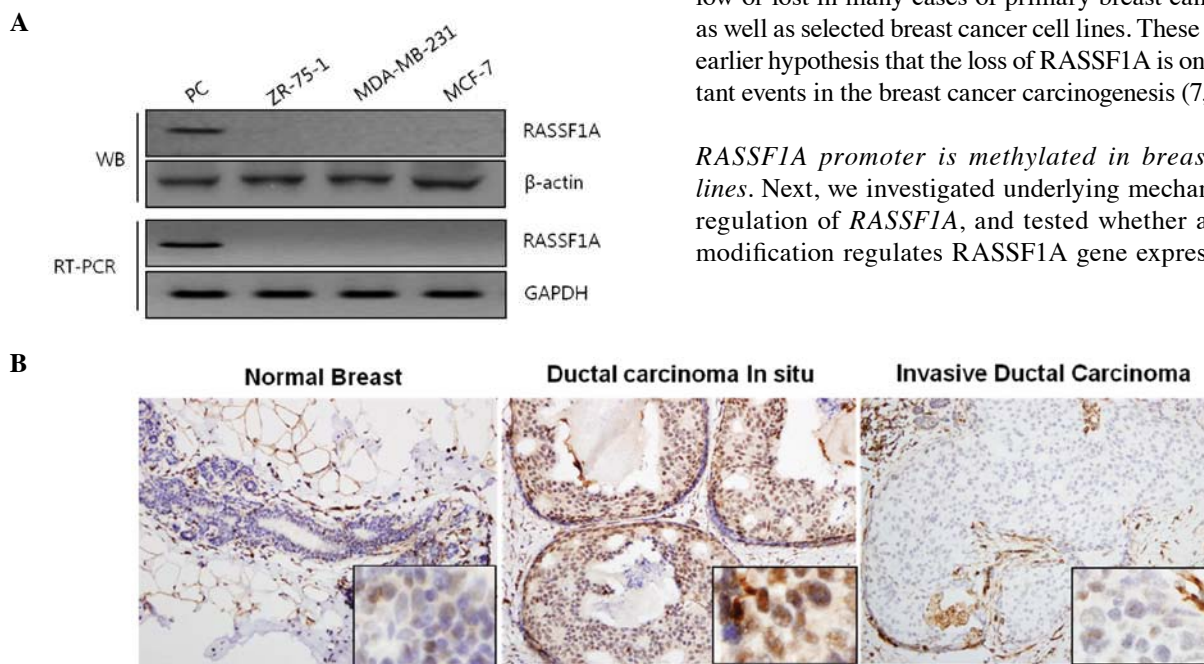

Figure 1. Decreased expression of RASSF1A in breast cancer. (A) Expression of RASSF1A in 3 breast cancer cell lines, determined by western blot analysis and RT-PCR analysis. β-actin and GAPDH were used as internal controls. (B) RASSF1A protein expression in normal breast, DCIS and invasive ductal carcinoma. Brown staining in immunohistochemistry is indicative of RASSF1A protein expression (magnification x100).

Table I. Correlation between RASSF1A gene methylation, protein expression, and clinicopathological characteristics of breast cancer patients (n=45).

Clinicopathological characteristics	*RASSF1A* methylation			RASSF1A expression		
	Number of patients (no.)	Level of methylation (%, mean±SD)	P	Positive (no.)	Negative (no.)	P
Age (years)						
<35	4	17.7±18.7	NS	1	3	NS
≥35	41	21.7±16.6		17	24	
Menopausal status						
Premenopausal	28	18.5±16.3	NS	7	21	0.013
Postmenopausal	17	27.0±16.2		6	11	
Primary tumor size (cm)						
<2.0	13	16.9±16.6	NS	6	7	NS
≥2.0	32	23.7±16.5		20	12	
Stage						
II-III	30	18.7±14.4	0.088	10	20	NS
IV, recurrent	15	27.7±16.4		8	7	
ER						
Positive	19	28.2±17.7	0.024	6	13	NS
Negative	26	17.0±14.4		12	14	
PR						
Positive	15	29.5±17.4	0.026	3	12	NS
Negative	30	17.9±15.1		15	15	
HER-2						
Positive	16	21.6±15.6	NS	9	7	NS
Negative	29	21.8±17.4		9	20	
Triple negativity						
Yes	15	14.4±11.9	0.041	5	10	NS
No	30	24.8±17.0		13	17	
Tumor grade						
G1	2	2.5±0.1	NS	0	2	NS
G2	19	21.7±19.1		7	12	
G3	24	23.3±15.4		11	13	
Ki-67 (%)						
≥20	35	22.0±17.5	NS	17	18	0.034
<20	10	21.6±16.7		1	9	
p53 (%)						
≥10	28	22.9±17.1	NS	10	18	NS
<10	17	19.8±16.1		8	9	
Response to chemotherapy						
Responders	32	20.1±11.2	0.042	14	17	0.343
Non-responders	13	30.6±8.5		3	10	

ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2; NS, not statistically significant.

cancer cell lines, as reported previously (28,29). RASSF1A mRNA and protein levels were restored after treatment with the demethylating agent in dose-dependent manner, implying that DNA methylation might be a mechanism involved in the *RASSF1A* inactivation in the selected cells (Fig. 2A, upper panel). Moreover, immunofluorescence study revealed that RASSF1A protein expression was restored by the demethyl-ating agent, mainly in the perinuclear area of the cancer cells (Fig. 2A, lower panel). Based on the results of the demethyl-ating agent, the methylation status of the *RASSF1A* promoter was determined by MSP analysis before and after treatment of 3 different breast cancer cell lines with 5-aza-deoxycytidine, and the demethylating agent induced the appearance of unmethylated alleles of the *RASSF1A* (Fig. 2B). Therefore, the
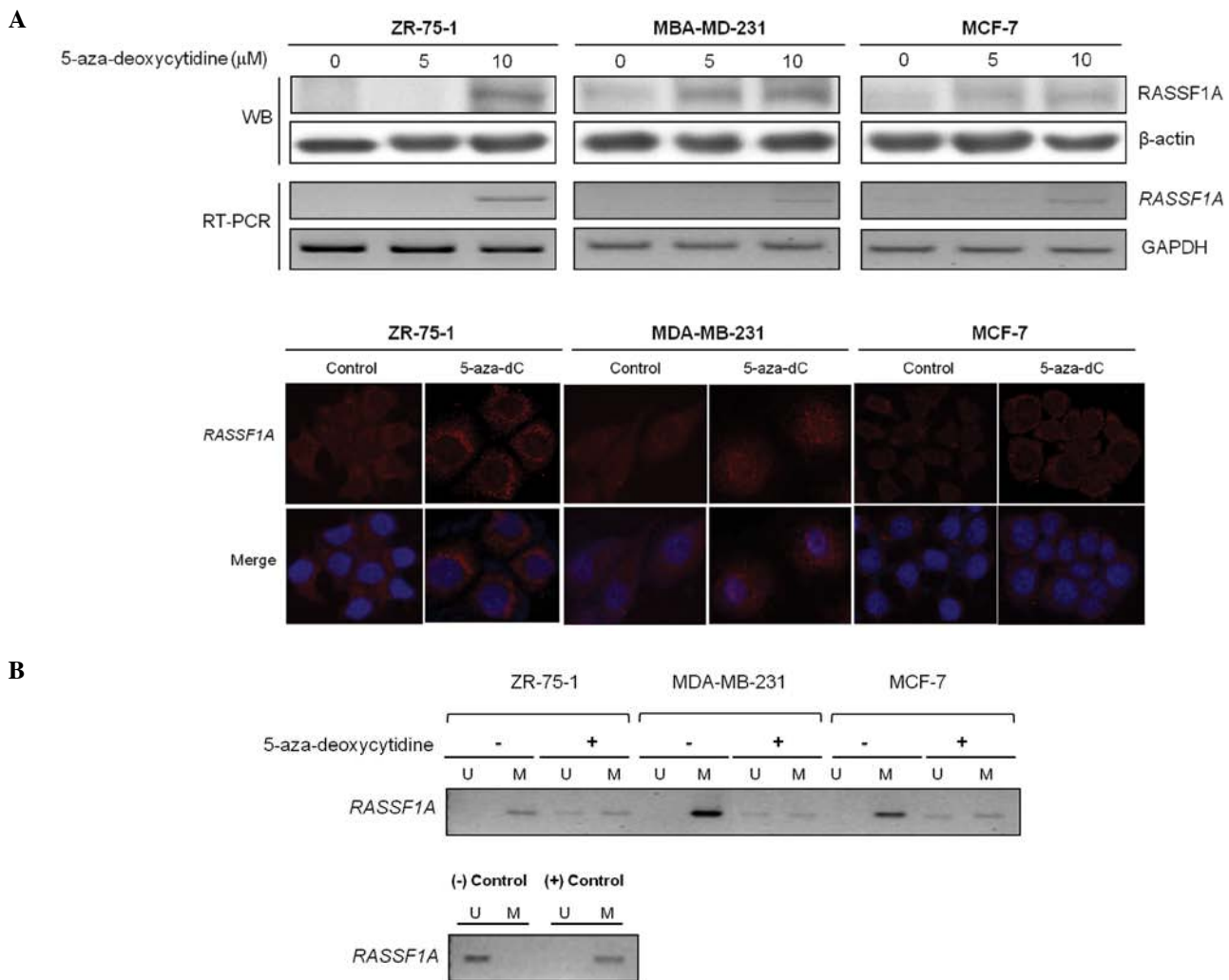
Figure 2. Suppression of RASSF1A by methylation in breast cancer. (A) Re-expression of RASSF1A by treatment of 3 different breast cancer cell lines with 5-azadeoxycytidine. Western blot analysis and RT-PCR show that RASSF1A is re-expressed by treatment with increasing concentrations of 5-azadeoxycytidine (upper panel). β-actin and GAPDH were used as internal controls. Immunofluorescence study confirms the re-expression of perinuclear RASSF1A (red) in breast cancer cells by 5-azadeoxycytidine treatment (x400, lower panel). (B) MSP analysis before and after treatment of ZR 75-1, MDA-MB-231 and MCF-7 cells with 5-azadeoxycytidine. Bisulfite-converted universally methylated human DNA standard served as a positive control for methylation, and human lymphocyte DNA was included as an unmethylated *RASSF1A*. M, methylated alleles; U, unmethylated alleles.

decreased *RASSF1A* gene expression was associated with the *RASSF1A* promoter methylation in breast cancer.

Quantitative analysis of RASSF1A methylation by pyrosequencing and relationship with clinicopathological characteristics of breast cancer patients. The associations between the promoter methylation status of *RASSF1A* and relevant demographic and clinicopathological characteristics are shown in Table I. The 7 CpG sites were analyzed by methylation-sensitive pyrosequencing and a representative pyrogram is shown in Fig. 3A. Pyrosequencing showed that the mean level of methylation in 7 CpG sites in the 45 primary breast cancers ranged from 1.08% to 59.8%, and 24 of 45 tumors (53.3%) exhibited a high level of methylation (>20%). Interestingly, triple negative tumors had significantly lower level of methylation in the promoter region of *RASSF1A* at 14.4±11.9% compared to non-triple negative tumors (24.8±17.0%, p=0.041; Fig. 3B). Furthermore, it is of an interest to note that the mean level of methylation in *RASSF1A* was significantly higher in

patients who did not respond to docetaxel-based chemotherapy (30.6±8.5 %) than patients with partial or complete response (20.1±11.2 %, p=0.042; Fig. 3C). Mean level of methylation in 7 CpG sites of the promoter region of *RASSF1A* in ER-positive tumors was significantly higher at 28.2±17.7% compared to ER-negative tumors (17.0±14.4%, p=0.024). Positive for PR showed the same pattern of association as observed in ER (positive: 29.5±17.4%, negative: 17.9±15.1%, p=0.026). However, there was no significant correlation between RASSF1A protein expression and the level of promoter methylation or any clinicopathological characterisitics in primary breast cancers in this study (Table I).

Multivariate analysis showed that low level of methylation (<20%) in *RASSF1A* was an independent predictor for response to chemotherapy after adjusted for ER (negative vs positive), PR (negative vs positive), HER-2 (negative vs positive), tumor grade (grade I/II vs III), p53 immunostaining (low; <20% vs high; ≥20%), and Ki-67 (low vs high) in these 45 patients (odds ratio = 15.99, 95% confidence interval = 1.16-219.29,
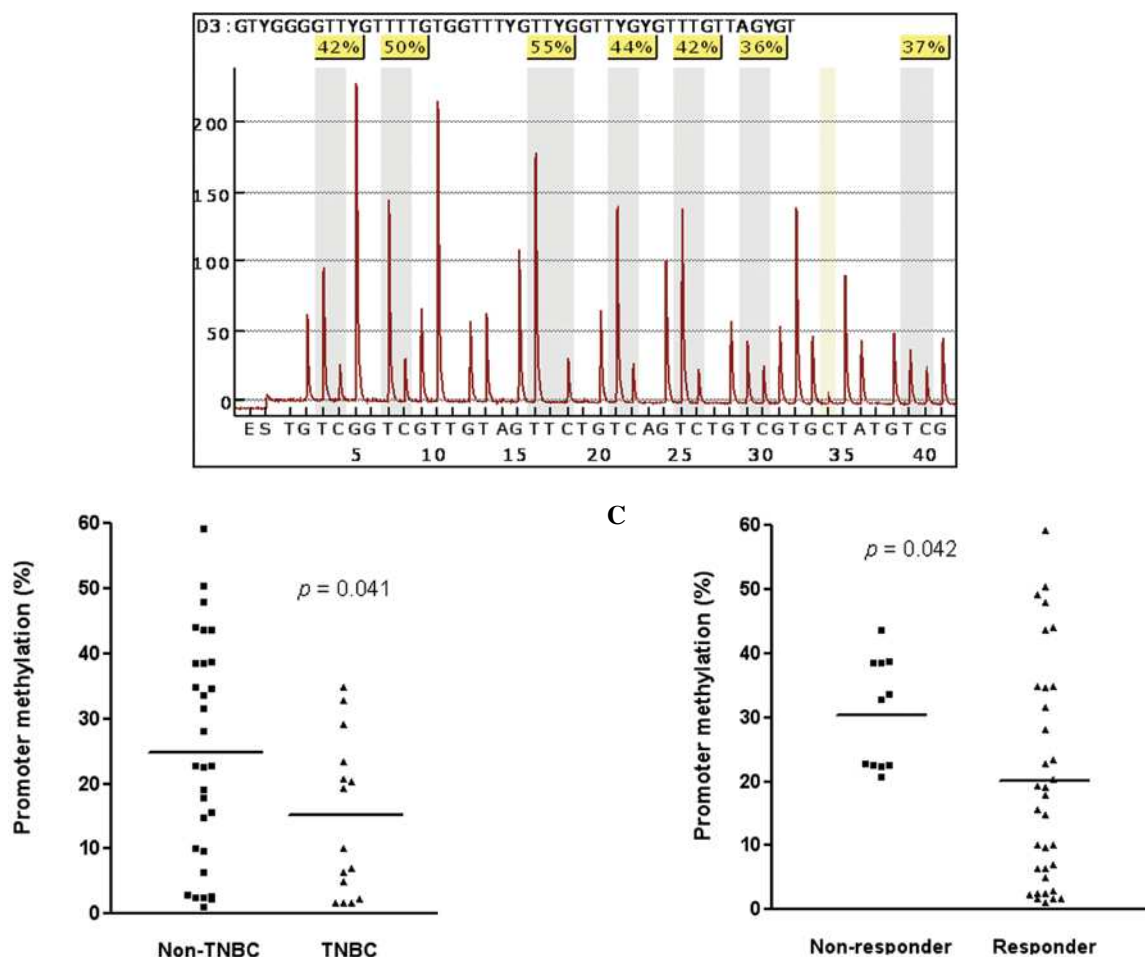
A

B

C

Figure 3. Quantitative analysis of *RASSF1A* methylation by pyrosequencing. (A) Pyrogram from quantification of methylation of 7 CpG sites at the *RASSF1A* promoter. During the pyrosequencing reaction, a 'C' is incorporated if the template CpG is methylated, while a 'T' is incorporated if the template CpG is unmethylated. In the resulting pyrogram, the proportion of C:T reflects the degree of methylation at the particular CpG sites assessed. (B) Comparison of mean level of methylation of RASSF1A between non-triple negative breast cancer (TNBC) and TNBC patients. Bar indicates mean level of methylation of RASSF1A in each group. P denotes significance of t-test. (C) Comparison of mean level of methylation of RASSF1A between non-responder and responder to chemotherapy. Bar indicates mean level of methylation of RASSF1A in each group.

Table II. Multivariate logistic regression model for response to chemotherapy.

Factor	Response to chemotherapy	
	OR (95% CI)	P
ER (negative vs positive)	0.00	0.99
PR (negative vs positive)	8.970E8	0.99
HER-2 (negative vs positive)	4.31 (.53-34.96)	0.17
Grade (I vs II/III)	0.61 (.016-23.35)	0.79
p53 immunoreactivity (low vs high)	49.02 (1.46-1639.25)	0.03
Ki-67 immunoreactivity (low vs high)	0.17 (.004-7.06)	0.35
RASSF1A methylation (low vs high)	15.99 (1.16-219.29)	0.03

OR, odds ratio; CI, confidence interval; ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2.

p=0.03; Table II). Patients with low level of p53 immunos-taining (<20%) were also more sensitive to docetaxel-based chemotherapy than the patients with tumors of high level of p53 immunostaining.

RASSF1A modulates docetaxel-induced cell death. Since the docetaxel-based chemotherapy seemed less effective in patients with methylated promoter region of *RASSF1A*, we examined potential interaction between *RASSF1A* and
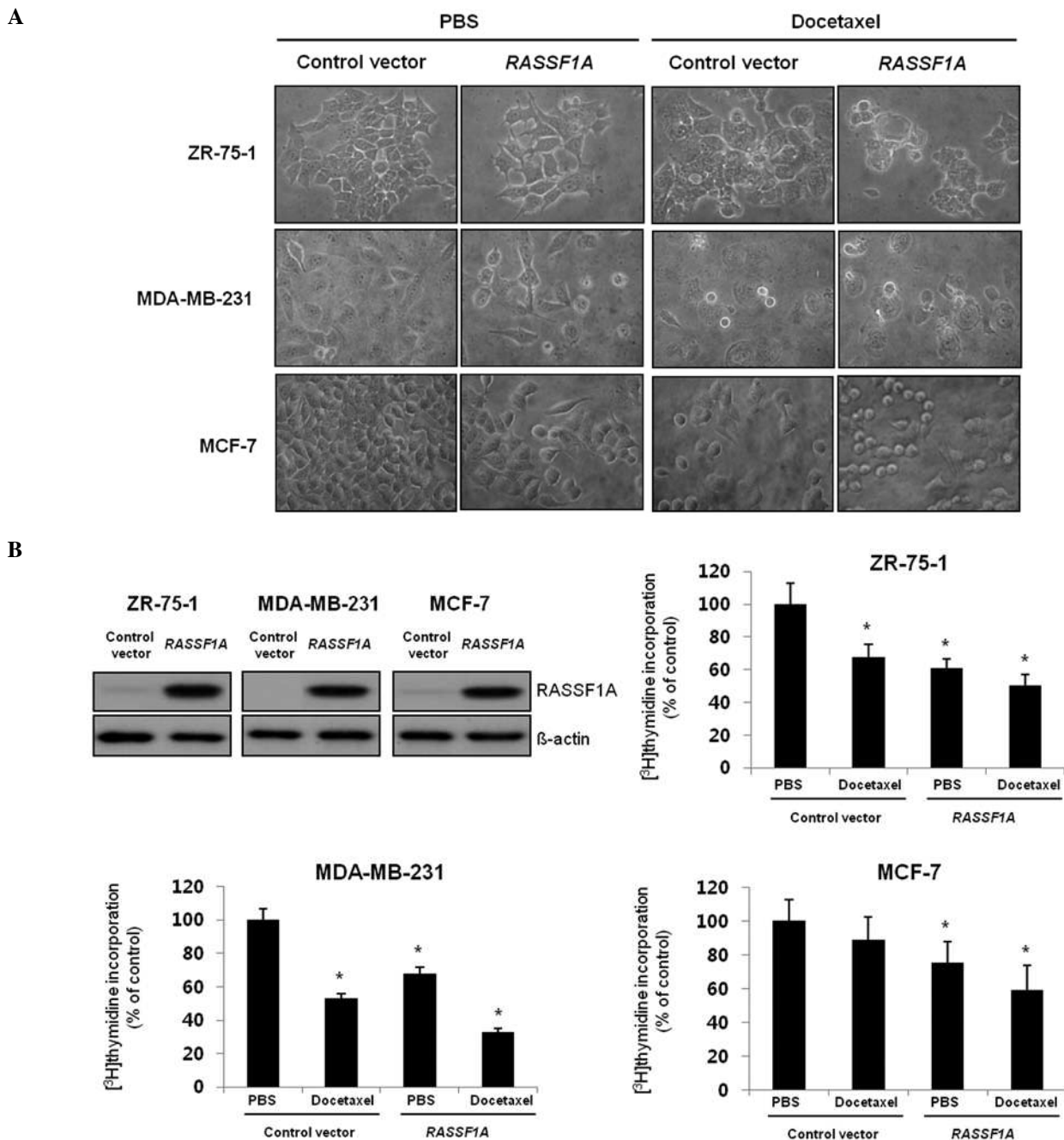
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Figure 4. RASSF1A modulates growth inhibitory effect of docetaxel. (A) The effect of RASSF1A on docetaxel-induced cell growth inhibition in 3 breast cancer cell lines was analyzed by phase contrast light microscopy. Control vector indicates pcDNA3.1. (B) Upper right panel, expression status of RASSF1A in RASSF1A and control vector stably transfected cells. Upper left and low panel, [³H]-thymidine incorporation assay in each stably transfected breast cancer cell line. Statistical analysis was performed on the basis of PBS-treated, control vector transfected cells in each cell type. $p<0.05$.

docetaxel. Therefore, we transfected *RASSF1A* or control vector to the breast cancer cells (ZR-75-1, MDA-MB-231, MCF-7), and investigated whether reintroduction of *RASSF1A* had any growth inhibitory effect on breast cancer cells. As shown in Fig. 4A, the number of round-shape cells which are typical features of apoptotic cells was increased in *RASSF1A* transfected cells compared with the control vector-transfected cells and the effect was greatly enhanced after treatment of docetaxel. Next, the cooperative effect was confirmed in a proliferation assay showing that docetaxel induced a marked reduction of [³H]-thymidine incorporation in the cells stably transfected with *RASSF1A*, compared with the control vector-transfected

cells (Fig. 4B). Therefore, these results indicate that the anti-proliferative effect of docetaxel was enhanced in the presence of *RASSF1A* in breast cancer cells.

RASSF1A enhances docetaxel-induced cell cycle arrest. The cooperative effects of *RASSF1A* and docetaxel in the cell survival and proliferation prompted us to investigate whether reintroduction of *RASSF1A*, in addition to docetaxel, has any impact on the cell cycle progression. When the *RASSF1A*-transfected breast cancer cells were treated with docetaxel for 24 h, they showed statistically significant increase of G2/M phase cell population, compared to those of empty
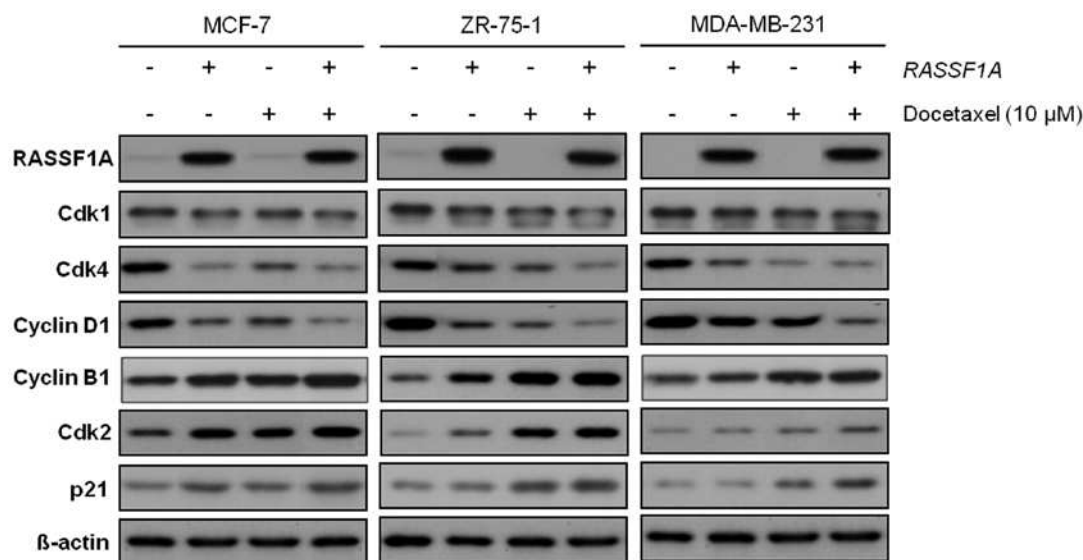
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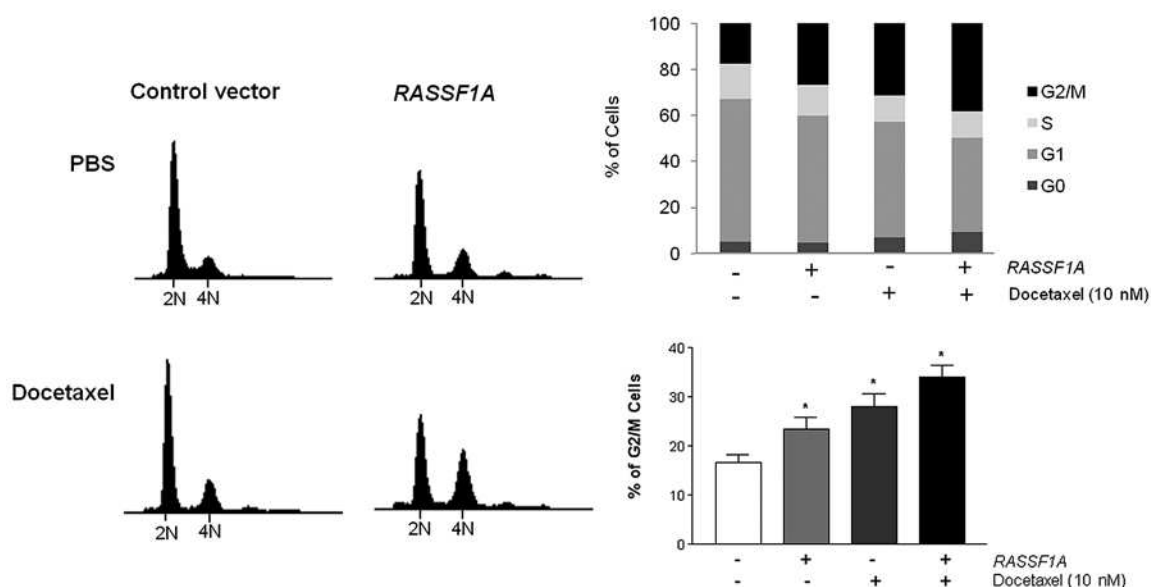
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Figure 5. RASSF1A enhances docetaxel-induced cell cycle arrest. (A) The effect of RASSF1A on docetaxel-induced cell cycle arrest was evaluated in MDA-MB-231 cells stably transfected with *RASSF1A* or control vector. Cells were analyzed for cell cycle progression. Positions of cell populations with 2N and 4N DNA content are indicated (upper panel). Histogram plotting of the cell cycle progression is shown in lower left, while comparison of cells in G2/M phase is shown in lower right. Columns, means (n=3); bars, SEM; *p<0.05. Expression of cell cycle regulating proteins is shown by western blot analysis. Three breast cancer cell lines transfected with RASSF1A and control vector were analyzed with or without exposure to docetaxel (10 nM).

vector transfected cells (p=0.02, Fig. 5A). The result indicates that the cooperative effect of *RASSF1A* and docetaxel is accompanied with cell cycle arrest at the G2/M phase. Further analysis using western blot analysis showed that the cell cycle arrest at the G2/M phase in the breast cancer cells was associated with the induction of cyclin B1, Cdk2 and p21 by both RASSF1A and docetaxel (Fig. 5B). Maximum cyclin B1, Cdk2 and p21 induction was observed when docetaxel was applied to the *RASSF1A*-transfected breast cancer cells, whereas Cdk1, Cdk4, and cyclin D1 were greatly decreased when breast cancer cells reintroduced with *RASSF1A* were treated with docetaxel. These findings indicate that the effects of *RASSF1A* and docetaxel on the G2/M phase cell cycle arrest are associated with accumulation of cyclin B1/Cdk2/p21 and suppression of Cdk1, Cdk4, and cyclin D1 in breast cancer cells.

Discussion

RASSF1A has been reported to be frequently silenced in many cancer types including breast cancer; however, the biologic relevance in clinical standpoint has not yet been clearly characterized. Because there is a probability that unsatisfactory response in breast cancer treatment may in part be due to silencing of gene, the role and clinical relevance of a frequently silenced tumor suppressor gene, *RASSF1A*, was examined in this study. In the current study, we found that the promoter region of *RASSF1A* was frequently methylated in the primary breast cancer samples from 45 locally advanced or metastatic cancer patients, in good agreement with previous reports. In addition, we showed that level of methylation of *RASSF1A* was independent factor for response to docetaxel-based chemotherapy in

this study. Moreover, RASSF1A enhanced docetaxel-mediated growth inhibition by inducing cell cycle arrest and altering the level of related proteins. Our results demonstrated that methylation status of *RASSF1A* modulates the efficacy of docetaxel chemotherapy and regulates docetaxel mediated cell cycle arrest in breast cancers.

There are a few studies suggesting the methylated *RASSF1A* as a potential biomarker for poor prognosis in breast cancer (12-14). However, no biologically relevant mechanism, thus far, has been presented. In this context, we showed herein that the methylation status of *RASSF1A* was significantly associated with response to docetaxel-based chemotherapy: cell line studies showed that *RASSF1A per se* had docetaxel-like effect in suppressing growth and proliferation of breast cancer cells via inducing cell cycle arrest. Furthermore, cooperative activity of *RASSF1A* in docetaxel-induced cell cycle arrest could be a potential mechanism for differential response among breast cancer patients.

Docetaxel is one of the prototype taxane and a front line chemotherapeutic agent in the treatment of breast, ovary, lung, and head and neck cancers. Despite of its widespread use and success in clinical practice, there are significant shortcomings including myelosuppression, peripheral neuropathy, and primary or secondary resistance (30). Nevertheless, there is no rational biomarker for the identification of patients who are most likely to respond to docetaxel. Of the predictive markers for the response to neoadjuvant chemotherapy, biology-based tumor types have been shown to be most consistent and reproducible among the studies (4). However, it is still unsatisfactory marker due to strong heterogeneity within the subgroups. More mechanistic markers such as Ki-67 (31) and topoisomerase IIα (32) have also been suggested as valuable predictive markers for response to anthracyclines and other chemotherapeutics. For the taxanes, however, no biologic marker has been suggested for the prediction of response based on mechanistic study. In the present study, we found a significant association between the methylation level of *RASSF1A* and response to docetaxel-based chemotherapy in patients with locally advanced or recurrent breast cancer. Additional multivariate analysis showed that low level of methylation (<20%) in the promoter region of *RASSF1A* was an independent factor for response to docetaxel-based chemotherapy after adjusted for ER, PR, HER-2, p53 immunoreactivity, and Ki-67 staining. Furthermore, the specific modulating effect of *RASSF1A* on docetaxel-induced cytotoxicity was demonstrated in the cell culture system. Therefore, the loss of *RASSF1A* for the prediction of docetaxel-based chemotherapy also seems to deserve further investigation.

It is now well known that taxanes exert their anti-cancer effect by inducing mitotic arrest of cancer cells. When cells are exposed to anti-mitotics, they are arrested in mitosis and then undergo one of several fates; death in mitosis, unequal division, or exit without division (33). However, little is known about how cells respond to this prolonged cell cycle delay. Recent studies suggested that commitment to mitotic exit is determined by the level of cyclin B1 in anti-mitotics exposed cells (34). Therefore, it seems important to define the factors that govern the degradation of cyclin B1 during a prolonged mitotic arrest. Notably, our present results showed that overexpression of *RASSF1A* in breast cancers induced the accumulation of cyclin B1, as observed in docetaxel-treated cells in consistent with previous studies (21,35). Furthermore, the accumulation of cyclin B1 in the *RASSF1A*-transfected cells was significantly enhanced by docetaxel. Thus, *RASSF1A* could be considered as one of the factors controlling degradation of cyclin B1 during the mitotic arrest which was induced by docetaxel in breast cancer cells. In the present study, the accumulation of p21 and decreased levels of Cdk 1, Cdk 4, and cyclin D1 were also found to be involved in the induction of cell cycle arrest and apoptosis by both docetaxel and *RASSF1A*. In addition, decreased expression of Cdk 1 by RASSF1A might also contribute to cell death after mitotic exit, since Cdk 1 has been shown to inhibit caspase-9 (36). Our present observations therefore show that RASSF1A might be an important biologic contributor to docetaxel-induced cell cycle arrest and, finally, cell death in breast cancer cells.

In conclusion, the data presented herein led us to propose that hypermethylated *RASSF1A* might be an important modulating factor for efficacy of docetaxel-based chemotherapy in breast cancers. We also provided mechanistic data that the tumor suppressor, *RASSF1A*, has a cooperative effect along with docetaxel in inducing cell cycle arrest in breast cancer. Our results are expected to contribute to the identification of biomarkers in predicting the response of breast cancer patients to docetaxel. Since our data are limited by the small sample size and locally advanced stage cancers in most of the cases, the statistical significances shown here are still marginal. Therefore, further research with a large number of clinical samples is needed to confirm our results.

Acknowledgements

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