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Journal of Cellular Biochemistry 113:322–328 (2012)

Journal of Cellular Biochemistry

Down-Regulation of the Cavin Family Proteins in Breast Cancer

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ABSTRACT

Caveolae are abundant membrane domain on the cell surface of many mammalian cell types and are implicated in a wide range of physiological processes. The caveolae structural protein caveolin-1 is often mutated or deregulated in cancer, and cavin family protein serum deprivation response factor-related gene product that binds to C-kinase (SRBC) has been found to be epigenetically inactivated in lung, breast, and gastric cancer. Both caveolin-1 and SRBC have been proposed to function as tumor suppressors. Polymerase 1 and transcript release factor (PTRF) is the essential component for caveolae formation. The regulation of PTRF expression in cancer has not been characterized. We report here that the cavin family protein PTRF, SRBC and serum deprivation response protein were down regulated in breast cancer cell lines and breast tumor tissue. We further show that down-regulation of PTRF in breast cancer cells was associated with the promoter methylation. As caveolin-1 and cavin family proteins are required for caveolae formation and function, the reported tumor suppression function of caveolin-1 and SRBC may be due to the deregulation of caveolae and its down-stream signaling. Thus, the caveolae is a potential therapeutic target and the expression of cavin family proteins could be a useful prognostic indicator of breast cancer progression. J. Cell. Biochem. 113: 322–328, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: CAVEOLAE; PTRF; SRBC; SDPR; CAVEOLIN-1; METHYLATION

Caveolae are specialized invaginations of the plasma membrane that are implicated in diverse cellular functions including signal transduction, lipid regulation, endocytosis, and tumorigenesis [Parton and Simons, 2007]. The major components of caveolae consist of the caveolin family (caveolin-1, caveolin-2, and caveolin-3) and the cavin family proteins (Polymerase 1 and transcript release factor (PTRF)/cavin-1, serum deprivation response protein (SDPR)/cavin-2, serum deprivation response factor-related gene product that binds to C-kinase (SRBC)/cavin-3, and MURC/cavin-4) [Parton and Simons, 2007; Bastiani and Parton, 2010; Hansen and Nichols, 2010]. Caveolin-1 and caveolin-2 are coexpressed and form a hetero-oligomeric complex in many cell types, whereas caveolin-3 is expressed exclusively in skeletal and cardiac muscles [Liu et al., 2002; Parton and Simons, 2007; Nabi, 2009]. The cavin family proteins can form a complex on caveolae and are important in concert with caveolin-1 for caveolae

Abbreviations: PTRF, polymerase 1 and transcript release factor; SDPR, serum deprivation response protein; SRBC, serum deprivation response factor-related gene product that binds to C-kinase; 5-azaC, 5-aza-2′-deoxycytidine

Lin Bai and Xiaoli Deng contributed equally to this work.

Competing interests: The authors declare that they have no competing interests.

Authors' contributions: LB and XD carried out the majority of the experiments and data analysis; QL participated the expression analysis; WA and DA participated immunochemical analysis; ZG, YX and YD carried out the tissue sample collection, LB, XD and YSC conceived and designed the experiments, and wrote the article.

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 30671065, 31171320; Grant sponsor: "973" Project of the Ministry of Science and Technology; Grant numbers: 2007CB507402, 2007CB914402, 2009CB18904.

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Received 4 May 2011; Accepted 6 September 2011 • DOI 10.1002/jcb.23358 • © 2011 Wiley Periodicals, Inc.
Published online 12 September 2011 in Wiley Online Library (wileyonlinelibrary.com).

biogenesis and function [Bastiani and Parton, 2010; Hansen and Nichols, 2010]. The genetic alterations and deregulation of caveolae component genes have been linked to the progression of breast cancer. For example, *caveolin-1* gene was mapped to a suspected tumor suppressor locus (D7S522/7q31.1) which is frequently lost in various carcinomas including breast cancer [Williams and Lisanti, 2005; Sotgia et al., 2006]. In addition, studies have proved that recombinant expression of caveolin-1 inhibited anchorage-independent growth and invasiveness in MCF7 breast cancer cell line, and inactivation of caveolin-1 accelerated mice mammary lesion development or hyperplasia [Fiucci et al., 2002; Williams et al., 2003, 2004]. Human *SRBC* located at 11p15.5–p15.4 chromosome region, where loss of heterozygosity is frequently observed in sporadic breast and other types of cancers [Xu et al., 2001; Zochbauer-Muller et al., 2005; Lee et al., 2008]. Another report recently found that *SDPR* is suppressed in breast tumor [Li et al., 2008].

PTRF has recently been demonstrated to be the essential component in the biogenesis and function of caveolae [Hill et al., 2008; Liu and Pilch, 2008]. Mice that are deficient in PTRF exhibit a global loss of caveolae, dyslipidemia, and glucose intolerance [Liu et al., 2008]. The human PTRF gene is localized at 17q21.2, its mutations have been recently identified to be associated with generalized lipodystrophy [Hayashi et al., 2009; Rajab et al., 2010]. These observations underscore the physiological importance of PTRF. We recently showed that PTRF is up-regulated in senescent human fibroblasts and regulates cellular senescence through caveolae/p53/p21 pathway [Bai et al., 2011]. The regulation and function of PTRF in cancers have not been characterized. In this report, we showed that cavin family proteins PTRF, SRBC, and SDPR were down regulated in breast cancer cell lines and breast tumors, which suggests that caveolae may function as tumor suppressor and expression of cavin family proteins may serve as an indicator of breast cancer progression.

RESULTS

DOWN-REGULATION OF THE CAVIN FAMILY PROTEIN EXPRESSION IN BREAST CANCER CELL LINES

Cavin family proteins can form a complex in cytosol and on caveolae and are required in concert with caveolin-1 for caveolae biogenesis and function. We examined the expression pattern of the cavin family proteins (PTRF, SRBC, and SDPR) and caveolin-1 in a panel of cancer cell lines by Western blot and found that cavin family proteins were down-regulated significantly in breast cancer cell lines (data not shown). We further analyzed their expression by RT-PCR (Fig. 1A), real-time PCR (Fig. 1B) and Western blot (Fig. 1C, D) in immortalized human mammary epithelial cell line MCF 10A and breast cancer cell lines MCF7 and MDA-MB-231. The results showed all the cavin family genes and caveolin-1 were highly expressed in MCF 10A but down regulated in MCF7 and MDA-MB-231. These data suggest the expression of cavin family members is down regulated in breast cancer cells.

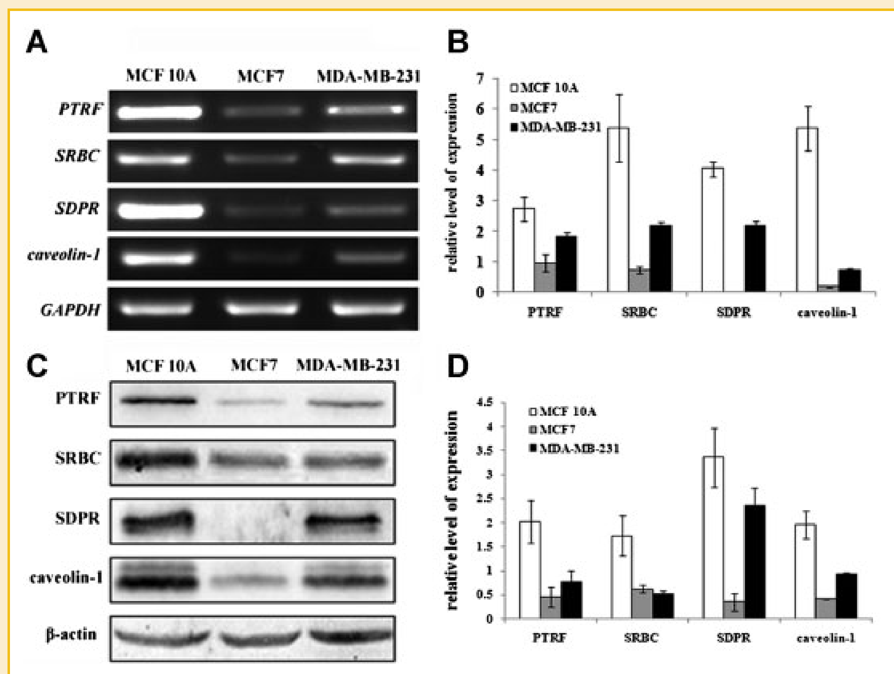


Fig. 1. Expression of cavin family proteins (PTRF, SRBC, and SDPR) and caveolin-1 in breast cell lines. The level of cavin family genes and *caveolin-1* were detected in immortalized breast cell line (MCF 10A) and breast cancer cell lines (MCF7 and MDA-MB-231) by RT-PCR (A) and real-time PCR (B). Expression of the cavin family proteins were detected by Western blot (C) and quantitative expression were shown in (D).

DOWN-REGULATION OF THE CAVIN FAMILY PROTEIN EXPRESSION IN BREAST CANCER TISSUE

To investigate the regulation of cavin family genes in breast tumor development, we examined their expression pattern in paired breast tumor and tumor-adjacent tissue. We found that both of PTRF and SRBC were down regulated in breast tumor tissue compared to their corresponding tumor-adjacent tissue in 7 out of the 10 pairs (Fig. 2). Similarly, we found that SDPR and caveolin-1 expression were also down regulated in breast tumor compared to their corresponding tumor-adjacent tissue. To further validate the Western blot data with a larger number of tissue samples, PTRF, SRBC, and SDPR expression were examined by immunohistochemistry assay in paraffin-embedded tissue array chips of 40 paired breast cancer and cancer-adjacent tissue. We graded the expression levels of PTRF, SRBC, and SDPR expression with a scale of 1+ to 3+ as low, medium, and high (Fig. 3A–C). Statistic analysis showed that PTRF, SRBC, and SDPR were significantly down regulated in cancer tissue compared to their cancer-adjacent counterparts. But there was no statistic difference between staining grade and tumor phase of the corresponding tissue samples (data not showed).

DOWN-REGULATION OF PTRF IN BREAST CANCER TISSUE AND CELL LINES IS ASSOCIATED WITH PROMOTER HYPERMETHYLATION

Several studies have found that down-regulation of SRBC was due to promoter hypermethylation in breast cancer cell lines [Xu et al., 2001; Tong et al., 2010]. We showed here that PTRF, SRBC, and SDPR were down regulated in breast cancer cells. We therefore analyzed the sequences in the promoter region (about 3,000 bp upstream of the first translation codon) of *PTRF* and *SDPR* genes. Similar to the *SRBC* gene, we found that there was a CpG island on *PTRF* promoters, but there is no typical CpG islands found in the 3,000 bp up-stream of the first translation code of *SDPR* gene (Fig. 4A), suggesting that methylation may be involved in the down-regulation of PTRF in breast cancer. Five pairs of breast tissue with apparent difference of PTRF expression at protein levels from Fig. 2 were chosen for analysis of *PTRF* expression by RT-PCR (Fig. 4B). The results further confirmed that down-regulation of PTRF in breast cancer tissue was at transcriptional level. To determine whether epigenetic mechanism is involved in the down-regulation of *PTRF* in breast cancer, the bisulfite genomic sequencing analysis of the *PTRF* promoter was performed in the paired breast cancer and cancer-adjacent tissue, and also in immortalized human mammary epithelial cell line MCF 10A and breast cancer cell lines MCF7 and MDA-MB-231. Correlated with the levels of *PTRF* expression, we found that the *PTRF* promoter was highly methylated in breast cancer tissue but barely methylated in cancer-adjacent counterparts. The methylation status of 25 CpG sites within 800 bp of the *PTRF* promoter (nucleotides −758 to −145) was evaluated using bisulfite genomic sequencing analysis. Ten PCR clones were sequenced to determine methylation frequency at individual CpG sites. The results indicated that the *PTRF* promoter was highly methylated in breast cancer tissue (88% in 503T, 36% in 511T, 92% in 540T, 92% in 551T, and 60% in 562T), but low methylated or unmethylated in cancer-adjacent counterparts (0% in 503N, 0% in 511N, 4% in 540N, 28% in 551N, and 12% in 562N). Similarly, the *PTRF* promoter was highly methylated in the breast cell lines MCF7 (56%) and MDA-MB-231 (36%), but PTRF promoter was low methylated in MCF 10A cell line (12%) (Fig. 4C). In addition, treatment with the demethylation reagent 5′-azacytidine induced the expression of *PTRF*, *SRBC*, and *caveolin-1* but not *SDPR* in MCF7 and MDA-MB-231 cells (Fig. 4D). Collectively, these data indicate that PTRF was down regulated in breast cancer through epigenetic mechanisms.

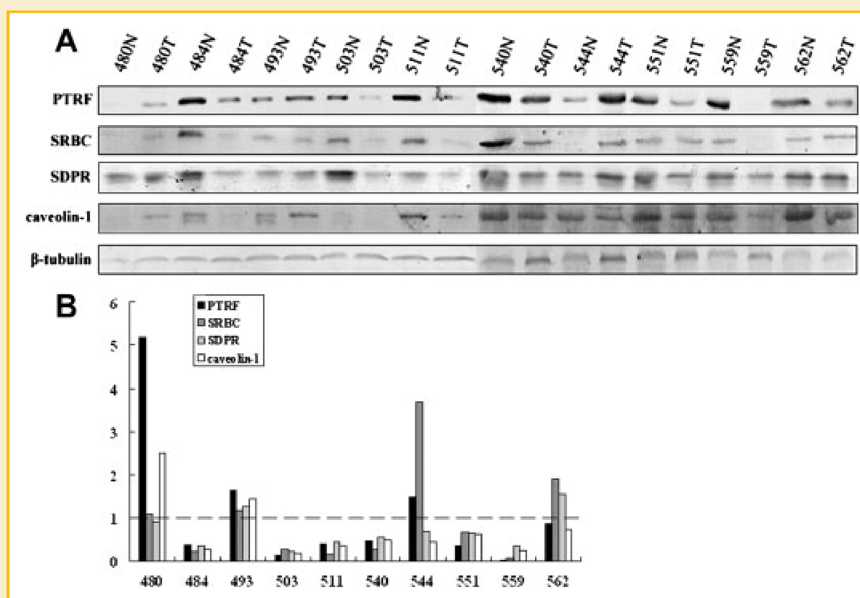
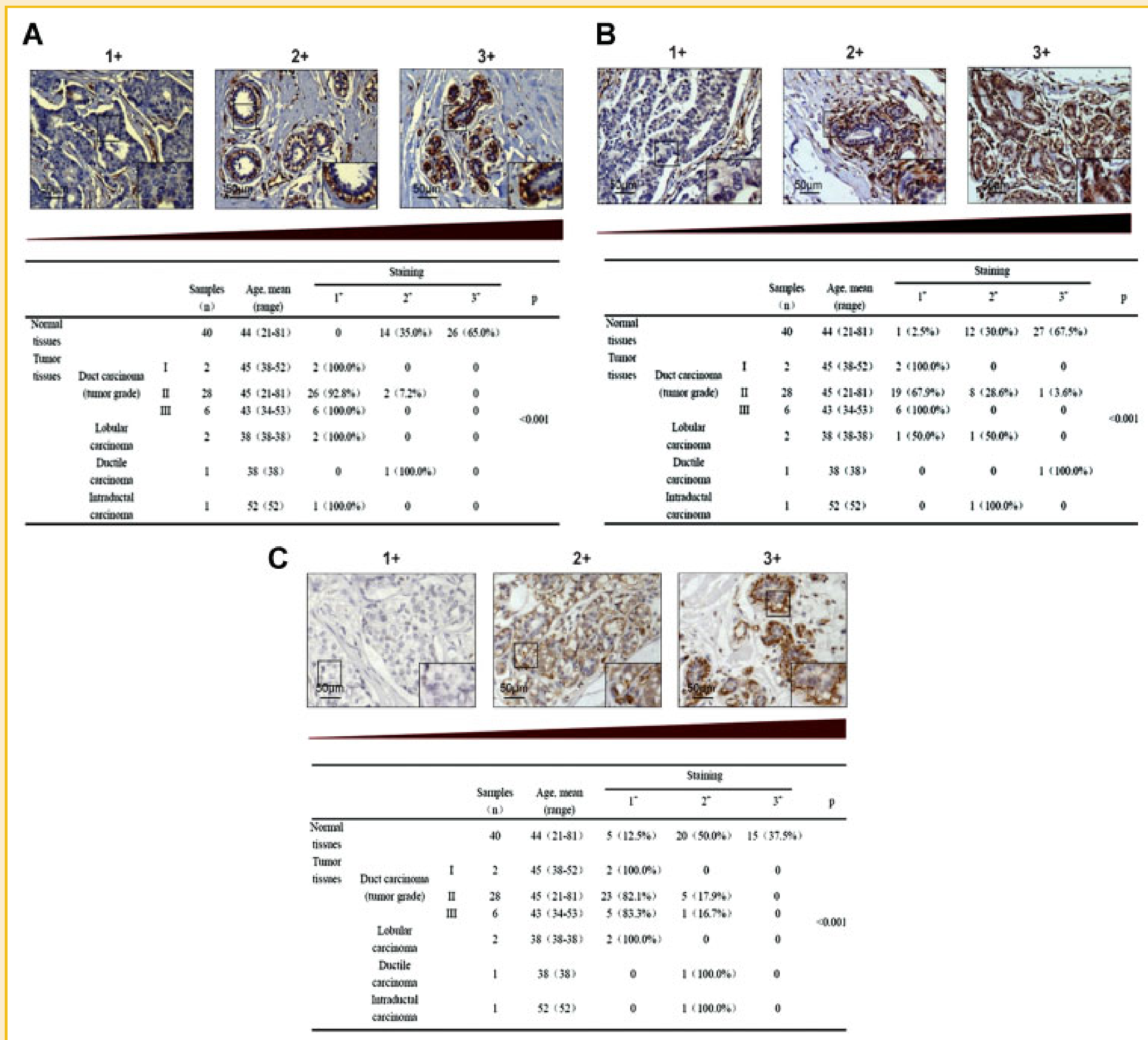


Fig. 2. Expression of the cavin family proteins and caveolin-1 in human breast tumor and tumor-adjacent tissue. Ten paired breast cancer and cancer-adjacent tissue were prepared for cavin family protein and caveolin-1 expression by Western blot (A). Relative expression of cavin family proteins and caveolin-1 in the each breast tumor compared to the tumor-adjacent tissue normalized by β-tubulin were shown in B, level of expression of each protein in the tumor-adjacent tissue was arbitrarily set as 1.



A

			Samples (n)	Age, mean (range)	Staining 1^+	Staining 2^+	Staining 3^+	p
Normal tissues			40	44 (21-81)	0	14 (35.0%)	26 (65.0%)	<0.001
Tumor tissues	Duct carcinoma (tumor grade)	I	2	45 (38-52)	2 (100.0%)	0	0	
		II	28	45 (21-81)	26 (92.8%)	2 (7.2%)	0	
		III	6	43 (34-53)	6 (100.0%)	0	0	
	Lobular carcinoma		2	38 (38-38)	2 (100.0%)	0	0	
	Ductile carcinoma		1	38 (38)	0	1 (100.0%)	0	
	Intraductal carcinoma		1	52 (52)	1 (100.0%)	0	0	

B

			Samples (n)	Age, mean (range)	Staining 1^+	Staining 2^+	Staining 3^+	p
Normal tissues			40	44 (21-81)	1 (2.5%)	12 (30.0%)	27 (67.5%)	<0.001
Tumor tissues	Duct carcinoma (tumor grade)	I	2	45 (38-52)	2 (100.0%)	0	0	
		II	28	45 (21-81)	19 (67.9%)	8 (28.6%)	1 (3.6%)	
		III	6	43 (34-53)	6 (100.0%)	0	0	
	Lobular carcinoma		2	38 (38-38)	1 (50.0%)	1 (50.0%)	0	
	Ductile carcinoma		1	38 (38)	0	0	1 (100.0%)	
	Intraductal carcinoma		1	52 (52)	0	1 (100.0%)	0	

C

			Samples (n)	Age, mean (range)	Staining 1^+	Staining 2^+	Staining 3^+	p
Normal tissues			40	44 (21-81)	5 (12.5%)	20 (50.0%)	15 (37.5%)	<0.001
Tumor tissues	Duct carcinoma (tumor grade)	I	2	45 (38-52)	2 (100.0%)	0	0	
		II	28	45 (21-81)	23 (82.1%)	5 (17.9%)	0	
		III	6	43 (34-53)	5 (83.3%)	1 (16.7%)	0	
	Lobular carcinoma		2	38 (38-38)	2 (100.0%)	0	0	
	Ductile carcinoma		1	38 (38)	0	1 (100.0%)	0	
	Intraductal carcinoma		1	52 (52)	0	1 (100.0%)	0	

Fig. 3. Immunohistochemical analysis of PTRF and SRBC in paired breast cancer and cancer-adjacent tissue. Forty-paired breast cancer and cancer-adjacent tissue were immuno-stained for PTRF (A), SRBC (B) and SDPR(C). Immunohistochemical staining results were presented as 1+ with low expression level and 2+, 3+ with higher protein expression level. The pictures in (A), (B), (C) were under 10× objective magnification. The enlarged images at the right-bottom of each picture were under 20× objective magnification. The tissue sample information was listed as bellows. Statistic analysis was done by nonparametric tests for two-related samples with significant difference.

DISCUSSIONS

In this study, we investigated the expression of cavin family proteins in breast cancer cell lines and breast tumor tissue. Data presented here suggest that PTRF, SRBC, and SDPR are coordinately down regulated in breast cancer cell lines and breast tumor tissue. Similar to *SRBC*, We showed that down-regulation of *PTRF* expression was associated with the promoter methylation.

Caveolae are the most abundant membrane domain devoted to endocytosis, lipid regulation, and signal transduction [Parton and Simons, 2007]. They contain high concentration of signaling molecules such as heterotrimeric G proteins, nonreceptor tyrosine kinases, platelet derived growth-factor receptor, and endothelial nitric-oxide synthase (eNOS) [Parton and Simons, 2007; Bastiani and Parton, 2010; Hansen and Nichols, 2010]. Caveolae may mediate interaction between different signaling pathways and are implicated in a range of different physiological processes. The caveolin-1 was first identified as a prominent structural component of caveolae and is required for the caveolae formation [Liu et al., 2002]. A number of studies have demonstrated that caveolin-1 is frequently mutated or deregulated in human breast carcinomas and has been proposed to function as a tumor suppressor [Fiucci et al., 2002; Williams and Lisanti, 2005; Sotgia et al., 2006]. Aberrant promoter methylation of caveolin-1 gene is indeed associated with its inactivation of expression in breast cancer [Chan et al., 2003; Chen et al., 2004]. Recent studies demonstrated that cavin family proteins PTRF, SRBC, and SDPR are required for caveolae formation and function [Nabi, 2009; Bastiani and Parton, 2010; Hansen and

Fig. 4. Epigenic inactivation of PTRF in paired breast cancer and cancer-adjacent tissue. (A) Sketch map indicates the CpG sites in the promoter regions of the cavin family genes. (B) Five pairs of breast tissue with apparent difference of PTRF expression at protein levels were chosen for PTRF expression at mRNA level by RT-PCR. (C) Bisulfite sequencing analysis of *PTRF* promoter in the paired breast cancer and cancer-adjacent tissue indicated in (B), and in immortal MCF 10A and cancer cell lines (MCF7 and MDA-MB-231). The amplified PTRF DNA region for sequencing analysis ranges from −758 to −145 nucleotides relative to the first nucleotide (set as the default +1) of exon 1. The methylation status of each CpG site is indicated by black square (>5 from 10 sequencing clones were methylated), gray square (≤5 and ≥3 from 10 sequencing clones were methylated) and unfilled square (<3 from 10 sequencing clones were methylated). (D) RT-PCR analysis of *PTRF*, *SRBC*, *SDPR*, and *caveolin-1* expression in breast cancer cell lines MCF7 and MDA-MB-231 treated with 5-aza-2′-deoxycytidine (5-azaC). *GAPDH* expression is shown as an internal control.

Nichols, 2010]. It has been shown that SRBC, a putative tumor suppressor, is epigenetically silenced in breast cancer [Xu et al., 2001], in gastric cancer [Lee et al., 2008], and lung cancer [Zochbauer-Muller et al., 2005]), and that the cavin family protein SDPR is also down-regulated in breast cancer [Li et al., 2008]. We compared the expression profiles of cavin family proteins in breast cancer cells and we found that PTRF, SRBC, and SDPR genes were down regulated in breast cancer cell lines and breast tumor tissue.

It has been previously shown that inactivation of SRBC is associated with the promoter methylation in breast cancer [Xu et al., 2001]. We analyzed CpG islands in promoter regions of *PTRF* and *SDPR* genes. We found typical CpG islands in the promoter region of *PTRF* gene. But no CpG island was found within 3,000 bp upstream of the first translation start codon in the promoter of the *SDPR* gene, suggesting other mechanisms might be involved in regulation of SDPR in breast cancer. We showed that the promoter region of *PTRF* was highly methylated in breast cancer cells and breast tumors, and treatment with the demethylation reagent 5′-azacytidine induced the expression of PTRF in MCF7 and MDA-MB-231 cells, suggesting that *PTRF* is epigenetically silenced in breast cancer.

Aberrant methylation of cancer-related genes has been associated with transcriptional inactivation of these genes and is the main mechanism for gene silencing in tumors [Jones and Baylin, 2007]. Loss of caveolin-1 and SRBC is associated with methylation of the promoters [Xu et al., 2001; Chen et al., 2004], similarly, we found in this study that down-regulation of PTRF was also due to methylation of the promoter. Both caveolin-1 and SRBC have been proposed to function as tumor suppressors in breast cancer [Xu et al., 2001; Chen et al., 2004]. It remains unclear how the cavin family genes are coordinately down regulated at transcriptional level in breast cancer. Given that PTRF is essential for caveolae formation and function [Hill et al., 2008], the findings that cavin family genes are inactivated in breast cancer suggest a tumor suppressor function of caveolae and possibility to develop anti-cancer therapies that target PTRF and caveolae in breast cancer.

MATERIALS AND METHODS

TISSUE ARRAY AND TISSUE SAMPLES

The human breast tissue arrays used in this study were purchased from Xi'an Ailina Biological Technology Co. Ltd, which consists of 40 pairs of breast cancer and cancer-adjacent specimens from 40 patients. Ten pairs of fresh-frozen breast cancer tissue and matched adjacent normal breast tissue were from Breast Center, Peking University Cancer Hospital.

ANTIBODIES

Anti-β-actin and anti-β-tubulin were from Cell Signaling (Beverly, MA); antibodies against PTRF and caveolin-1 were from BD Pharmingen (San Jose, CA); anti-SRBC was from Abnova (Swampscott, MA); anti-SDPR was from ProteinTech Group (Cambridge, UK).

CELL CULTURE

Breast cancer cells (MCF7, MDA-MB-231) were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin. MCF 10A Cells were cultured in DMEM/F12 supplemented with 5% horse serum, 0.5 pg/ml hydrocortisone, 10 pg/ml insulin, 2 ng/ml EGF, 0.1 pg/ml cholera enterotoxin, 2 mM L-glutamine, 100 U/ml penicillin–streptomycin, and 0.5 pg/ml fungizone. Cells were cultured at 37°C in 5% CO_2.

WESTERN BLOT ANALYSIS

For Western blot, cell lysates were prepared in RIPA buffer (50 mM Tris–Cl, pH 8.0, 100 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40), and a protease inhibitor cocktail (Roche). Total protein concentrations were measured using BCA kit, and immunoblotting with clarified cell extracts was performed in 12% SDS–polyacrylamide gels. Proteins were transferred to PVDF membrane (Millipore) and incubated at 4°C overnight with primary antibodies. Alkline phosphatase-conjugated anti-mouse or anti-rabbit secondary antibodies were used for detection using a BCIP/NBT color development substrate (Promega, Madison).

RT-PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Genes of interest were amplified from 2 μg DNase I-treated total RNAs using M-MLV Reverse Transcriptase (Promega) and poly dT primer.

The primers used for PCR were as follows: *PTRF* (forward: 5′-ACGCCACCACGAGCAATAC-3′; reverse: 5′-CGGCAGCTTCACTTC-ATCC-3′, $T_m = 60$°C, 26 cycles), *SRBC* (forward: 5′-AAGCTCCACG-TTCTGCTCTTC-3′; reverse: 5′-GATCTTCCTCGGTGTCCTGC-3′, $T_m = 64$°C, 27 cycles), *SDPR* (forward: 5′-TCCCTGCCAGCGTGTTTGTG-3′; reverse: 5′-AAAATGCTTTCTTGAGGCTATCCAC-3′, $T_m = 62$°C, 27 cycles), *caveolin-1* (forward: 5′-AAGCTCCACGTTCTGCTCTTC-3′; reverse: 5′-GATCTTCCTCGGTGTCCTGC-3′, $T_m = 62$°C, 27 cycles) and *GAPDH* (forward: 5′-TGCTAAGCAGTTGGTGGTGCAGGA-3′; reverse: 5′-CGGAGTCAACGGATTTGGTCGTAT-3′, $T_m = 60$°C, 22 cycles). After amplification, the PCR products were separated in 1.5% agarose gels and visualized by ethidium bromide staining. For real-time PCR, amounts of transcripts were quantified using iQ5 real-time PCR detection system (Bio-Rad, CA) and iQ™ SYBR® Green Supermix (Bio-Rad). Experiments were repeated at least three times in triplicate, *GAPDH* as an internal control.

IDENTIFICATION OF THE CPG ISLAND OF PTRF GENE

The genomic DNA sequences including the upstream promoter sequence of *cavin* gene were obtained from the GenBank Database of the National Center of Biotechnology Information. CpG-rich regions at the *cavin* gene locus were analyzed using the criteria and algorithm of online CpG Island Searcher (http://cpgislands.usc.edu/) and the software (Methyl Primer Express v1.0).

BISULFITE SEQUENCING ANALYSIS

DNA was isolated from cells and tissue (TIANamp Genomic DNA kit, TIANGEN) and modified with sodium metabisulfite (EZ DNA Methylation-Gold Kit™, ZYMO RESEARCH). PCR was performed with 5 μl of sulfonated DNA. Primers used for PCR were as follows: the forward primer 5′-GGGTAATTTTAAAAAGAATTTAGAG-3′ and reverse primer 5′-CCCTTACCCTTTACAATATTTTCTA-3′ (Tm = 56°C, 30 cycles) for amplification of the promoter region (nucleotides −758 to −566 upstream ATG start codon); and the forward primer 5′-TGGTTGGGGTTGGTGATGAGG-3′ and reverse primer 5′-TCCCCAACTCCCGAACCAAAC-3' (Tm = 55°C, 30 cycles) for amplification of the promoter region (nucleotides −580 to −145 upstream ATG start codon). The PCR products were subcloned into

pGEM-T (pGEM-T Easy Vector System, Promega) and sequenced with a T7 promoter sequencing primer.

IMMUNOHISTOCHEMISTRY

The immunohistochemical staining was carried out in paraffin-embedded tissue sections purchased from Xi'an Ailina Biological Technology Co. Ltd (The tissue-chip number is BR804). All sections were dried at 60°C for 4 h. Antigen retrieval was done by steaming the slides in 10 mM citrate buffer (pH 6.0) for 5 min. After antigen retrieval and H_2O_2 (3%; v/v) treatment to block endogenous peroxidase, the sections were washed with PBS, and then incubated with PTRF or SRBC antibody (1:100 in PBS) at 4°C overnight. After washing twice with PBS, HRP-conjugated secondary antibody was added. Immunoreactivity was detected with the DAB staining system (Cymed). After staining, the sections were counterstained with Gill's hematoxylin, dehydrated with graded alcohol and xylene, and mounted with coverslips. Photos were taken by Zeiss Upright Fluorscence Microscope.

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Natural Science Foundation of China [30671065,31171320] and grants from the "973" Project of the Ministry of Science and Technology [2007CB507402, 2007CB914402, 2009CB18904, 2011CB911203].

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