

Identification of novel epigenetically inactivated gene *PAMR1* in breast carcinoma

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Abstract. Development of cancer is a complex process involving multiple genetic and epigenetic alterations. In our microarray analysis of 81 breast carcinoma specimens, we identified *peptidase domain containing associated with muscle regeneration 1* (*PAMR1*) as being frequently suppressed in breast cancer tissues. *PAMR1* expression was also reduced in all tested breast cancer cell lines, while *PAMR1* was expressed moderately in normal breast tissues and primary mammary epithelial cells. DNA sequencing of the *PAMR1* promoter after sodium bisulfite treatment revealed that CpG sites were hypermethylated in the breast cancer tissues and cell lines. *PAMR1* expression was restored by 5-aza-2' deoxycytidine treatment, demonstrating that promoter hypermethylation contributed to *PAMR1* inactivation in the breast cancer cells. In addition, ectopic expression of *PAMR1* markedly suppressed cancer cell growth. In summary, our study identified *PAMR1* as a putative tumor suppressor which was frequently inactivated by promoter hypermethylation in breast cancer tissues.

Introduction

Cancer is the leading cause of death in most developed countries, and breast cancer is one of the leading causes of cancer-related mortality among women (1). Although surgery and follow-up treatment have been successful in improving the prognosis of breast cancer patients, patients with metastatic tumors still suffer from poor prognosis. Therefore, developing novel therapeutics for breast cancer is an absolute necessity. For this purpose, understanding the molecular mechanism of breast carcinogenesis is essential. Microarray technology

which provides quantitative genome-wide gene expression profiling has been widely used to analyze the pathways associated with cancer development and progression. (2). Through the screening of genes which showed enhanced expression in breast cancer tissues, we identified several molecular targets that are essential for breast cancer cell proliferation (3-6). For example, brefeldin A-inhibited guanine nucleotide-exchange protein 3 (BIG3), which was found to be frequently upregulated in breast cancer tissues, interacts with prohibitin 2/repressor of estrogen receptor activity (PHB2/REA) protein. This binding inhibits PHB2/REA nuclear translocation and subsequently activates ER α signaling pathways (7). In addition, a synthesized peptide which inhibits the interaction between BIG3 and PHB2/REA is able to suppress E2-dependent breast cancer cell growth (8).

Similarly, identification of genes which exhibit low expression in cancer tissues is also important for the understanding of human carcinogenesis. Tumor-suppressor genes (TSGs) act as guardians against malignant transformation. Genomic alteration or promoter hypermethylation are common causes of TSG inactivation. In breast cancer tissues, hypermethylation of TSGs is considered to be an early event during tumorigenesis (9). Overexpression of DNA methyltransferase (*DNMT*) 1, 3a, and 3b is frequently observed in breast fibroadenoma (22-44%) (10), which may result in TSG promoter hypermethylation including *APC*, *BRCA1*, *p16*, *p21* and *TIMP3* (11-13). Several studies have demonstrated that hypermethylated DNA of TSGs in serum could be a potential biomarker for disease prediction and therapeutic response in breast cancer (14). In addition, DNMT inhibitors are used for the treatment of myelodysplastic syndrome and solid cancers (15-17). Therefore, identification of novel TSGs would not only provide a fundamental understanding of cancer biology, but may also contribute to breast cancer diagnosis or more effective therapeutics. Recently, we reported a TSG candidate, *HSPB7*, which was found to be downregulated in renal cancer samples by epigenetic abnormalities (18). In the present study, we used microarray technology and identified *peptidase domain containing associated with muscle regeneration 1* (*PAMR1*) whose expression was frequently suppressed in breast cancer tissues by promoter hypermethylation.

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Table I. List of primers used in the present study.

	Forward primer	Reverse primer
Cell line RT-PCR		
<i>C2orf88</i>	GCTTAATCACAATGCCCTCAAC	CTGAACTAATGCCCCACAGCTC
<i>CSRNP3</i>	AGTGGGGACAGTGTCAATCC	CCTTGCCTCCTGGTGAAGTA
<i>PAMR1</i>	CCTTCTCATCTCGTCCTTGC	AACCCACGACTTCCCTCTTT
<i>PDLIM3</i>	CTCAGGGGGCATAGACTTC	ATCTCCAGGACACAGGTTGG
<i>PPP1R12B</i>	TGACCAGCCGTGTAGAAGAAG	CTGGGCTTCCTGAAGTTTTG
<i>SAMD5</i>	GCTACCCCAAACCTGAAGCTG	AGCGGCTCTGTGATGACTTC
Tissue RT-PCR	AGGGAAGATCTGGGCTTCATG	GGGAAGGAAAAGGACCAGAC
Cloning PCR	TTAAGAATTGCGGCAAGGATGGAGCTGGG	CGCGCTCGAGTTTCATATTTCTTTCAATCC
Isoform PCR	TTACAAGTGTGCCTGCTTGG	GCCCCCTGTTATTTTCTGGT
Bisulfite sequencing	TTAATTTGTGATTATTTGGAGTAA	CTCATCTAAAAAAACCACCTTCAA

Materials and methods

Breast cancer cell lines and clinical cancer samples. Human breast cancer cell lines including BSY1, BT-20, BT-474, BT-549, HBC4, HBC5, HBL-100, HCC1143, HCC1395, HCC1500, HCC1599, HCC1937, MCF7, MDA-MB-231, MDA-MB-435s, MDA-MB-453, OCUB-F, SK-BR-3, T-47D, YMB-1 and ZR-75-1 were obtained and cultured as previously reported (4). The cell lines BST1, HBC4 and HBC5 were kindly provided by Dr Takao Yamori of the Division of Molecular Pharmacology, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research. The other cell lines were purchased from the American Type Culture Collection (ATCC, USA). Human mammary epithelial cells (HMECs) were purchased from Lonza Switzerland and were cultured in mammary epithelial cell growth medium supplemented with bovine pituitary extract, hEGF, hydrocortisone, GA-1000 and insulin (Lonza). The HMECs used for all experiments were under passage 15. All cells were maintained at 37°C in an atmosphere of humidified air with 5% CO₂ except for MDA-MB-231 and MDA-MB-435s which were maintained at 37°C in an atmosphere of humidified air without CO₂. Primary breast normal and cancer tissues were obtained with informed consent from patients who received treatment at the Department of Breast Surgery, Cancer Institute Hospital, Tokyo. All tissue samples underwent laser-microbeam microdissection (19).

Plasmid construction. The two *PAMR1* isoforms were amplified from HMEC cDNA by KOD plus DNA polymerase (Toyobo, Japan). The sequences of the cloning primers are listed in Table I. The amplified DNAs were then subsequently cloned into the pCAGGS vector with HA-tagged at the C-terminal.

cDNA microarray. cDNA microarray analysis was performed as previously described (19). In brief, tumor cells obtained from 81 breast cancer patients (12 ductal carcinomas *in situ* and 69 T2 invasive ductal carcinomas) underwent laser microbeam microdissection. The total RNAs were extracted using the RNeasy Mini kit (Qiagen, Germany) and treated with

DNase I digestion according to the manufacturer's manual. The RNAs were then reverse-transcribed and hybridized with the microarray slide. The microarray slide contained 23,040 cDNAs selected from the UniGene database (build #131), including 52 housekeeping genes and two types of negative control genes. A mixture of normal breast ductal cell RNAs isolated from 15 pre-menopausal breast cancer patients was used as the normal control.

Real-time quantitative PCR. The mRNAs of human normal tissues were purchased from Takara (Takara Bio, Japan). Total RNAs from the cell lines were extracted using the RNeasy Mini kit and reverse transcribed into cDNA by SuperScript III (Life Technologies, USA) according to the manufacturer's instructions. Real-time quantitative PCR (qPCR) was performed using SYBR-Green I Master Mix on LightCycler 480 (Roche, Germany). The primer sequences are listed in Table I.

DNA isolation, sodium bisulfite treatment and DNA sequencing. Genomic DNAs were isolated by DNeasy Blood & Tissue kit (Qiagen) according to the instruction manual. Bisulfite treatment and DNA sequencing was performed as previously reported (20). In brief, 2 µg of DNA was digested by *XhoI* for 16 h at 37°C. The digested DNA was then denatured by 0.3 M NaOH and treated with 3.12 M sodium bisulfite and 0.5 mM hydroquinone for 16 h at 55°C. Following incubation, DNA was purified and desulfonated by 0.3 M of NaOH at 37°C for 20 min, followed by ethanol precipitation. Finally the DNA was amplified by PCR with the specific primers (Table I) and subcloned into the pCR 2.1 vector by TA cloning kit (Invitrogen, USA). The cloned plasmids were transformed into competent cells. For each treated DNA, 10 individual colonies were chosen and plasmid extractions were performed. DNA sequencing of the isolated plasmids was performed by the ABI sequencing system (Applied Biosystems, USA) according to the manufacturer's instructions.

Demethylation drug treatment. The demethylation drug 5-aza-2' deoxycytidine (5-aza-dC) was purchased from Sigma (Sigma-Aldrich, USA). The drug was dissolved in

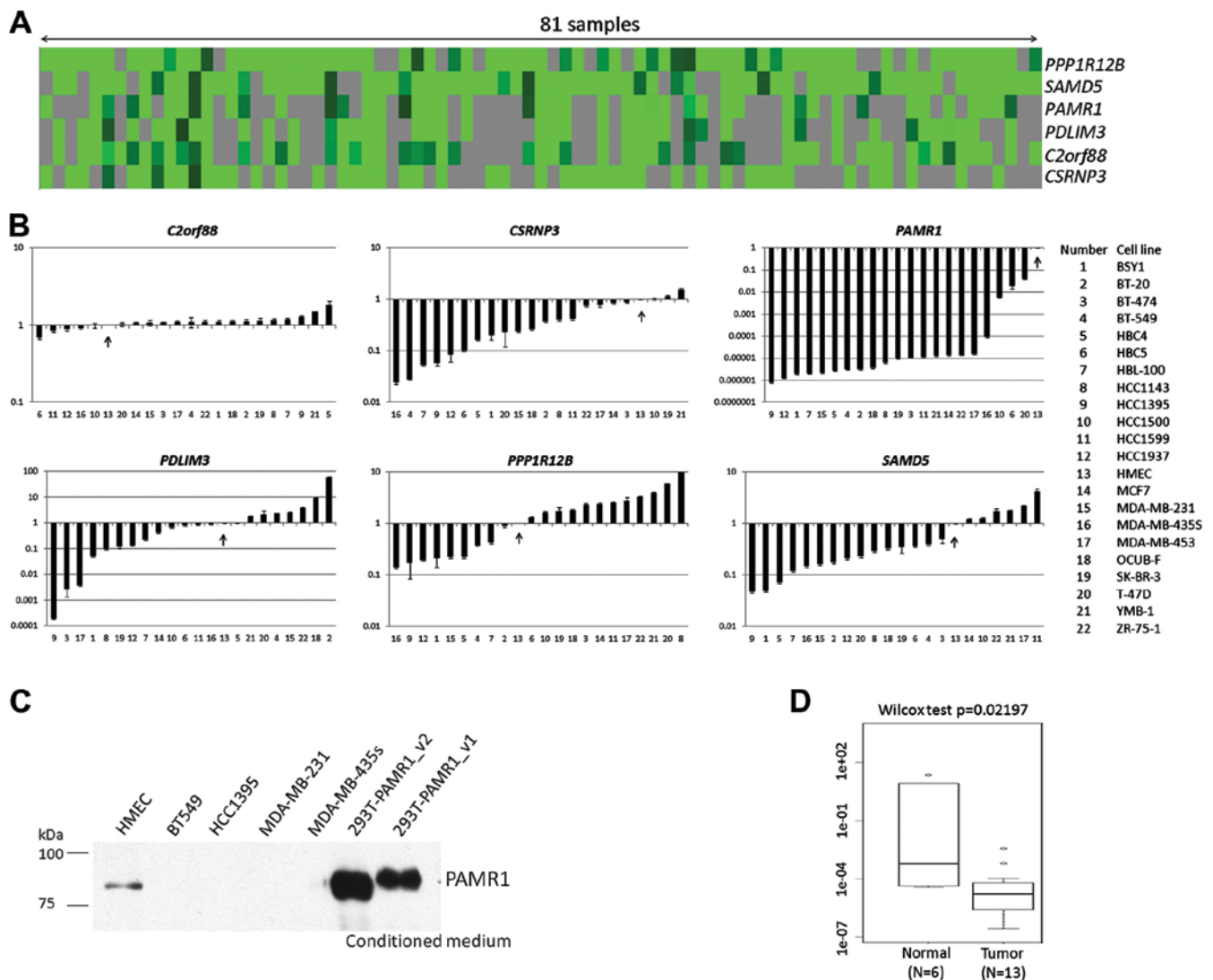


Figure 1. Microarray analysis identified *PAMR1* which exhibited low expression in breast cancer. (A) Heatmap showing the microarray results of the 6 down-regulated genes in the breast cancer samples. (B) qPCR results showing the relative expression of the 6 candidate genes compared with HMECs (indicated by an arrow) in 21 breast cancer cell lines. Data represent means \pm SD. The list of cell lines are shown in the right panel. (C) Endogenous *PAMR1* expression in HMECs and breast cancer cell lines. Conditioned medium was collected and separated by 8% SDS-PAGE. *PAMR1* secreted protein was detected by sheep anti-*PAMR1* antibody. (D) Boxplot representing the expression of *PAMR1* in normal and tumor specimens from breast cancer patients.

dimethyl sulphoxide (DMSO) and freshly prepared before use. Breast cancer cells were cultured in 6-well plates one day before drug treatment. Fresh medium containing various concentrations of 5-aza-dC was replaced daily for 3 consecutive days. The RNA from each treated cell line was isolated 72 h post drug treatment. Cells treated with DMSO served as the negative controls.

Western blotting. Breast cancer cells (5×10^5) were cultured in a 60-mm dish under normal conditions and allowed to attach for 24 h. The culture medium was then removed and the cells were washed twice by PBS. A total of 2 ml of fresh medium without FBS was then replaced, and the cells were allowed to grow for another 24 h. After incubation, 1 ml of conditioned medium was collected from each sample, followed by centrifugation at 15,000 rpm for 15 min at 4°C twice to remove all floating cells. The conditioned medium was then mixed with an equal volume of ice-cold acetone and stored at -80°C for 1 h. The protein was

harvested by centrifugation at 15,000 rpm for 15 min at 4°C. The precipitated protein was dissolved using Laemmli sample buffer and analyzed by western blotting following standard protocols (Bio-Rad, USA). Rat anti-HA antibody (Roche) and sheep anti-*PAMR1* antibody (R&D Systems, USA) were used to detect *PAMR1* protein in the conditioned medium. Mouse anti- β -actin antibody (Santa Cruz, USA) was used as the loading control.

Colony formation assay. Breast cancer cells were cultured in 6-well plates for 24 h before transfection. One hundred and fifty million copies of plasmid from the vector alone control (pCAGGS), and two variants of *PAMR1* were transfected into each well individually by FuGene HD (Roche) in a 1:3 ($\mu\text{g}:\mu\text{l}$) ratio. Transfection was performed according to the user manual. G418 (Life Technologies) was added to the cells one day after transfection. The drug-resistant cells were allowed to grow for three weeks until colonies formed.

Table II. Microarray study results of the 6 novel candidate genes with downregulated expression in breast cancer tissues.

Gene	Valid sample (n)	Ratio <0.2 (n)	Downregulated (%)
<i>C2orf88</i>	54	54	100
<i>CSRNP3</i>	46	45	98
<i>PAMR1</i>	46	42	91
<i>PDLIM3</i>	44	42	95
<i>PPP1R12B</i>	67	63	94
<i>SAMD5</i>	70	66	94

Finally the cells were fixed by 10% formamide and stained with 0.1% crystal violet solution. The number of colonies was counted by Image J software.

Results

Identification of genes frequently downregulated in breast cancer tissues. We previously performed cDNA microarray

analyses of 81 breast tumor samples (19). All the tumor cells and normal breast epithelial cells were purified by laser micro-beam microdissection. In order to identify novel genes which are commonly downregulated in breast cancer tissues, we screened the cDNA microarray database consisting of 23,040 probes using the following criteria: i) genes for which we were able to obtain expression signal in >50% of total examined samples; ii) genes whose expression ratio (cancer/normal) was <0.2 in more than 90% of informative samples; iii) genes whose association with human carcinogenesis had not been reported to date. Finally, we selected 6 candidate genes, namely *chromosome 2 open reading frame 88 (C2orf88)*, *cysteine-serine-rich nuclear protein 3 (CSRNP3)*, *PAMR1*, *PDZ and LIM domain 3 (PDLIM3)*, *protein phosphatase 1 regulatory subunit 12B (PPP1R12B)* and *sterile a motif domain containing 5 (SAMD5)* (Fig. 1A, Table II).

Downregulation of PAMR1 in breast cancer cell lines and tissues. We then examined the expression of these genes in 21 breast cancer cell lines by qPCR analyses (Fig. 1B). Human mammary epithelial cells (HMECs) served as a normal control. Among the 6 candidate genes, *PAMR1* expression was reduced in all breast cancer cell lines. To confirm this result, we conducted western blot analysis using conditioned

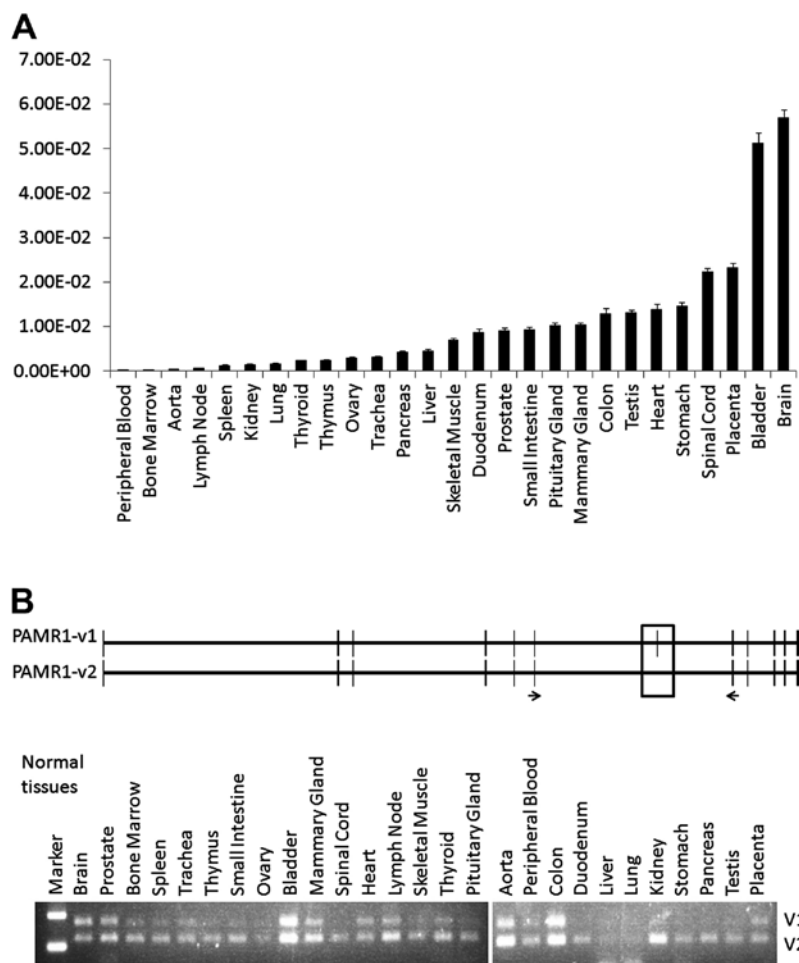


Figure 2. *PAMR1* expression in normal tissues. (A) qPCR results showing the expression of *PAMR1* in 27 normal human tissues. Data represent means \pm SD. (B) Genomic structure of *PAMR1* variant 1 (v1) and variant 2 (v2) (upper panel). The exon 7 of variant 1 is absent in variant 2. A pair of primers (arrow) flanking exons 6 and 8 of variant 1 was designed to distinguish each variant. The result of gel electrophoresis indicating the expression of *PAMR1* variants in 27 different normal tissues (lower panel).

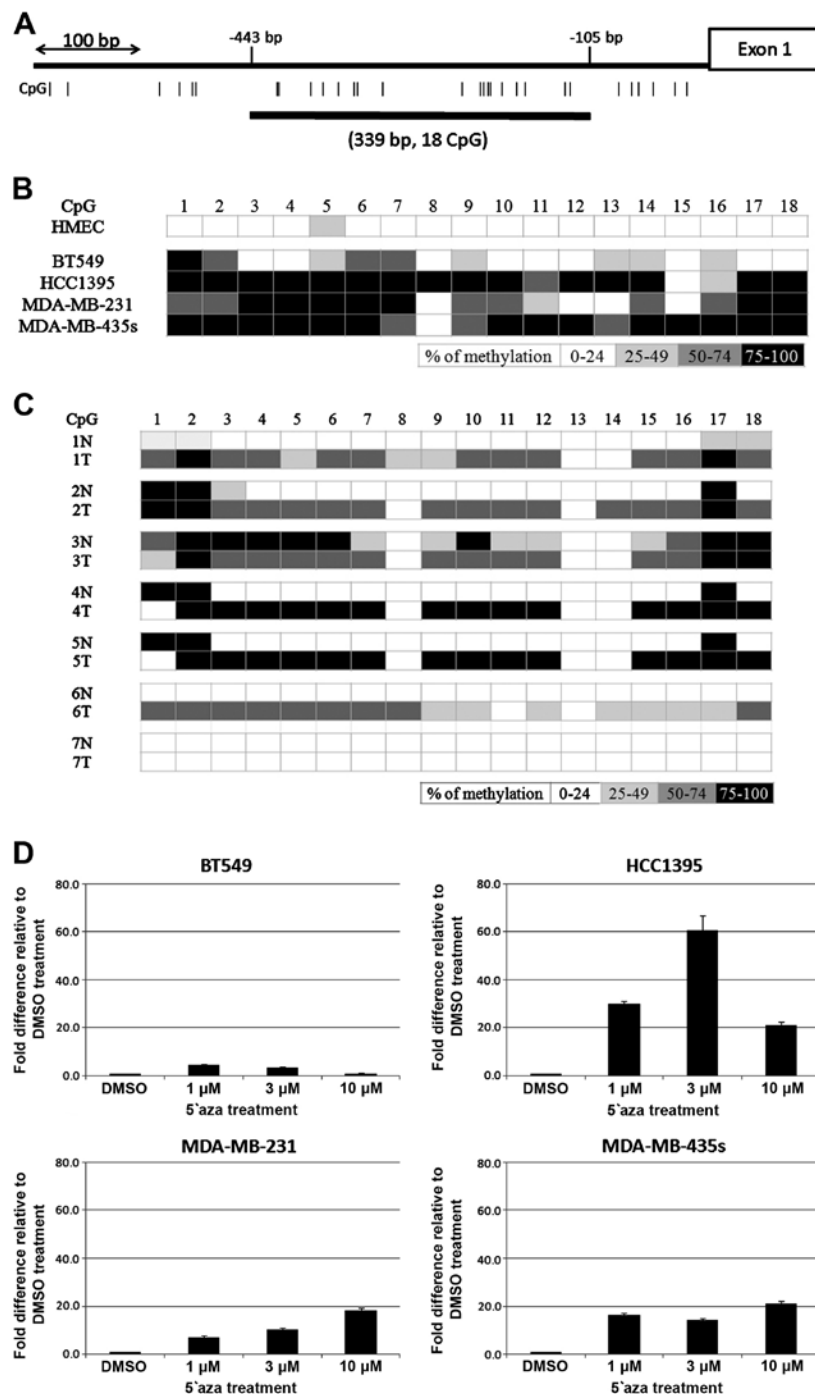


Figure 3. Hypermethylation of the *PAMR1* promoter in breast cancer. (A) *In-silico* prediction of 18 CpG sites located at the promoter region. (B and C) Methylation status of CpG in the *PAMR1* promoter. A 339-bp fragment including 18 CpG sites was analyzed by bisulfite sequencing in HMECs and 4 breast cancer cell lines (B) as well as 7 breast primary tissues and the corresponding normal tissues (C). (D) qPCR analysis of *PAMR1* expression after 3 days of 5-aza-dC treatment. Data represent means \pm SD.

medium from the cultured cell lines, as *PAMR1* was shown to be a secreted protein (21). As a result, *PAMR1* protein was detectable only in the culture medium of HMECs but not in those of the cancer cell lines (Fig. 1C). The conditioned media from HEK293T cells transfected with the plasmid designed to express *PAMR1* were used as a positive control. We also examined *PAMR1* expression in 13 breast cancer tissues and 6 normal breast tissues by qPCR analysis. The cancer tissues showed reduced expression of *PAMR1*, concordant with the result of the cDNA microarray analysis (Fig. 1D).

Expression of *PAMR1* in mammary gland. *PAMR1* was originally identified as a regulator of muscle regeneration. *PAMR1* was found to be downregulated in the muscles of Duchenne muscular dystrophy (DMD) patients and DMD mice (22). Our qPCR analysis revealed that *PAMR1* showed the highest expression in brain tissue and moderate expression in breast and skeletal muscle tissues among the 27 normal human tissues (Fig. 2A), concordant with a previous report (22). Therefore, we hypothesized that *PAMR1* may have unique functions in different tissues. *PAMR1* has two isoforms, and

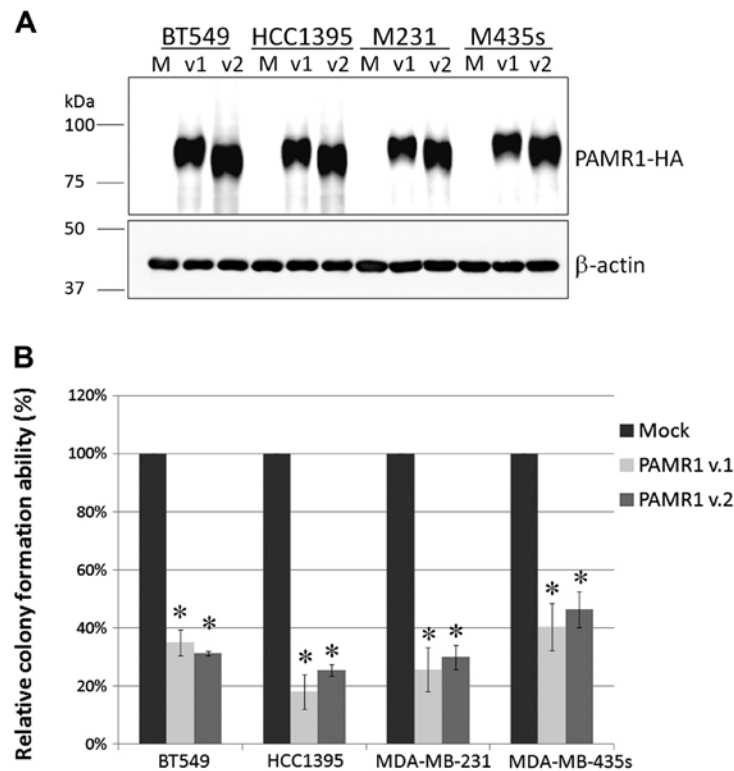


Figure 4. Suppression of breast cancer cell growth by PAMR1. (A) Expression of PAMR1 in cells transfected with the plasmid encoding HA-tagged PAMR1. (B) Relative number of colonies in cells transfected with the plasmid encoding PAMR1 or mock. Data represent means \pm SD. * $P < 0.05$ by Student's t-test.

variant 2 which lacks exon 7 (51 bp) encodes a 17-amino acid shorter protein compared with variant 1. To investigate expression of the two isoforms in each tissue, we designed a pair of primers flanking exons 6 and 8. After PCR amplification and gel electrophoresis, DNA fragments corresponding to variant 1 and variant 2 showed similar intensity in the brain, prostate, bladder, heart, colon and placenta, while the intensity of the DNA fragment corresponding to variant 2 was dominant in the other tissues including the mammary gland (Fig. 2B).

Promoter hypermethylation of PAMR1 in breast cancer tissues and cell lines. To further investigate the molecular mechanism of PAMR1 inactivation in breast cancer tissues, we sequenced all exons of PAMR1 in 21 breast cancer cell lines. However, we did not identify any mutations in our tested samples. We, then, considered whether epigenetic inactivation could cause PAMR1 downregulation. Although we were not able to identify any CpG island within the PAMR1 locus including a 10-kb region encompassing its 5' flanking region by *in-silico* analysis (23), a CpG-rich region was found within -443 to -105 bp of the PAMR1 promoter region (Fig. 3A). From the result of the bisulfite treated DNA sequencing analysis, hypermethylation was found in 3 cancer cell lines, namely HCC1395, MDA-MB-231, and MDA-MB-435s among the 4 cancer cell lines examined. Moreover, the PAMR1 promoter was also found to be moderately methylated in the BT549 cancer cells but not in normal HMECs (Fig. 3B). We, then, analyzed 7 pairs of normal and tumor tissues from breast cancer patients and found tumor-specific promoter hypermethylation in 5/7 tumor samples (Fig. 3C).

We treated the breast cancer cell lines with demethylating agent 5-aza-2'-deoxycytidine (5-aza-dC) and examined PAMR1 expression by qPCR analysis. The expression of PAMR1 was recovered after drug treatment by 4.2-, 62.7-, 18.1- and 20.8-fold in the BT549, HCC1395, MDA-MB-231 and MDA-MB-435s cells, respectively (Fig. 3D). The expression of PAMR1 in the BT549 cells showed the least degree of restoration compared to the other cell lines, concordant with the low degree of DNA methylation in the BT549 cells (Fig. 3B). Taken together, promoter hypermethylation is one of the mechanisms contributing to the inactivation of PAMR1 in both breast cancer cell lines and tumor tissues.

Suppression of tumor cell growth by ectopic expression of PAMR1. To investigate the role of PAMR1 in breast carcinogenesis, we constructed plasmids expressing variant 1 or variant 2 of PAMR1. We confirmed the expression of PAMR1 protein in all cancer cell lines examined (Fig. 4A). We next conducted colony formation assays and observed a significant decrease in colony number (18-46%) for all PAMR1-introduced cells (Fig. 4B), indicating the growth-suppressive function of PAMR1.

Discussion

In the present study, we identified PAMR1 as a putative breast cancer tumor suppressor by a screening of the gene expression profiling of 81 breast cancer tissues. Although we did not find mutations of PAMR1 in 21 breast cancer cell lines, promoter hypermethylation was frequently observed in both breast cancer tissues and cell lines. The PAMR1 gene is located at

chromosome 11p13, which is frequently lost in breast cancer samples (20.8-58.3%) (24-26). Therefore, both genetic and epigenetic inactivation would contribute to the downregulation of *PAMR1* in breast cancer.

PAMR1 was first identified as a gene which was down-regulated in myoblastic cells isolated from DMD mice. The expression of *PAMR1* was induced in gastrocnemius muscle cells after crush injury, reaching the highest expression on day 4 and was reduced to a normal level on day 14. *PAMR1* induction was only observed in the regenerating muscle fibers by *in situ* hybridization but not in normal muscle cells. Thus, *PAMR1* is considered to be involved in the regeneration of skeletal muscles (22). *PAMR1* was expressed in various tissues such as skeletal muscle, brain, and mammary gland. Moreover, our microarray analyses indicated that *PAMR1* expression was reduced in several types of cancers including breast, bladder, liver cancers and osteosarcoma (data not shown). Therefore, *PAMR1* may have as yet unidentified roles other than muscle regeneration.

Although the molecular mechanism whereby *PAMR1* suppresses tumor cell growth has not yet been clarified, *PAMR1* contains putative signal peptides at the N-terminal, a CUB domain, two EGF domains, two Sushi domains and an inactive trypsin-like serine protease domain. The secreted *signal peptide CUB-EGF domain-containing protein 2 (SCUBE2)* which contains CUB and EGF domains was shown to suppress breast cancer cell growth (27). Functional domain analysis revealed that the CUB domain bound to bone morphogenetic protein (BMP) and antagonized BMP signaling to suppress cell differentiation and proliferation. Moreover, the EGF-like repeats of SCUBE2 interact with E-cadherin to inhibit the β -catenin pathway (27-29). Since overexpression of *PAMR1* in breast cancer cell lines significantly suppressed cancer cell growth, secreted *PAMR1* might exert a tumor-suppressive function by antagonizing growth signals through the interaction with growth factors or their receptors.

In conclusion, our study demonstrated that *PAMR1* may be a novel TSG for breast cancer. We provide evidence that promoter hypermethylation plays an important role in *PAMR1* inactivation during breast carcinogenesis. Although further functional studies and pathway analyses are necessary, identification of its downstream pathway would lead to the development of novel breast cancer therapy by using recombinant soluble *PAMR1* protein.

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