Human Cancer Biology

BRCA1 5083del19 Mutant Allele Selectively Up-Regulates Periostin Expression *In vitro* and *In vivo*

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Abstract

Purpose: The aim of this study was to explore the gene expression pattern produced by the cancer-associated BRCA1 5083del19 founder mutation by using a microarray analysis. Such a mutation, identified in a subset of familial breast cancer patients, involves a deletion at the 3′ end of the BRCA1 messenger leading, in the mature protein, to the ablation of the BRCT tandem domain.

Experimental Design: We generated HeLa cells stably expressing both exogenous wild-type (HeLa/[wt]BRCA1), used as a control, and 5083del19 BRCA1 (HeLa/[5083del19]BRCA1) alleles; gene chips were then used to investigate any changes in the transcription profile induced by the 5083del19 BRCA1 mutant compared with controls.

Results: Among the genes showing perturbation of their expression, periostin was found to be up-regulated in HeLa/[5083del19]BRCA1 cells to an extent of 72-fold versus HeLa/[pcDNA3.1/empty] and 76-fold versus HeLa/[wt]BRCA1 cells. This finding was validated both *in vitro* in breast cancer cell lines harboring mutations of BRCA1 and *in vivo* by immunohistochemistry of breast cancer specimens bearing the 5083del19 BRCA1 mutation as well as by Western blot analysis of sera obtained from patients and healthy carriers of the same mutation.

Conclusions: Our results suggest that periostin overexpression, whose product is released from cells in the extracellular fluids, might be a potential marker for early cancer detection in a specific subset of hereditary breast carcinomas triggered by cancer-associated BRCA1 mutations that affect the BRCT tandem domain.

Hereditary breast/ovarian cancer is characterized by a high degree of clinical and genetic heterogeneity. Approximately 10% to 15% of ovarian carcinomas occur secondary to an inherited mutation in BRCA1 or BRCA2 (1, 2). Inherited mutations in BRCA1 confer lifetime risks of breast cancer of 60% to 80% and of ovarian cancer of >25% to 50% (3, 4). A better understanding of the molecular events of breast/ovarian transformation would facilitate improved surveillance, prevention, and therapy.

On the other hand, the development of human cancers is a multistep complex process by which cancer cells acquire the ability to overcome the restraints imposed by the surrounding normal tissue internal environment (5). This process is believed to be driven by the intrinsic genomic instability of cancer cells to express genes that confer selective advantages under the adverse growth conditions associated with a rapidly expanding tumor mass.

Recently, gene expression pattern differences between various types of human cancers and their corresponding normal tissues have been determined by using the serial analysis of gene expression and gene array analyses (6–10).

In particular, in breast cancer, such investigations have led to the application of gene array analysis in the surveillance, prevention, prognosis, and therapy of patients according to the molecular signatures of the individual tumors (9, 11–13).

BRCA1 is a tumor suppressor gene whose germ-line mutations predisposes to breast and ovarian cancer (1, 14) and regulates multiple nuclear processes including DNA repair and recombination, checkpoint control of the cell cycle, transcription, chromatin remodeling, and ubiquitination (15–19).

BRCA1 encodes a nuclear phosphoprotein of ~ 220 K_d, functionally organized in domains located at the NH_2 and COOH termini of the molecule (20). In particular, the COOH-terminal BRCT domain is an evolutionarily conserved region characterized by hydrophobic clusters of amino acids that are thought to stabilize the three-dimensional structure of the protein (21). Along with the role in the stability of protein conformation, the BRCT domain is involved in protein-protein interactions with many different molecules (22). The majority of

Translational Relevance

In this study, the gene expression pattern produced by the breast cancer-associated BRCA1 5083del19 founder mutation was studied by microarray analysis with the aim to shed light on the features of inherited breast cancer through the identification of new, more specific, and sensitive tumor biomarkers that could be rapidly translated in the clinical practice, contributing to improve early diagnosis and design patient-tailored therapies. Among the genes whose expression was modified, a periostin up-regulation was detected in HeLa/^{5083del19}BRCA1. *In vitro* and *in vivo* experiments confirmed this finding, underscoring its clinical relevance. In fact, based on previous findings showing that periostin is a secreted protein and on our observation on the sera from breast cancer patients, it can be hypothesized that the periostin product might be searched in the biological fluids, thus enhancing the possibility of an earlier diagnostic tool of familial breast cancer-bearing BRCA1 mutations.

cancer-associated BRCA1 mutations affect the BRCT tandem, resulting in truncated products lacking one or two BRCT domains (23). These findings together with the observation that deletion of the BRCA1 BRCT domains is responsible for tumor development in mice (24) show that BRCT domains play a central role in the BRCA1 tumor suppressor function. Our group recently identified a BRCA1 mutation consisting of a heterozygous frame shift mutation causing a large 19-bp deletion in exon 16, leading to a stop codon at position 1,670 that was indicated as BRCA1 founder mutation (5083del19; ref. 25).

The aim of this study was to explore the gene expression pattern of this cancer-associated BRCA1 5083del19 founder mutation by using a microarray analysis. Here, we show that periostin, whose product is released from cells in the extracellular fluids (26), was significantly up-regulated both in HeLa/^{5083del19}BRCA1 cells compared with HeLa/$^{pcDNA3.1/empty}$ cells and HeLa/^{wt}BRCA1 cells and in mammary tumor specimens and sera from individuals harboring the BRCA1 5083del19 mutation.

Materials and Methods

Cell culture and DNA transfections. HeLa, human cervix adenocarcinoma cells (parental and stable clones), and MCF-7 and MDA-MB-231, human sporadic breast cancer cells, were grown in DMEM (Life Technologies) supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin. HCC1937$^{-/-}$, human hereditary breast cancer cells, were grown in RPMI 1640 (Life Technologies) containing 15% fetal bovine serum and 1% streptomycin/penicillin. All cells were grown at 37°C in a 5% CO_2 atmosphere.

HeLa cells were stably transfected with pcDNA3.1/BRCA1^{5083del19} (HeLa/^{5083del19}BRCA1) using the calcium phosphate precipitation method and subsequently selected in 400 µg/mL G418 (Invitrogen Life Technologies). Moreover, HeLa stable clones (HeLa/$^{pcDNA3.1/empty}$, HeLa/^{wt}BRCA1) and HCC1937 stable clones (HCC1937$^{-/-pcDNA3.1/empty}$, HCC1937/^{wt}BRCA1) were generated as described previously (27, 28).

Plasmids. The entire open reading frame of BRCA1, cloned into pcDNA3.1 vector (Invitrogen Life Technologies), was already available in our laboratory. Site-directed mutagenesis of the BRCA1 insert was obtained using the QuickChange Kit (Stratagene). The BRCA1 mutant (pcDNA3.1/BRCA1^{5083del19}) was generated with the following primers: forward 5′-gtccatggtggtgtctttatgctcgtcgtacaag-3′ and reverse 5′-cttgtacac-gagcataaagacaccaccatggac-3′. The construct was verified by DNA sequencing.

Gene expression profiling. Total RNA was extracted from triplicate plates of from HeLa cells (HeLa/$^{pcDNA3.1/empty}$, HeLa/^{wt}BRCA1, and HeLa/^{5083del19}BRCA1) with TRIzol method (Invitrogen Life Technologies) according to the manufacturer's instructions. RNA was further cleaned up using RNeasy mini-kit (Qiagen), and quality was assessed with a Agilent Bioanalyzer 2100 (Agilent Technologies). Three RNA samples each from HeLa/$^{pcDNA3.1/empty}$, HeLa/^{wt}BRCA1, and HeLa/^{5083del19}BRCA1 cells were labeled and hybridized to Affymetrix GeneChip U133 Plus 2.0 that contains 54,000 probe sets for >47,000 characterized genes and expressed sequence tags. Sample labeling and processing, GeneChip hybridization, and scanning were done according to Affymetrix protocols. Briefly, double-stranded cDNA was synthesized from total RNA with the SuperScript Choice System (Invitrogen), with a T7 RNA polymerase promoter site added to its 3′-end (Genset). Biotinylated cRNAs were generated from cDNAs *in vitro* and amplified by using the BioArray T7 RNA polymerase labeling kit (Enzo Diagnostics). After purification of cRNAs by the RNeasy mini kit (Qiagen), 20 µg cRNA was fragmented at 94°C for 35 min. Fragmented cRNA (~12.5 µg) was used in a 250 µL hybridization mixture containing herring-sperm DNA (0.1 mg/mL; Promega) plus bacterial and phage cRNA controls (1.5 pmol/L BioB, 5 pmol/L BioC, 25 pmol/L BioD, and 100 pmol/L Cre) to serve as internal controls for hybridization efficiency. Aliquots (200 µL) of the mixture were hybridized to arrays for 18 h at 45°C in a GeneChip Hybridization Oven 640 (Affymetrix). Each array was washed and stained with streptavidin-phycoerythrin (Invitrogen) and amplified with biotinylated anti-streptavidin antibody (Vector Laboratories) on the GeneChip Fluidics Station 450 (Affymetrix). Arrays were scanned with the GeneArray G7 scanner (Affymetrix) to obtain image and signal intensities.

BRB-ArrayTools version 3.5.0 (Research Branch, National Cancer Institute; ref. 29) were used to import Affymetrix CEL files and Robust Multichip Average method was done. Robust Multichip Average uses background correction on the Perfect Match data and then applies a quantile normalization and summarizes the probe set information using Tukey's median polish algorithm (30).

The Significance Analysis of Microarrays software version 3.0 (31) that employs the standard *t* test and permutation was used to draw inferences to identify differentially expressed mRNA. The cutoff for significance is determined by a variable called delta, which is the difference between the observed score and the average expected score (*t* statistics for the randomly permutated data) and is chosen based on the false discovery rate. False discovery rate is computed as a median or 90th percentile of the number of falsely called genes divided by the number of genes called significant. Here, we applied two classes unpaired comparison (ZD versus ZS) with *t* statistics. The value of delta was set so that the estimated *q* value was between 0 and 5%. *q* value is the lowest false discovery rate at which that gene is considered significant. The *q* value is similar to the well-known *P* value, except that it is a measure of significance in terms of the false discovery rate rather than the false-positive rate. The use of *q* value avoids a flood of false.

For hierarchical clustering, we used average linkage metrics and centered Pearson correlation (Cluster 3.0). Java Treeview 1.1.1 was used for tree visualization.

Western blot analysis. Western blotting analysis for detection of BRCA1 (wild-type and mutant) proteins was done on HeLa total extract as described previously (32). Total protein extract (60 µg) was separated by SDS-PAGE and transferred to nitrocellulose membranes (Immobilon P; Millipore). After addition of the blocking mixture [5% (w/v) milk in PBS (pH 7.4) and 0.05% Tween 20], the membrane was incubated with a 1:200 dilution of mouse anti-human BRCA1 antibody (SD118-Oncogene). The signal was detected with anti-mouse horseradish

peroxidase-conjugated secondary antibody (1:5,000; Santa Cruz Biotechnology). The membrane was developed by enhanced chemiluminescence-Western blot detection reagents according to the manufacturer's instructions (Santa Cruz Biotechnology). Moreover, the membrane was incubated with a goat anti-γ-tubulin antibody HRP-conjugated (C-20; Santa Cruz Biotechnology) to ensure uniform gel loading.

Western blotting analysis for detection of periostin protein was done on cytosolic fraction from HeLa cells (HeLa/[pcDNA3.1/empty], HeLa/[wt]BRCA1, and HeLa/[5083del19]BRCA1), HCC1937 cells (HCC1937[-/-pcDNA3.1/empty], HCC1937/[wt]BRCA1, and HCC1937^{-/-}), MCF-7 and MDA-MB-231 as described previously (33). Cytosolic fraction was diluted in 1 mL with buffer A [10 mmol/L sodium phosphate buffer and 0.15 mol/L sodium chloride (pH 7.14)] at final concentration of 1 μg/μL and was loaded on 100 μL heparin Sepharose (Pharmacia) equilibrated previously with buffer A and then incubated overnight at +4°C.

Subsequently beads were collected by centrifugation at 3,000 rpm for 2 min and washed two times with 1 mL buffer A. Elution was done at +4°C platform with 10 mmol/L sodium phosphate buffer and 1 mol/L sodium chloride (pH 7,14).

The fractions collected from different cell lines were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Immobilon P; Millipore). After addition of the blocking mixture, the membrane was incubated with a 1:2,000 dilution of rabbit anti-human periostin antibody (Abcam). The signal was detected with anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5,000) (Santa Cruz Biotechnology) and by enhanced chemiluminescence (Santa Cruz Biotechnology). Coomassie blue-stained was used as a control for quantity of protein extracts loaded.

Moreover, Western blot analysis for detection of periostin protein was done on 100 μg serum proteins resolved by SDS-PAGE and transferred to nitrocellulose membranes (Immobilon P; Millipore). Serum protein concentration for each sample was measured in triplicate using the dye-binding protein (Bio-Rad) with bovine serum albumin as standard curve. An internal control was not used because, as suggested by others, it is probably impossible to find a "housekeeping" protein in the serum that could be used as a constant reference; in fact, all serum proteins are subjected to significant changes between individuals (34).

The periostin protein was further detected in the conditioned medium of HeLa/[5083del19]BRCA1cells. The conditioned medium was concentrated 6-fold using a Centriprep YM-30 (Millipore) and than transferred to sterile tubes containing cold TCA (20%, w/v) and the proteins were precipitated for 30 min on ice followed by centrifugation at 15,000 rpm for 15 min at 4°C. The supernatant was decanted, and the pellet washed with chilled acetone followed by removal of all the acetone. The pellet was subsequently solubilized in LB [31.25 mmol/L Tris-HCl (pH 6.8), 1.25% SDS, 6.25% glycerol, 0.06% bromophenol
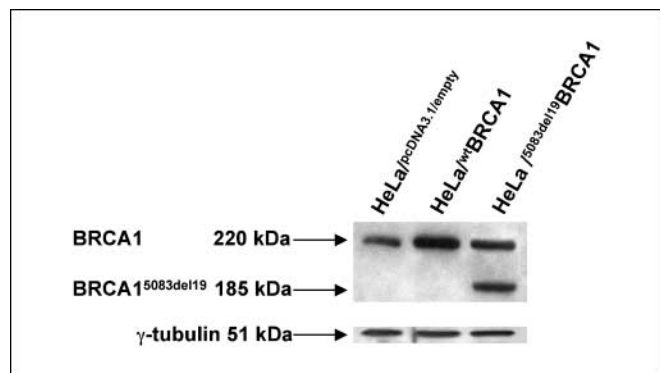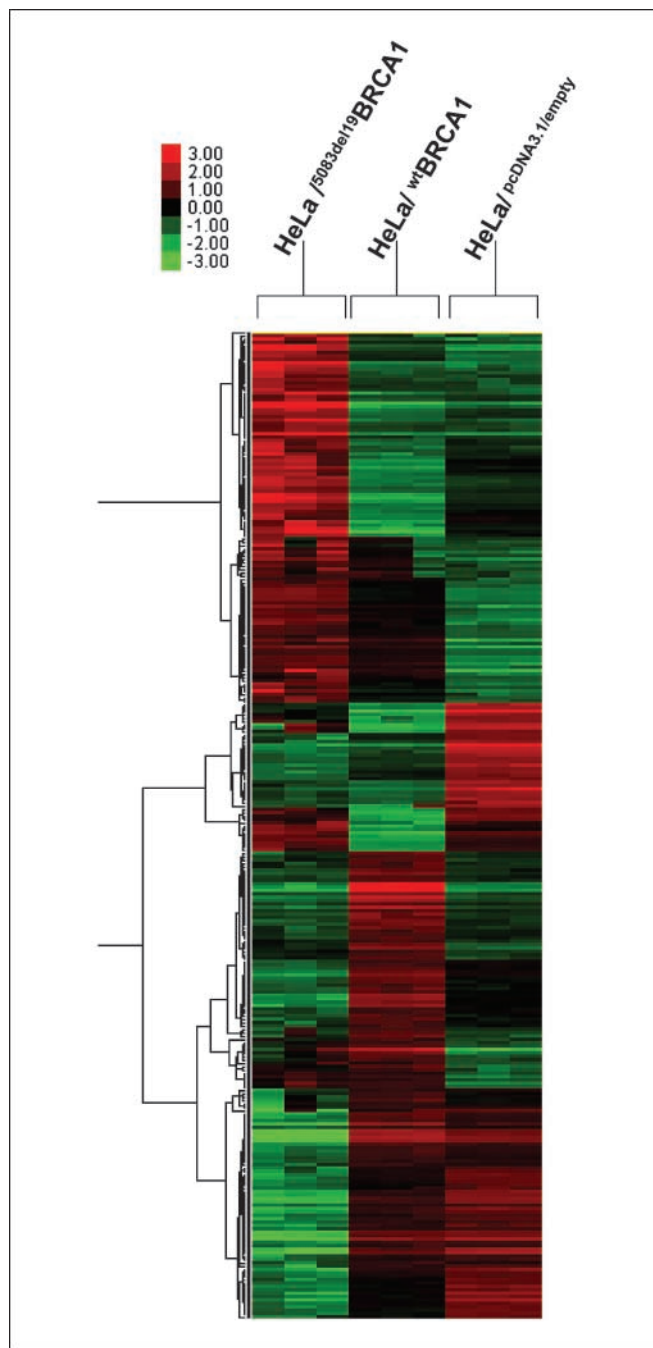

Fig. 2. Clustering analysis of gene expression profile in HeLa/[5083del19]BRCA1 (*lane 1*) cells compared with control HeLa/[wt]BRCA1 (*lane 2*) and HeLa/[pcDNA3.1/empty] cells (*lane 3*).

blue, and 5% β-mercaptoethanol], resolved by SDS-PAGE, and transferred to nitrocellulose membranes.

RNA extraction and semiquantitative reverse transcription-PCR. Total RNA extraction for semiquantitative reverse transcription-PCR (RT-PCR) was done from HeLa cells (HeLa/[pcDNA3.1/empty], HeLa/[wt]BRCA1, and HeLa/[5083del19]BRCA1), HCC1937 cells (HCC1937[-/-pcDNA3.1/empty], HCC1937/[wt]BRCA1, and HCC1937^{-/-}), MCF-7, and MDA-MB-231 cells at 80% to 90% confluence with TRIzol reagent (Life Technologies) according to the manufacturer's protocol. A total of 5 μg DNase-treated RNA was reverse transcribed into first-strand cDNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) with random hexanucleotide primers. cDNA (2 μL) was

Fig. 1. Western blot analysis of total protein extracts of HeLa/[pcDNA3.1/empty] (*lane 1*), HeLa/[wt]BRCA1 (*lane 2*), and HeLa/[5083del19]BRCA1 (*lane 3*). A mouse anti-BRCA1 monoclonal antibody was used.

amplified for periostin gene with the following primers: forward 5′-gtctttgagacgctggaagg-3′ and reverse 5′-agatccgtgaaggtggtttg-3′. A human glyceraldehyde 3-phosphate dehydrogenase cDNA fragment was amplified as the internal control for the amount of cDNA in the PCR.

Immunohistochemical analysis. We studied 12 consecutive invasive paraffin-embedded breast cancer samples, grouped in four categories based on the immunohistochemical expression of hormonal receptors and cErbB2 protein: four cases of RE-, RPg-, cErbB2-, four cases of RE+, RPg+, cErbB2-, three cases of RE-, RPg-, cErbB2 +, and one case of RE+, RPg+, cERbB2+. All patients were in perimenopausal or postmenopausal age, with a median average of 64 years. Two of them were referred as carriers for the 5083del19 deletion in the BRCT domain. Sections of 5 μm of formalin-fixed, paraffin-embedded tumor tissue were stained with a rabbit anti-human periostin antibody (Abcam); for each sample, antigen retrieval was tested both by enzymatic digestion with peroxidase enzyme and microwave treatment (800 W for three 5-min periods) using decreasing dilutions ranging from 1:1,000 to 1:200. Immunodetection was done by the ChemMate Dako EnVision Detection Kit Peroxidase/DAB Rabbit/Mouse (DAKO). All samples were tested without primary antibody as control. The best results were obtained with a dilution of 1:400 and with MW antigen retrieval.

Results

Microarray analysis of HeLa/[5083del19]BRCA1 cells. The primary goal of this study was to identify genes whose expression could be modulated by a cancer-associated BRCA1 founder mutation (5083del19). To test whether BRCA1 5083del19 mutation could activate or inactivate potential target genes, HeLa cells were stably transfected with the 5083del19 BRCA1 mutant cDNA (HeLa/[5083del19]BRCA1; see Materials and Methods); the expression levels of the BRCA1 5083del19 protein were assessed by Western blot analysis (Fig. 1). HeLa cells either transfected with the backbone vector (HeLa/[pcDNA3.1/empty]) or wild-type BRCA1 cDNA (HeLa/[wt]BRCA1; ref. 27) were used as controls. Total RNA purified from three samples of each HeLa cell line (HeLa/[pcDNA3.1/empty], HeLa/[wt]BRCA1, and HeLa/[5083del19]BRCA1, respectively) was used to hybridize a GeneChip arrays (Affymetrix; see Materials and Methods).

We then compared gene expression profiles of HeLa/[5083del19]BRCA1 versus HeLa/[wt]BRCA1 and versus HeLa/[pcDNA3.1/empty], respectively. The profile of genes transcriptionally responsive to BRCA1 5083del19 mutation is shown in Fig. 2. Significant analysis of microarrays was done by SAM, PAM, and BRB ArrayTools (see Materials and Methods). A partial list of genes up-regulated and down-regulated is shown in Supplementary Table S1 (HeLa/[5083del19]BRCA1 versus HeLa/[pcDNA3.1/empty]) and Supplementary Table S2 (HeLa/[5083del19]BRCA1 versus HeLa/[wt]BRCA1). In Supplementary Table S3 are shown genes that were overexpressed at least >2-fold, common to HeLa/[5083del19]BRCA1 versus HeLa/[pcDNA3.1/empty] and HeLa/[5083del19]BRCA1 versus HeLa/[wt]BRCA1, and categorized by function. No gene was found underexpressed >2-fold (see Supplementary Tables S1 and S2).

Among the genes putatively responsible for pathologic progression and associated with late stages of human cancer development, we found that periostin expression (also termed OSF-2 for osteoblast-specific factor-2) was >72- and 76-fold in HeLa/[5083del19]BRCA1 versus HeLa/[pcDNA3.1/empty] and in HeLa/[5083del19]BRCA1 versus HeLa/[wt]BRCA1, respectively, with a score that represents the value of the *t* statistic, of ∼ 19 and ∼ 20, respectively (see Supplementary Table S3).

Interestingly, periostin was originally defined as a secreted factor associated with osteoblastic cell function during bone development (6, 35, 36) and its overexpression was found in a broad range of human cancer types, including breast cancer (37 – 40).

Periostin expression is positively regulated in vitro ***and*** in vivo ***by [5083del19]BRCA1 mutation.*** To validate the data obtained with the microarray analysis, we did both RT-PCR and Western blot analysis. Periostin overexpression was initially assessed by RT-PCR carried out on an aliquot of the same RNA used in the microarray analysis. As shown in Fig. 3A, two independent assays confirmed that the transcript of the gene periostin is significantly increased in the HeLa/[5083del19]BRCA1 cells. Glyceraldehyde 3-phosphate dehydrogenase cDNA was used as control of quantify and quality of RNA preparation.

To determine if the higher levels of periostin mRNA expression revealed in the HeLa/[5083del19]BRCA1 cells by gene array analysis and confirmed by RT-PCR analysis were paralleled by an analogous increase in the protein level, we did Western blot analysis on cytosolic proteins extracted from HeLa cell lines (HeLa/[pcDNA3.1/empty], HeLa/[wt]BRCA1, and HeLa/[5083del19]BRCA1) by using a periostin antiserum (see Materials and Methods). As shown in Fig. 3B, periostin is highly expressed in HeLa/[5083del19]BRCA1 cells. Coomassie blue-stained was used as a control for quantity of protein extracts loaded (data not shown).

To enforce the idea that periostin overexpression may be intimately linked to cancer-associated BRCA1 mutations located within the BRCT tandem domain, we evaluated its expression by RT-PCR and Western blot analysis in a panel of breast cancer cell lines (HCC1937^{-/-}, HCC1937[-/- pcDNA3.1/empty], HCC1937/[wt]BRCA1, MCF-7, and MDA-MB-231; see Materials and Methods). As shown in Fig. 4A and B, periostin expression is increased, at both mRNA and protein levels, in HCC1937^{-/-} cells. Conversely, periostin was not detectable in HCC1937/[wt]
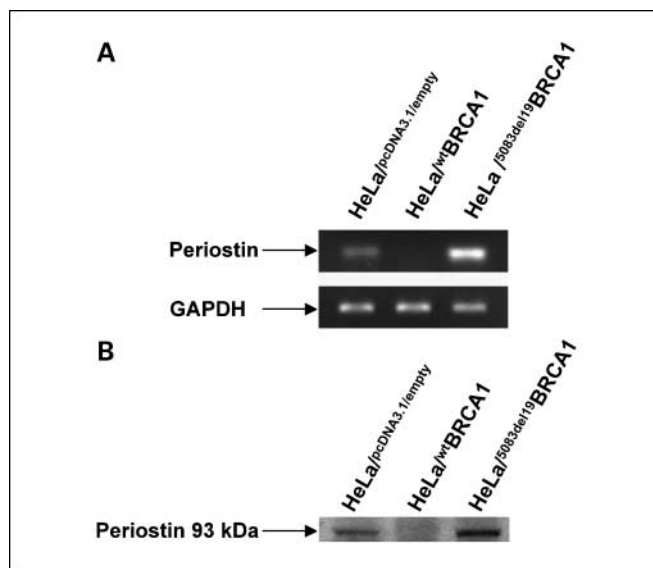

Fig. 3. *A,* RT-PCR amplification done using RNA from HeLa cells [HeLa/[pcDNA3.1/empty] (*lane 1*), HeLa/[wt]BRCA1 (*lane 2*), and HeLa/[5083del19]BRCA1 (*lane 3*)]. Following nested PCR amplification, the products were separated by agarose gel and stained by ethidium bromide. *B,* cytosolic fractions from HeLa/[pcDNA3.1/empty] (*lane 1*), HeLa/[wt]BRCA1 (*lane 2*), and HeLa/[5083del19]BRCA1 (*lane 3*) were analyzed by Western blot using a specific polyclonal anti-periostin antibody.
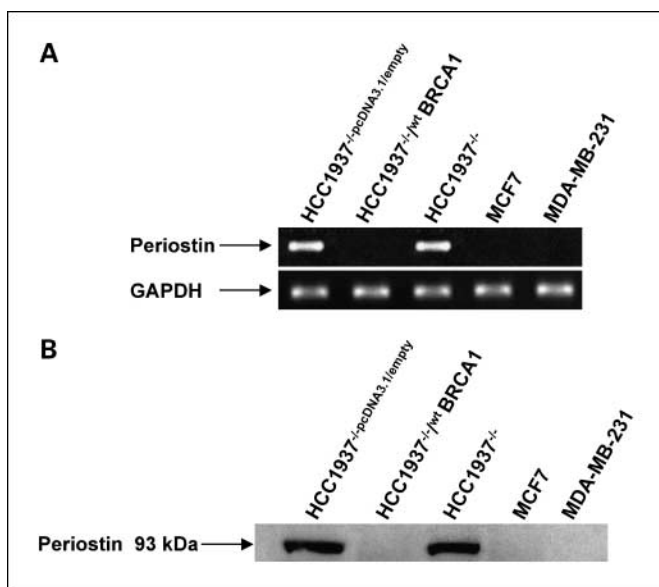
Fig. 4. *A,* RT-PCR amplification done using RNA from HCC1937[-/-pcDNA3.1/empty] (*lane 1*), HCC1937/[wt]BRCA1 (*lane 2*), HCC1937^{-/-} (*lane 3*), MCF-7 (*lane 4*), and MDA-MB-231 cells (*lane 5*). *B,* Western blot analysis of cytosolic fractions from HCC1937[-/-pcDNA3.1/empty] (*lane 1*), HCC1937/[wt]BRCA1 (*lane 2*), HCC1937^{-/-} (*lane 3*), MCF-7 (*lane 4*), and MDA-MB-231 cells (*lane 5*) using the same anti-periostin antibody of Fig. 3B.

BRCA1 cell, where wild-type BRCA1 protein expression was reconstituted, and in MCF-7, MDA-MB-231 cell lines, consistent with previously reported data (41).

Furthermore, the presence of the periostin protein was assessed in conditioned medium from HeLa/[5083del19]BRCA1 cells. As shown in Fig. 5A, periostin is not present at 12, 24, and 36 h, whereas it can be detected after 48 h following seeding of the cells. The higher molecular weight of periostin in lane 9 is due to glycosylation of this protein at Asn[599] (42).

To confirm this finding in cancer patients, periostin expression was analyzed in sera as well as in tissue from these individuals. In particular, Western blot analysis was done on serum from two patients bearing the BRCA1 5083del19 mutation, two healthy carriers, and two unaffected controls.

Remarkably, a high level of expression of the periostin protein was observed in cancer patients, whereas the periostin was less expressed in the two healthy carriers. No expression was detected in healthy controls (Fig. 5B).

Immunohistochemical analysis of periostin expression in invasive ductal breast cancer. Periostin expression was assessed in 12 samples obtained from individuals with sporadic and inherited breast cancer. Although only an aspecific periostin staining of the stroma was observed in enzymatic digestion-treated samples (Fig. 6A), a remarkable staining for this protein was detected in cancer cells following microwave treatment in the two samples positive for BRCA1 gene mutations (Fig. 6B). Negative staining was observed in normal ductal cells as well as in the absence of the primary antibody (Fig. 6C and D).

All positive cases belong to the triple-negative subgroup (ER-, PgR-, cErbB2-) of breast cancer. They were high-grade invasive ductal cancer according to Elston and Ellis (43), with pushing growing margins, necrosis, and a rich lymphocyte infiltrate. In one case, lymph node metastasis was present, and in two cases, neoplastic peritumoral embolism and muscle tissue infiltration were detected.

Discussion

Despite the tremendous improvement in clinical management of breast cancer achieved in the past decade, several questions on the molecular mechanisms underlying BRCA1-driven tumorigenesis are still open. On the other hand, studies of the pathologic features of hereditary breast cancer suggest that cancers with underlying germ-line mutations differ from each other and from cancers that do not carry these mutations. In this scenario, a better understanding of this process, through the identification of new, more specific/sensitive tumor biomarkers, could be rapidly translated in the clinical practice, contributing to ameliorate early diagnosis, to design patient-tailored therapies, and to eventually lower mortality rate.

Since 1995, when the first article on cDNA microarray technology was published (44), thousands of reports have shown the enormous potentials of this tool. In this report, to investigate the role the cancer-associated BRCA1 founder mutation (5083del19; ref. 25) in mammary tumorigenesis, we studied the changes in the cellular transcription profile induced by this mutant allele by microarray technology and confirmed our findings by different strategies.

Microarray analysis done on three distinct cell lines (HeLa/[pcDNA3.1/empty], HeLa/[wt]BRCA1, and HeLa/[5083del19]BRCA1) allowed for the identification of several genes whose expression was modified by the exogenous BRCA1[5083del19] compared with wild-type BRCA1. Among the genes known to play a role in pathologic tumor progression and to be associated with late stages of human cancer development, we found that periostin expression was significantly up-regulated in HeLa/[5083del19] BRCA1 cells compared with both HeLa/[pcDNA3.1/empty] and HeLa/[wt]BRCA1 cells. These data were confirmed by semiquantitative RT-PCR and Western blot analysis in all cell lines used in this study.

Moreover, we were able to show that periostin is detected only in the conditioned medium of HeLa/[5083del19]BRCA1 cells.

Periostin, an osteoblast-specific secreted protein known to be associated with cell adhesion activity for bone formation and development, by the epithelial cell-derived tumors, leads to a significant enhancement in angiogenesis and tumor progression (41). Periostin overexpression has been found in a broad range of human cancer types, including breast cancer (45, 46). Moreover, it has been reported that increased
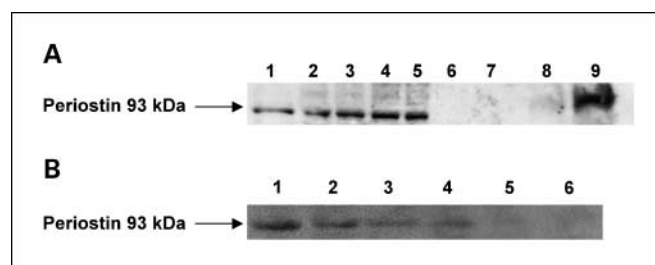

Fig. 5. *A,* cytosolic fractions and conditioned medium from HeLa/[5083del19]BRCA1 cells were analyzed by Western blot using a specific polyclonal anti-periostin antibody. *Lane 1,* heparin Sepharose-purified periostin; *lanes 2* to *5,* cytosolic fractions (at 12, 24, 36, and 48 h from seeding, respectively); *lanes 6* to *9,* conditioned medium at 12, 24, 36, and 48 h, respectively. *B,* Western blot analysis of serum proteins using the same anti-periostin antibody. *Lanes 1* and *2,* breast cancer patients carrying the 5083del19 mutation in the BRCA1 gene; *lanes 3* and *4,* healthy BRCA1 5083del19 carriers; *lanes 5* and *6,* unaffected controls.
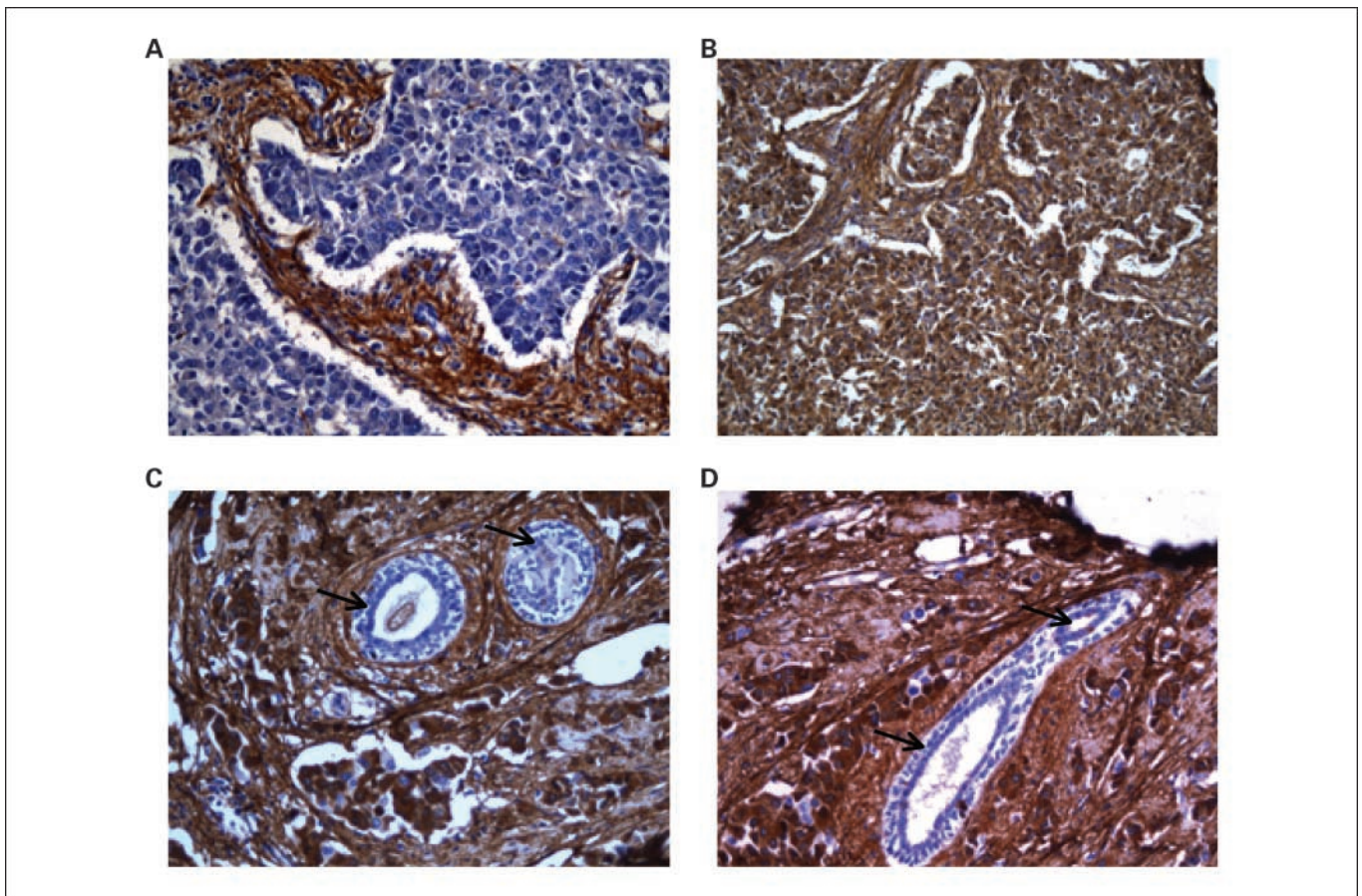
Fig. 6. Immunohistochemical analysis of ductal breast cancer tissue from a patient with a germ-line 5083del19 BRCA1 mutation. *A,* enzymatic digestion antigen retrieval produces an aspecific periostin staining of the stroma (*brown*; ×20). *B,* with the microwave treatment antigen retrieval method, a strong intracellular staining for periostin is observed (×10). *C* and *D,* normal ductal structures within the tumor (*arrows*) are negative for periostin staining (×20).

expression of periostin is associated with advanced stage and cell proliferation, adhesion, and migration (37, 39, 40, 47–52).

Based on our results, we speculate that periostin overexpression may be intimately linked to cancer-associated BRCA1 mutations that affect the BRCT tandem. In this study, in fact, we evaluated periostin expression by RT-PCR and Western blot in a panel of breast cancer cell lines (HCC1937$^{-/-}$, HCC1937$^{-/-pcDNA3.1/empty}$, HCC1937/^{wt}BRCA1, MCF-7, and MDA-MB-231). Periostin expression is increased, at both mRNA and protein levels, in HCC1937$^{-/-}$ cells. Conversely, periostin was not detectable in HCC1937/^{wt}BRCA1 cells, where wild-type BRCA1 protein expression was reconstituted, and in the MCF-7 and MDA-MB-231 sporadic breast cancer cell lines, consistent with previously reported data (41).

To assess the potential role of periostin as a molecular biomarker for the specific subset of breast cancer patients bearing BRCA1 gene mutations, we did a Western blot analysis of serum from two patients with the BRCA1 5083del19 mutation and compared the findings with sera from two healthy carriers of the same mutation and two unaffected controls.

Periostin was detected at a significant level in the serum from breast cancer patients, whereas a lower expression was found in healthy carriers. Western blot analysis was negative in the control individuals.

Finally, immunohistochemical analysis done on paraffin-embedded tissue samples obtained from 12 breast cancer patients highlighted a remarkable staining for periostin in cancer cells of two specimens, both carrying the BRCA1 5083del19 mutation, supporting the hypothesis that this protein can be considered as a specific marker for BRCA1-linked breast cancer.

These data appear of great interest, taking into account the fact that this is the first report showing a direct and strong correlation between hereditary breast cancer and periostin expression. Furthermore, based on previous findings showing that periostin is a secreted protein and on our observation on the sera from breast cancer patients, we also propose that the periostin product might be searched in the biological fluids, thus enhancing the possibility of an earlier diagnostic tool of familial breast cancer-bearing BRCA1 mutations.

Taken together, our data lend support to the general hypothesis of an important role played by periostin in cancer development (53), providing new insights into the molecular mechanisms underlying breast cancer tumorigenesis, with a special focus on the cascade of events triggered by specific mutations in the BRCA1 gene.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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