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Rhomboid domain containing 2 (*RHBDD2*): A novel cancer-related gene over-expressed in breast cancer

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ABSTRACT

In the course of breast cancer global gene expression studies, we identified an uncharacterized gene known as *RHBDD2* (*Rhomboid domain containing 2*) to be markedly over-expressed in primary tumors from patients with recurrent disease. In this study, we identified *RHBDD2* mRNA and protein expression significantly elevated in breast carcinomas compared with normal breast samples as analyzed by SAGE ($n=46$) and immunohistochemistry ($n=213$). Interestingly, specimens displaying RHBDD2 over-expression were predominantly advanced stage III breast carcinomas ($p=0.001$). Western-blot, RT-PCR and cDNA sequencing analyses allowed us to identify two *RHBDD2* alternatively spliced mRNA isoforms expressed in breast cancer cell lines. We further investigated the occurrence and frequency of gene amplification and over-expression affecting *RHBDD2* in 131 breast samples. *RHBDD2* gene amplification was detected in 21% of 98 invasive breast carcinomas analyzed. However, no *RHBDD2* amplification was detected in normal breast tissues ($n=17$) or breast benign lesions ($n=16$) ($p=0.014$). Interestingly, siRNA-mediated silencing of *RHBDD2* expression results in a decrease of MCF7 breast cancer cells proliferation compared with the corresponding controls ($p=0.001$). In addition, analysis of publicly available gene expression data showed a strong association between high *RHBDD2* expression and decreased overall survival ($p=0.0023$), relapse-free survival ($p=0.0013$), and metastasis-free interval ($p=0.006$) in patients with primary ER-negative breast carcinomas. In conclusion, our findings suggest that *RHBDD2* over-expression behaves as an indicator of poor prognosis and may play a role facilitating breast cancer progression.

1. Introduction

In the course of recent breast cancer global gene expression studies we identified a distantly related rhomboid-like gene known as *RHBDD2* (*Rhomboid domain containing 2*) located at the 7q11.23 chromosomal region, to be markedly over-expressed in primary invasive carcinomas from patients that recurred within 6 years of follow-up [1].

The human genome contains several rhomboid-like genes which can be phylogenetically grouped in three major classes [2]. The first class, includes the true active rhomboids genes that are subdivided into secretase (*e.g. RHBDL-1/-2/-3* and *RHBDD1* genes) and *PARL*-type subfamilies. The second class is composed of novel inactive rhomboids members, recently named as iRhoms group (*e.g. RHBDF-1/-2* genes). The third group includes a small number of other distant evolutionary related and uncharacterized genes (*e.g. RHBDD-2/-3*) for which there is no evidence that they are active proteases. Rhomboid-like proteins function in diverse processes including quorum sensing in bacteria, mitochondrial membrane fusion/apoptosis (*PARL*) and stem cell differentiation in eukaryotes [3,4]. Also, rhomboid-like proteins have been recently linked to human diseases, including early-onset blindness, diabetes, and parasitic diseases [5]. However, the biological functions of the mammalian rhomboid-like family remains to be determined.

Here we present data supporting the role of *RHBDD2* as a novel breast cancer-related gene. We demonstrate that *RHBDD2* is over-expressed at the mRNA and protein levels in breast cancer samples and in some of these cases due to gene amplification. Interestingly, analysis of publicly available breast cancer gene expression databases indicates that *RHBDD2* is over-expressed in estrogen receptor-negative breast carcinomas from patients with poor prognosis. Finally, we show that *in vitro RHBDD2* silencing regulates cell proliferation of breast cancer cells.

2. Material and methods

2.1. Serial analysis of gene expression database mining

To perform a comparative analysis of the human Rhomboid-like family members expressed in breast tissue, we analyzed 46 breast

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SAGE (serial analysis of gene expression) libraries: 4 normal breast epithelium, 8 ductal carcinoma *in situ* (DCIS), and 33 invasive ductal carcinomas (IDC). To this end, we combined 29 breast cancer SAGE libraries generated by us at a resolution of 100,000 tags per library (Aldaz Laboratory) with 17 SAGE libraries (generated at the Polyak Laboratory, Dana-Farber Cancer Institute, Boston, MA, USA) downloaded from the Cancer Genome Anatomy Project — SAGE Genie database (http://cgap.nci.nih.gov/SAGE/). SAGE data management and tag-to-gene matching for *RHBDD1* (AGGGCAGGGA), *RHBDD2* (TTGTCTGCCT), *RHBDD3* (CTGCCCTAGT), *RHBDL1* (TGGTGGCCGC), *RHBDL2* (AGTTCAAGAC), *RHBDL3* (TTGCTCCCCG), *RHBDF1* (TGGCCAATAA), *RHBDF2* (GATTAAATAA), and *PARL* (GCTATGCTCC) were performed with a suite of web-based SAGE library annotation tools developed by us (http://spi.mdacc.tmc.edu/bitools/about/sage_lib_tool.html). To enable the visualization and illustration of our analyses, we used the TIGR MultiExperiment Viewer (MeV 3.0) software (The Institute for Genomic Research, Rockville, MD, USA). This tool was employed for normalization and average clustering of the SAGE data.

2.2. RHBDD2 antibody production, Western-blot and immunofluorescence analyses

A polyclonal antibody against RHBDD2 was generated by sequential immunizations of two rabbits with three purified KLH-conjugated peptides (GenScript Corp., NJ, USA). Peptides were synthesized based on RHBDD2 protein sequence (NP065735) corresponding to residues 30–43 (EDRQPASRRGAGTT), residues 253–266 (ASGAEARSDLPLQP), and residues 393–406 (HQGLQAPRSPPGSP). The polyclonal antibody was purified from the immune serum by affinity chromatography. The primary antibody specificity was further demonstrated by Western-blot, immunofluorescence and siRNA analyses using breast cell lines (see below Figs. 2 and 6B).

Total protein extracts were prepared from a set of 7 breast normal and cancer cell lines (HME87, MCF10, MCF7, ZR75-30, T47D, BT47A, and BT549). As normal controls we also included human breast epithelial organoid protein extracts, obtained from three independent cosmetic mammoplastic specimens (B26, B27, and B28). Total cell protein lysates were made from frozen tissues using RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS) containing protease inhibitor cocktail (Roche, Mannheim, Germany). For Western blot, 50 µg of total protein was separated by 12.5% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA). Immunodetection was performed using Protein Detector™ (KPL, Gaithersburg, MD) Western blotting reagents as described by the manufacturer. RHBDD2 protein was detected using an anti-RHBDD2 polyclonal primary antibodies and HRP conjugated anti-rabbit secondary antibody (KPL, 1:2000) followed by chemiluminescence autoradiography. Beta-actin protein (ACTB) was detected using monoclonal anti-actin antibody (ICN Biomedicals, Burlingame, CA, 1:1000) and HRP conjugated anti-mouse secondary antibody (KPL, 1:5000).

For immunofluorescence analysis, MCF7 and MCF10 smears from exponentially growing cells were prepared on glass slides and then fixed in cold acetone for 10 min. Anti-RHBDD2 polyclonal antibody was added to the slides at a 1:250 dilution, and incubated at room temperature during 1 h. Detection was performed using FITC-labeled secondary anti-rabbit IgG antibodies following standard immunofluorescence methods.

2.3. RT-PCR and cDNA sequencing analyses

Total RNA was isolated from eleven frozen normal breast tissue derived from cosmetic mammoplastic specimens and three breast cancer cell lines (MCF7, T47D and ZR75) using TRI Reagent® (MCR Inc., USA). Quality control of RNA integrity was made by running out RNA samples onto formaldehyde denaturing 1.2% agarose gel and ethidium bromide staining. Following DNAse I digestion, template cDNAs were synthesized using SuperScript™ First-strand Synthesis System (Invitrogen, USA). For mRNA expression analysis, we designed three combinations of PCR primers (Forward-1/Reverse, Forward-2/Reverse, and Forward-3/Reverse) that spanned all exons of the *RHBDD2* transcript. The primer sequences are as follows: Forward-1 5′-CATCCGCCACCTTCTTCACT-3′; Forward-2 5′-GATCTTCGCCATCTTCTCCG-3′; Forward-3 5′-GCCTGATGAGGAGGATATCCG-3′; and Reverse 5′-AATACACCGTGCCAGGGCT-3′.

The isolated RT-PCR products were sequenced in both directions using the DYEnamic ET Dye Terminator Cycle Sequencing Kit and analyzed on a MEGABACE 1000 DNA sequencer (GE Healthcare Biosciences), according to the manufacturer's instructions. The obtained DNA sequences were further aligned and compared with the *RHBDD2* genomic sequence (accession no. NT007933.14) to determine the exon–intron structure of *RHBDD2*. Experiments were performed in triplicate for each data point and beta-actin (*ACTB*) mRNA was used as reference.

2.4. Inmunohistochemistry analyses of RHBDD2 protein expression in tissues

Formalin-fixed paraffin-embedded breast tissue samples were obtained from the MD Anderson Cancer Center — USA (61 samples), and from different hospitals associated to the National University of La Plata — Argentina (152 samples). The use of human tissue blocks and clinical records was approved by the appropriate institutional committees. By pooling both sample sets we were able to analyze a total of 213 cases including: 44 normal breast tissues (18 normal tissues from individuals without breast cancer, and 26 normal tissues adjacent to invasive breast cancers), 18 benign breast disease (7 benign mammary dysplasia, and 11 breast fibroadenomas), 26 ductal carcinomas *in situ* (DCIS), 109 primary invasive ductal carcinomas (IDC) and 16 lymph node metastasis specimens (LNM). Stage at time of diagnosis was based on the TNM classification.

Prior to immunostaining, endogenous peroxidase activity was blocked with 3% H_2O_2 in water for 10 min; heat-induced epitope retrieve was performed with 10 mM Citrate buffer pH 6.0 for 10 min in a microwave oven followed by a 20 min cool down. In order to block non-specific antibody binding, the slides were incubated with 10% goat serum in PBS for 30 min. Primary polyclonal RHBDD2 antibody was used at 1:250 dilution. Inmunodetection was performed with the DakoCytomation LSAB+ System-HRP (Dako, Denmark). Sections were counterstained with hematoxylin (Sigma, USA) and examined by light microscopy. Staining intensity was graded as negative (−), weak (+), moderate (++), or strong (+++). The number of optical fields in a specimen that were positively stained was expressed as a percentage of the total number of optical fields containing tissue. A reaction was considered positive when more than 5% of the breast epithelial cells were stained. The staining of cytoplasm, plasma membrane and nucleus was evaluated; cells were considered positive when at least one of these components was stained. IHC analysis of ESR1 (ER-alpha) was performed by using a primary monoclonal ERα antibody (ER-6F11, Novocastra — UK) at 1:50 dilution as was previously described [6].

2.5. RHBDD2 gene amplification analysis

DNA was isolated from 131 formalin-fixed paraffin-embedded samples (17 normal samples, 16 benign lesions and 98 invasive breast carcinomas) previously analyzed for RHBDD2 protein expression by IHC. *RHBDD2* gene amplification was estimated using a competitive PCR method [7,8]. In the duplex PCR reactions, *RHBDD2* gene located at 7q11.23, and Solute carrier family 13A1 gene (*SLC13A1*, gene not known to be associated with cancer) located at 7q31–q32, is used as the intra-chromosomal arm reference gene. *SLC13A1* was chosen as reference gene because its chromosome

location (7q31–32) is lost in less than 2% of breast cancer cases based on array-CGH Progenetix database [9]. Duplex PCR amplification was done in a final volume of 50 µl using 10 ng of cDNA, 1.25 U of Taq DNA polymerase (Promega, Madison, Wisconsin — USA), 2.5 mM $MgCl_2$, 200 mM of each dNTP and 12.5 pmol of both primer pairs in PCR buffer (20 mM, Tris–HCL pH 8.4, and 50 mM KCL). The primers for both genes are RHBDD2-Forward 5′-CATCCGCCACCTTCTTCACT-3′, RHBDD2-Reverse 5′-TGGTGATGAGGACCGAGACA-3′ (amplicon of 259 bp), *SLC13A1*-Forward 5′-TCGCCGATTTCTCTTCGTG-3′ and *SLC13A1*-Reverse 5′-GCCAGGCAGTTAAACAGCAAA-3′ (amplicon of 157 bp). The reactions were cycled as follows: 1 cycle of 94 °C for 2 min; 25 cycles of 40″ at 92 °C, 40″ at 57 °C, and 40″ at 72 °C. Detection of the amplified fragments was made by electrophoresis onto a 2% agarose gel and SYBR-Safe™ DNA staining (Invitrogen — USA). The Kodak Digital Science 1D Image Analysis Software was used to determine the ratio between net intensity bands of *RHBDD2* and *SLC13A1* amplicons. Samples were considered to be affected by genomic amplification for *RHBDD2* when the ratio between net intensity bands was greater than +3 SD (99% confidence interval, $p<0.001$) relative to the average value determined from 11 normal breast control samples. Experiments were performed in triplicate for each data point.

2.6. In silico RHBDD2 gene expression and clinical data analysis

To further investigate correlations of transcriptional up-modulation of *RHBDD2* gene and clinicopathologic parameters on larger breast carcinoma sets, data were obtained from a publicly available breast cancer microarray study [10]. Clinicopathologic and gene expression data from 295 primary invasive breast carcinomas (226 ER-positive and 69 ER-negative carcinomas) were collected from the Rosetta Inpharmatics website (http://www.rii.com/publications/2002/nejm.html). Two groups of patients were derived by using the median expression value of the Log2 ratio overall distribution for RHBDD2 probe (NM_020684) (RHBDD2 median = 0.017; high expression, greater than 0.017; and low expression, less than 0.017). Kaplan–Meier analysis was assessed with both groups using the Van de Vijver et al. data by means of SPSS® statistic software (SPSS Inc., Chicago).

2.7. RHBDD2 gene silencing and cell proliferation assay

Three siRNAs of 19-mer against *RHBDD2* mRNA corresponding to coding sequences starting at positions 1326 (siRNA-R1: 5′-CUGUGUUGGGUACUUUGAUdTdT-3′), 340 (siRNA-R2: 5′-GUCUACGAGAAUCCCAUCUdTdT-3′) and 1050 (siRNA-R3: 5′-GCAGAACCACUUUGGUCCAdtdt-3′) relative to the cDNA sequence AF226732 were synthesized (Bioneer Inc, Korea). The AccuTarget™ biotin-labeled negative control siRNA (siRNA-NegCt: 5′-CCUACGCCACCAAUUUCGUdTdT-3′) (Bioneer Inc, Korea) that exhibits no homology to any human genome sequence was used as a non-silencing reference. In addition, we included a positive control siRNA sequence (Termo Scientific Dharmacon, USA),

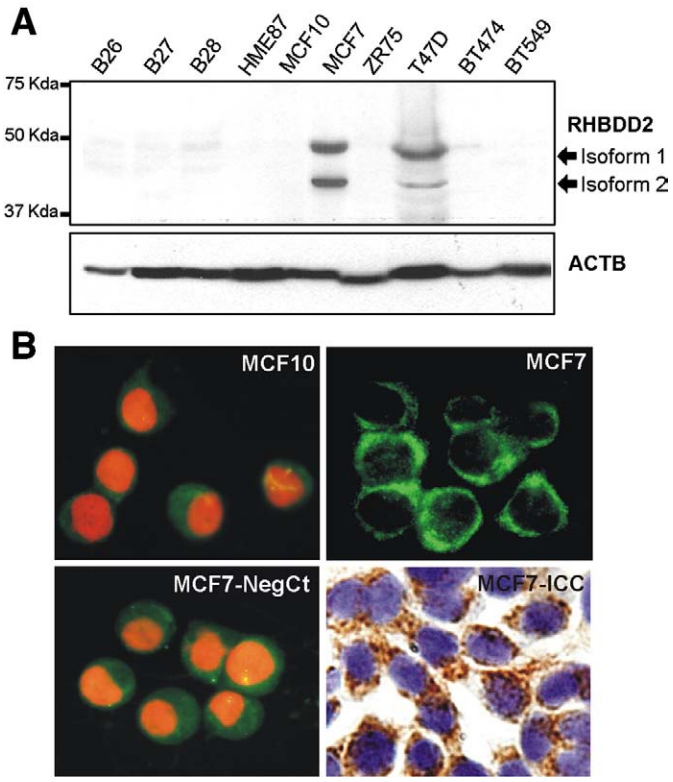

Fig. 2. RHBDD2 protein expression in normal and breast cancer cell lines. (A) Protein extracts were separated by 12.5% SDS-PAGE and transferred to PVDF membranes. RHBDD2 protein was detected using a polyclonal anti-RHBDD2 antibody developed by our laboratory. The membrane was then probed with mouse monoclonal anti-actin antibody for normalization of differences in protein loading. Three normal human protein extracts prepared from breast organoids were included (B25, B26, and B27). The full-length RHBDD2 protein (isoform 1) and the predicted splicing variant (isoform 2) are indicated. (B) Immunofluorescence and immunocytochemistry detection of RHBDD2 displayed a vesicular pattern compatible with an ER-like distribution in MCF7 cells (see Supplementary Fig. 1). MCF10 cell smears were used as the negative control demonstrating non cross-reactivity of the primary antibody. MCF7 cells without RHBDD2 polyclonal antibody was also used as negative control of the reaction (MCF7-NegCt). The nuclei were counterstained with propidium iodide (red fluorescence).

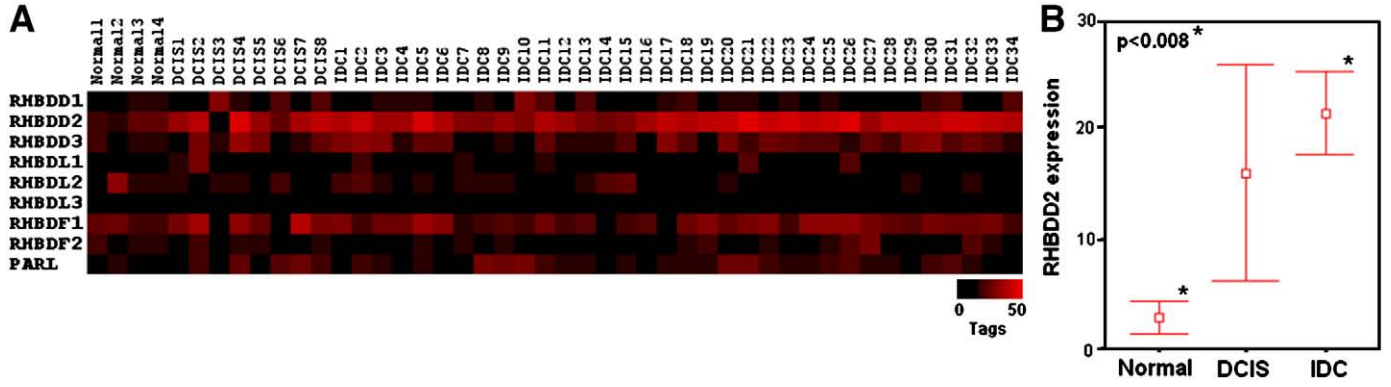

Fig. 1. Digital Northern analysis of human rhomboid-like family members based on SAGE data in 46 breast samples (4 normal breast epithelium, 8 ductal carcinoma *in situ* and 34 invasive ductal carcinomas) (A). The color scale at the bottom of the picture is used to represent relative expression level based on numbers of transcripts (tags) per libraries. (B) ANOVA test of *RHBDD2* expression among normal breast tissue, ductal carcinoma *in situ* (DCIS) and primary invasive breast carcinomas (IDC). High expression levels of *RHBDD2* tag were detected in IDC compared with normal breast tissue group ($p<0.008$).

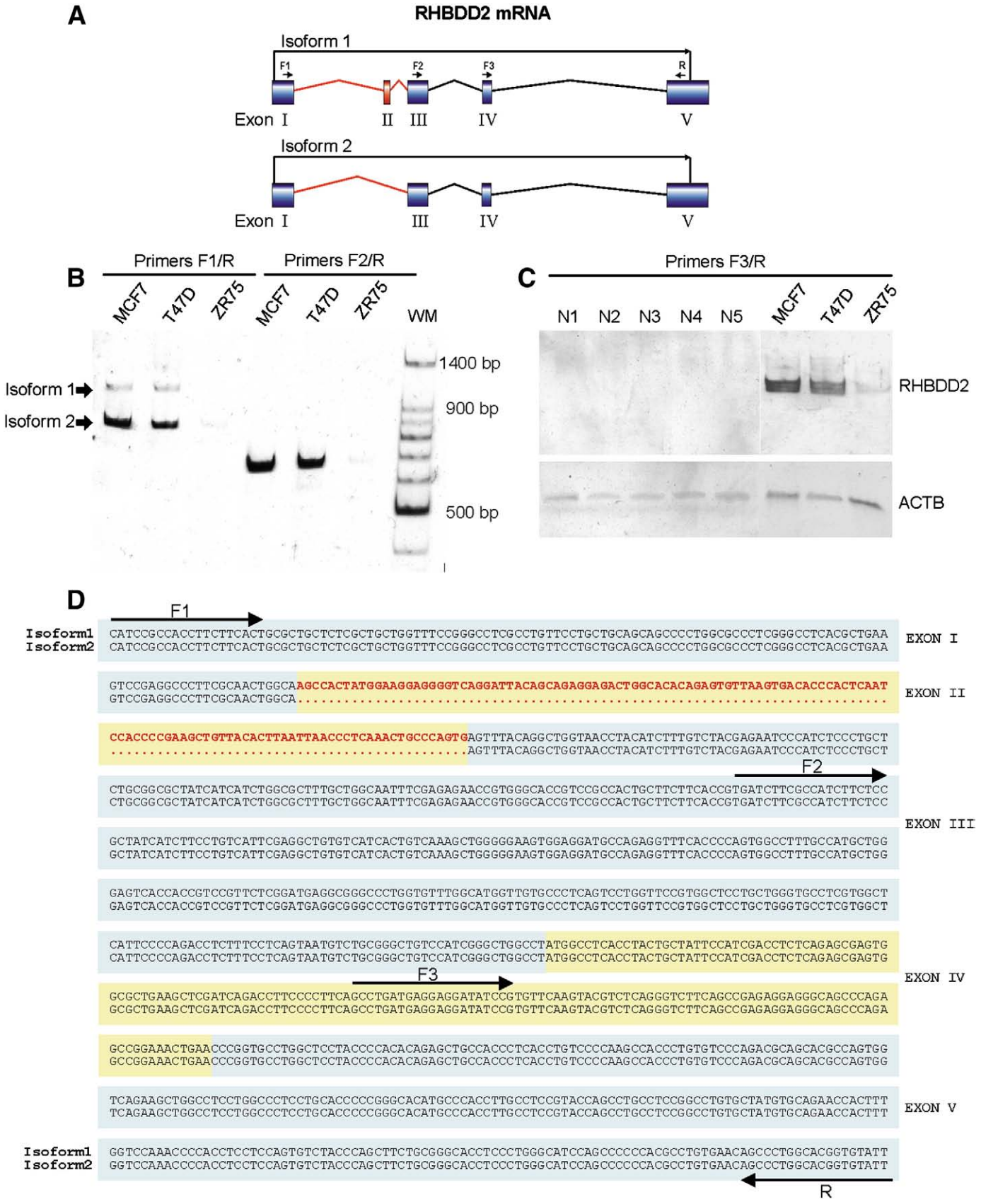

Fig. 3. Identification of two RHBDD2 mRNA isoforms. (A) Schematic representation of human RHBDD2 genomic structure and the corresponding mRNA isoform structure predicted *in silico* based on RHBDD2 EST database searching. (B) RT-PCR analysis in MCF7, T47D, and ZR75 breast cancer cell lines. Three pairs of PCR primers (F1 / R, F2 / R and F3 / R), based on EST sequences were used to amplify the RHBDD2 transcript from cDNA. Alternative splicing involving exons 1 and 2 generates the isoform 2. (C) *RHBDD2* RT-PCR of five normal breast tissue (N1–N5) and three breast cancer cell lines (MCF7, T47D, ZR75). (D) Alignment of the MCF7 cDNA sequences among *RHBDD2* isoforms.

which significantly reduces the lamin A/C protein level by >70% without affecting the MCF7 cell phenotype or viability. MCF7 cells were seeded on 12 well plate format in Opti-MEM I Reduced Serum Medium (Invitrogen, USA), when cells reached 40% confluence, they were transfected with 40 pmoles/μl of siRNA mixed with TransIT-TKO® Transfection Reagent according to the manufacturer's protocol (Mirus, USA). Transfection efficiency was monitored using biotinylated siRNA and FITC-avidin for detection. *RHBDD2* mRNA and protein levels and effects on cells were analyzed by RT-PCR and immunofluorescence at 48 h post-transfection respectively. Cell proliferation was assessed using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, USA), that is a colorimetric method for determining the number of viable cells in proliferation. In addition, cell viability was monitored daily by the trypan blue exclusion test.

2.8. Additional bioinformatics and statistical analyses

We employed the ASAP online database resource (Alternative Splicing Annotation Project) for the *in silico* analysis of RHBDD2 exon–intron structure (http://www.bioinformatics.ucla.edu/ASAP). ASAP is based on human genome-wide analyses of alternative splicing events based on detailed alignment of EST/cDNA sequences onto the genomic sequence [11].

To enable visualization and illustration of the 7q11.2 chromosome region in some breast cancer cell lines from Pollack's array-CGH data [12], we used the VAMP software (Visualization and Analysis of array-CGH, transcriptome and other Molecular Profiles) (http://bioinfo-out.curie.fr/actudb). VAMP is a graphical user interface for visualization and analysis of genomic profiles [13]. The chromosome 7 array-CGH profiles previously generated by Pollack et al. [12] from MCF7, T47D and ZR75 breast cancer cell lines were download from the ACTuDB database at the same website [14]. The frequency of DNA gains/losses affecting the chromosome region 7q11.23 was determined from a cumulative set of 552 IDC analyzed by array-CGH based on a publicly available Progenetix database (http://www.progenetix.net) [9].

Univariate analysis of clinical–pathological parameters based on *RHBDD2* gene amplification/expression was determined by Fisher's exact test. Ordinal-by-ordinal associations have been assessed by Kendall's tau-b test. The basic significance level was fixed at $p<0.05$ and all data were analyzed using SPSS® statistic software (SPSS Inc., Chicago).

3. Results

3.1. Human rhomboid family members expressed in breast epithelial cells

We analyzed the rhomboid-like family member gene expression profile in a set of normal and breast cancer SAGE libraries. Among the rhomboid family members, *RHBDD2* and *RHBDF1* were the most frequently expressed rhomboid-like genes in breast SAGE libraries (Fig. 1a). Specifically, *RHBDD2* was detected as being expressed in 100% of breast cancer cases (34 out of 34) according to SAGE database analyses. interestingly, among the rhomboid genes only *RHBDD2* was identified as over-expressed in primary invasive breast carcinomas compared with normal breast samples ($p<0.008$) (Fig. 1b). No association was detected between the expression of this transcript and ERα status ($p>0.05$).

3.2. RHBDD2 expression analysis in normal and breast cancer cell lines and identification of alternatively spliced mRNA isoforms

We performed Western-blot analysis in normal and breast cancer cell lines using a custom-design polyclonal antibody against the RHBDD2 protein. We identified the expected 47 kDa product as well as a smaller protein product at approximately 40 KDa. MCF7 and T47D cell lines showed high levels of RHBDD2 protein expression, while normal breast organoids (B26, B27, and B28) and non-transformed breast cell lines (MCF10A and HME87) showed weak and undetectable levels of RHBDD2 protein expression respectively (Fig. 2A). RHBDD2 immunostaining displayed a granulate/vesicular pattern compatible with an endoplasmic reticulum-like distribution with accumulation in the perinuclear space of MCF7 and T47D cells (Fig. 2B and Supplementary Fig. 1). MCF10 cell smears were used as the negative control of the reaction demonstrating non cross-reactivity of the primary antibody (Fig. 2B).

We decided to investigate whether the different protein products were results of alternatively spliced *RHBDD2* transcripts (isoform). *RHBDD2* alternative splicing prediction based on ASAP (Alternative Splicing Annotation Project) bioinformatics analysis revealed two putative isoforms, here named as isoform 1 (full-length *RHBDD2* mRNA) and isoform 2 (alternatively spliced isoform) (Fig. 3A). To validate these findings, we first performed an RT-PCR analysis in MCF7, T47D and ZR75 breast cancer cell lines. Three pairs of PCR primers, based on EST sequences were used to amplify the *RHBDD2* transcript from cDNA, and the products were sequenced. MCF7 and T47D cell lines showed high expression levels of *RHBDD2* with any combination of PCR primers used for RT-PCR analysis (Fig. 3B, C). Interestingly, primer pairs F1/R located on the first and last exons of *RHBDD2* mRNA respectively, result in two PCR products of approximately 1100 and 900 bp in MCF7 and T47D cell lines (Fig. 3A). The luminal-tumor derived cell line ZR75 showed weak RHBDD2 mRNA expression. However, primer pairs F2/R and F3/R result in a unique RT-PCR product of 720 bp and 369 bp respectively as it was expected (Fig. 3B, C) and confirmed by direct DNA sequencing (data not shown). Bi-directional sequence analysis was performed on each purified RT-PCR product from the MCF7 cell line. Comparison of these sequences with the publicly available *RHBDD2* genomic sequence (accession no. NT007933.14) confirmed gene identity for each PCR fragment and revealed that these two transcripts detected with the primer pairs F1/R were generated by alternative RNA splicing of exons 1 and 2 of *RHBDD2* (Fig. 3D). These data suggest that the low protein product observed in the Western-blot analysis represents true RHBDD2 protein, demonstrating that the antibody used in our study detected the full-length RHBDD2 protein (isoform 1) as well as the alternative splicing variant (isoform 2) (Fig. 2). In addition, this RT-PCR analysis showed undetectable or low expression of *RHBDD2* gene in normal breast tissues analyzed (Fig. 3C). These data corroborate the SAGE analysis showing that *RHBDD2* mRNA/protein expression is highly elevated in some breast cancer cells compared with normal breast samples.

3.3. Increased RHBDD2 protein expression in breast cancer clinical specimens

To further investigate the relevance of RHBDD2 protein expression in breast cancer, we analyzed 213 breast tissue sections by immunohistochemistry (Table 1 and Fig. 4). We identified a statistical significant increase in RHBDD2 protein expression from normal

Table 1
RHBDD2 protein expression in breast samples as related to histopathological characteristics.

Histopathology	Protein expression *n* (%)				Cases
	Absent	Weak	Moderate	Strong	
Normal epithelium	13 (72%)	4 (22%)	0 (0%)	1 (6%)	18
Normal adjacent IDC	4 (15%)	4 (15%)	12 (46%)	6 (23%)	26
Benign lesions	6 (33%)	6 (33%)	4 (22%)	2 (11%)	18
DCIS	1 (4%)	7 (27%)	11 (42%)	7 (27%)	26
IDC	31 (28%)	34 (31%)	23 (21%)	21 (20%)	109
LNM	0 (0%)	4 (25%)	4 (25%)	8 (50%)	16
Total	55	59	54	45	213

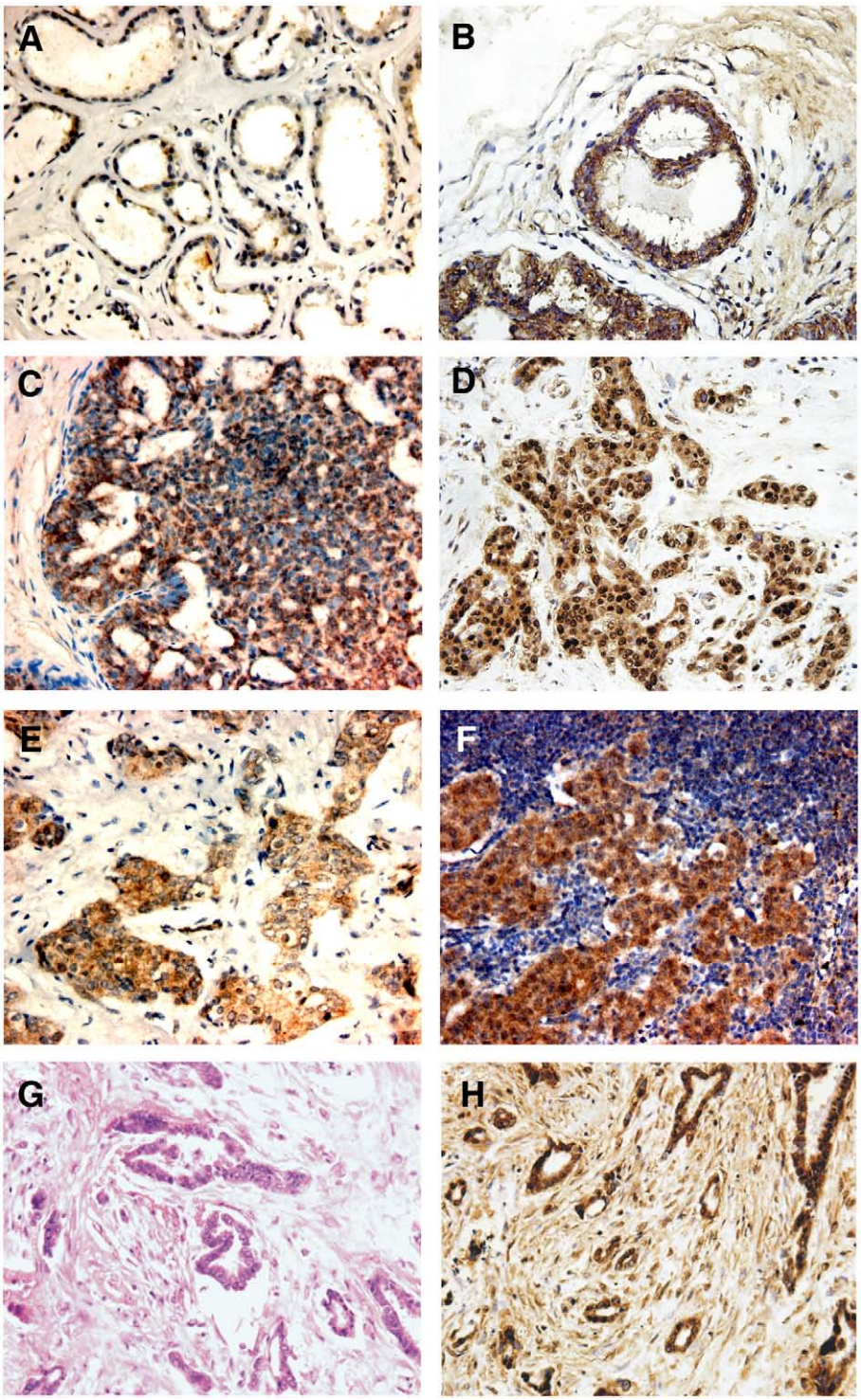

Fig. 4. RHBDD2 immunohistochemical staining in (A) normal breast adjacent tumor (×100), (B) hyperplastic breast lesion (×100), (C) ductal carcinoma *in situ* (×400), (D, E) ERα-negative invasive ductal carcinomas with *RHBDD2* gene amplification (×100 and ×400 respectively), and (F) lymph node metastasis sample (×400). As negative control for immunohistochemical staining we used a tissue sample, without primary antibody incubation (G), derived from serial sections of a RHBDD2-positive invasive breast carcinoma (H).

breast tissues to breast cancer metastasis specimens analyzed (p-trend = 0.01).

RHBDD2 protein expression was detected in 61% (27 out of 44) of normal breast samples analyzed. Interestingly, 95% (17 out of 18) of normal breast epithelium obtained from cosmetic mammoplasties, showed negative or weak RHBDD2 protein expression (Table 1). In contrast, 69% (18 out of 26) of normal breast tissue adjacent to invasive ductal carcinomas showed moderate or strong RHBDD2 expression, and only 31% (8 out of 26) of these samples showed negative or weak RHBDD2 immunostaining (Table 1). Statistical analysis of RHBDD2 protein expression between normal breast epithelium and normal breast adjacent IDC showed highly significant differences ($p = 0.0001$). RHBDD2 immunostaining showed a granulate reaction that was mostly localized in the perinuclear space and in the membrane.

Benign mammary dysplasia and fibroadenoma lesions displayed negative or weak RHBDD2 protein expression in approximately 66% (12 out of 18) of the analyzed cases, while moderate or strong

RHBDD2 protein expression was predominantly detected in 69% (18 out of 26) of the ductal carcinomas *in situ* analyzed ($p<0.01$) (Table 1).

Among IDC samples, negative RHBDD2 expression was detected in 28% of the cases (31 out of 109). However, when RHBDD2 expression and tumor stage were considered, we observed a statistically significant correlation between high levels of RHBDD2 and more clinically advanced cancer ($p=0.001$). In this sense, 92% of IDC negative for RHBDD2 expression were early-stage (I and II) breast carcinomas while only 9% of tumor stage III were negative for RHBDD2 protein expression. Furthermore, 50% of IDC with tumor stage III showed moderate or strong RHBDD2 protein expression. Interestingly, RHBDD2 protein expression was detected in all breast lymph node metastasis samples (16 out of 16) displaying a moderate or strong RHBDD2 expression in approximately 75% (12 out of 16) of the analyzed samples (Table 1 and Fig. 4). Non-statistical significant association was detected between RHBDD2 protein expression and ERα status as well as histologic tumor grade ($p>0.05$).

Overall, these data are in agreement with the SAGE profiling analysis, indicating that a high proportion of invasive breast carcinomas expressed significantly increased levels of RHBDD2 protein compared with normal breast samples. More importantly, strong RHBDD2 protein expression was highly associated with primary invasive breast carcinomas with axillary lymph node metastases.

3.4. RHBDD2 gene is amplified in correlation with over-expression in breast cancer cells

Microarray-based comparative genomic hybridization evidence is available reporting the amplification of the chromosomal region 7q11.23 in breast cancer cell lines and primary breast carcinomas [12,15]. To investigate the frequency of DNA gains/losses affecting the chromosome region 7q11.23, we performed a pooled re-analysis of 552 invasive breast carcinomas previously analyzed by array-CGH, available at Progenetix online database. Pooled estimates showed gain of chromosome region 7q11.23 in 11% of the analyzed cases (61 out of 552 IDC). The Visualization and Analysis of array-CGH, transcriptome and other Molecular Profiles (VAMP) [13] resource was employed for microarray-CGH data analysis of chromosome region 7q11.23 from MCF7, T47D and ZR75 breast cancer cell lines. *In silico* analysis of Pollack's data [14] identified a gain of chromosome regions 7q11.22–7q11.23 including the *RHBDD2* gene region in MCF7 and T47D cell lines (Fig. 5A). These data are in agreement with our Western-blot observations.

We used a competitive PCR method based on duplex PCR of *RHBDD2* (target) and *SLC13A1* (control) genes for the quick interrogation of human breast tumor DNA for the presence or absence of genomic amplification of specific genes affecting the region of interest. We analyzed DNA obtained from breast cancer cell lines and 131 samples (17 normal tissues, 16 benign lesions, 98 IDC) previously analyzed for protein expression by IHC. The DNAs from all samples were adequately amplified by the *SLC13A1* control primers. Competitive PCR analysis showed *RHBDD2* gene amplification in MCF7 and T47D breast cancer cell lines, while no amplification was detected for the ZR75 cancer line, in agreement with the array-CGH data and validating the method (Fig. 5B). It is important to note, that *RHBDD2* mRNA and protein expression are highly elevated in both breast cancer cell lines (MCF7 and T47D), thus confirming a direct association between *RHBDD2* gene amplification and over-expression. *RHBDD2* genomic amplification was detected in 21%

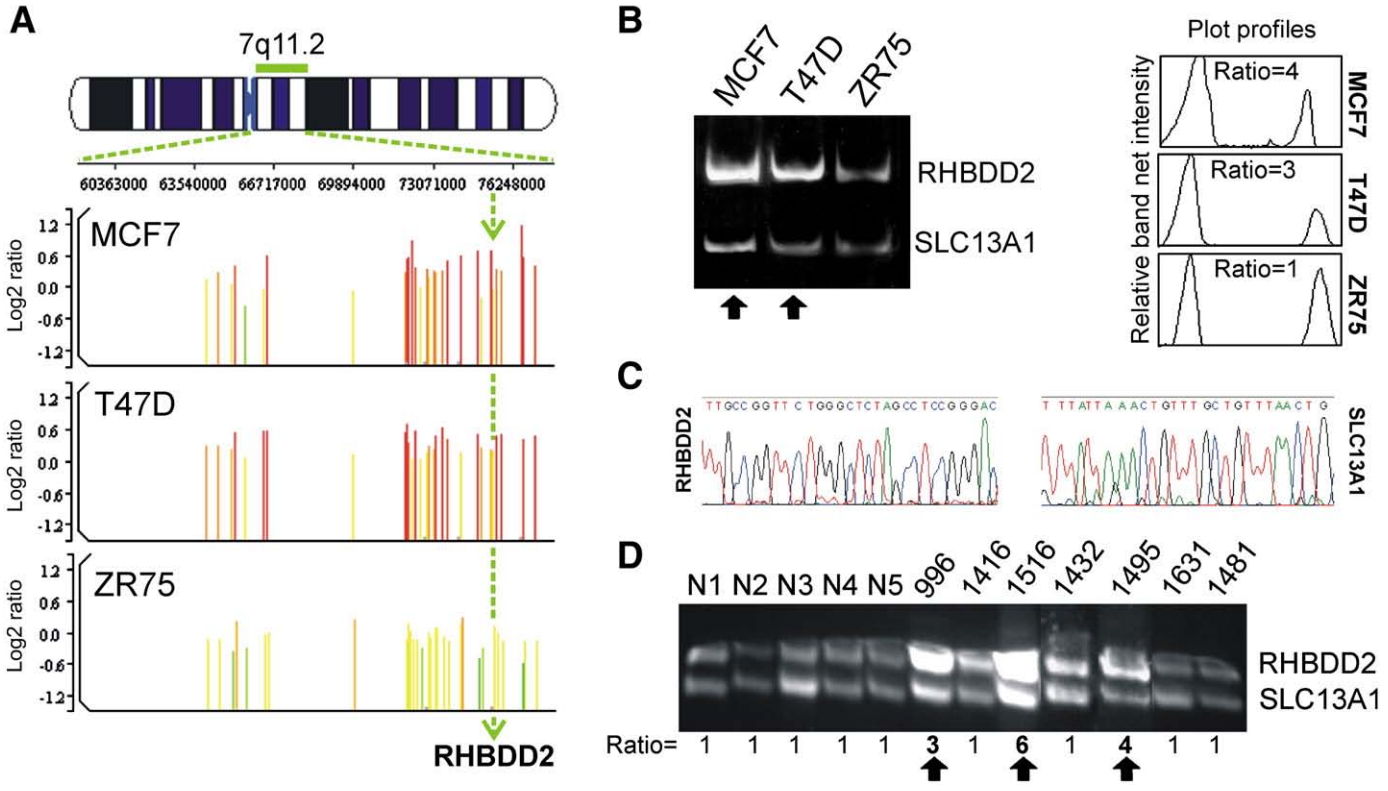

Fig. 5. *RHBDD2* gene amplification analysis in breast samples. (A) Visualization of 7q11.2 microarray-based CGH profile of MCF7, T47D and ZR75 breast cancer cell lines obtained from Pollack et al. study [12]. Red lines indicate chromosomal regions of gain. These data suggest a region of genomic gain located at 7q11.23 involving the *RHBDD2* gene. (B) RG-PCR assay showed *RHBDD2* gene amplification in MCF7 and T47D breast cancer cell lines in agreement with Pollack data. (C) DNA sequence chromatogram of isolated PCR amplicons confirming the *RHBDD2* and *SLC13A1* gene sequence identities. (D) RG-PCR analysis in normal breast tissues (N1–N5), and seven primary invasive breast carcinomas. Note that three invasive breast carcinomas (samples 996, 1516 and 1495) showed *RHBDD2* gene amplification (black arrows).

(21 out of 98) primary invasive breast carcinomas analyzed (Fig. 5D). However, no amplification in *RHBDD2* was identified in any of 17 normal and 16 benign tissue samples analyzed. Statistical analysis identified a significant positive association between *RHBDD2* gene amplification and RHBDD2 protein expression assessed by IHC studies ($p<0.05$).

3.5. RHBDD2 gene silencing in vitro leads to reduced cell proliferation rate

To investigate whether *RHBDD2* gene expression plays any role associated to cell proliferation; a knock-down assay was performed with specific siRNA sequences targeting *RHBDD2* mRNA in MCF7 breast cancer cell line. Cells were treated with optimal concentrations of *RHBDD2* siRNA and examined the number of viable cells 48 h after transfection.

RT-PCR and immunofluorescence analyses demonstrated an effective depletion of RHBDD2 at mRNA and protein levels in MCF7 by using the siRNA-R1 and siRNA-R3 sequences compared with the negative control siRNA (Fig. 6A and B). MCF7 cells in the control group were treated with the siRNA-Ct, which had a randomized nucleotide sequence that had no significant homology to the human genome. siRNA-R1 and siRNA-R3 treated cells showed a significant decrease in proliferation 48 h after transfection compared to negative or positive control siRNAs (Fig. 6C). In addition, siRNA-R1 and siRNA-R3 treatments did not affect cell viability or morphology of MCF7 breast cancer cells. This result suggests that RHBDD2 function is related to regulation of cell proliferation in MCF7 breast cancer cells.

3.6. RHBDD2 expression analysis and its clinical relevance as poor-prognosis marker

To further explore the clinical relevance of gene expression in human breast carcinomas, we evaluated information from publicly available breast cancer gene expression data sets (microarrays). Because the estrogen receptor plays a critical role in breast cancer, we first analyzed gene expression profiles of *RHBDD2* relative to ERα status. Statistical analysis showed that *RHBDD2* expression was not associated with ERα status of primary breast carcinomas ($p=0.148$). This result is in agreement with our previously described data regarding *RHBDD2* gene/protein expression in primary breast carcinomas obtained by SAGE and IHC studies.

Next, we analyzed *RHBDD2* expression with patient's outcome using the microarray data set of Van de Vijver et al [10]. Interestingly, we identified a significant association between high expression of *RHBDD2* and short time relapse-free survival among 295 patients ($p=0.018$; Fig. 7A). Kaplan–Meier analysis revealed that *RHBDD2* over-expression was particularly associated with shorter overall survival ($p=0.0023$; Fig. 7B), metastasis-free interval ($p=0.006$; Fig. 7C), and relapse-free survival ($p=0.013$; Fig. 7D) but surprisingly only in patients with ERα-negative breast carcinomas. Non-statistically significant associations were found for *RHBDD2* expression and follow-up in patients with ERα-positive carcinomas ($p>0.05$).

4. Discussion

In a recent study using SAGE, we performed a comparative global gene expression profile of primary invasive breast carcinomas [1].

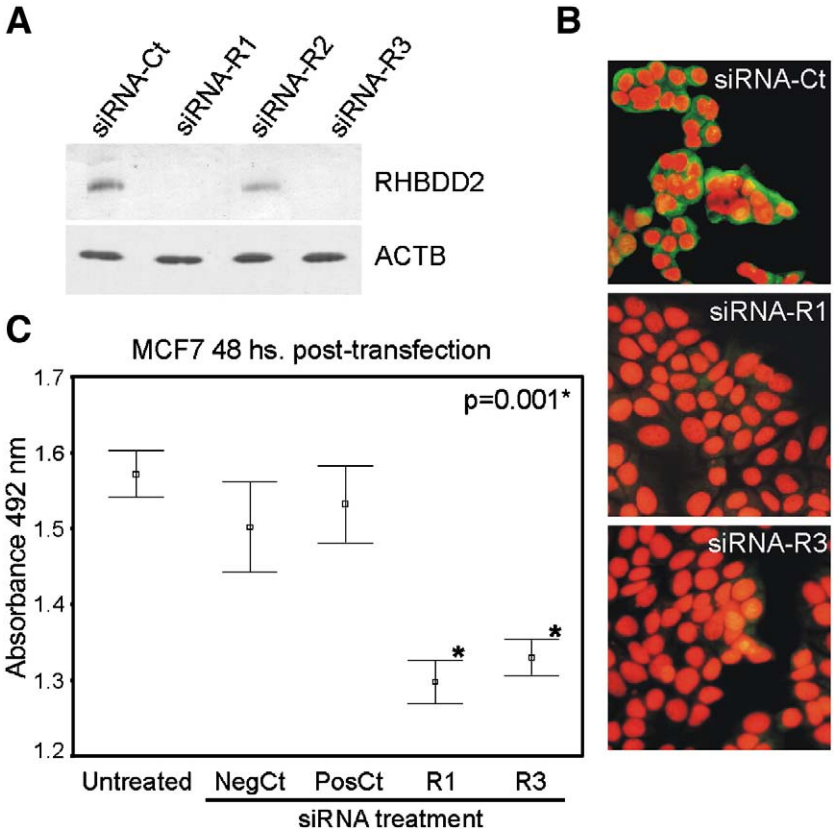

Fig. 6. *RHBDD2* knock-down analysis in MCF7 breast cancer cell line. (A) RT-PCR analysis of *RHBDD2* and β-actin (*ACTB*) mRNAs in AccuTarget™ negative control siRNA (siRNA-Ct) and three *RHBDD2* siRNA sequence treatments (siRNA-R1, siRNA-R2 and siRNA-R3). (B) Immunofluorescence analysis of RHBDD2 protein expression (green fluorescence) in siRNA-Ct, siRNA-R1 and siRNA-R3 treatments. The nuclei were counterstained with propidium iodide (red fluorescence). (C) Analysis of relative proliferation rates in MCF7 breast cancer cells at 48 h after siRNA transfection, expressed as absorbances at 492 nm measured using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, USA). Each point represents the mean ± 2 SE of 12 replicates.

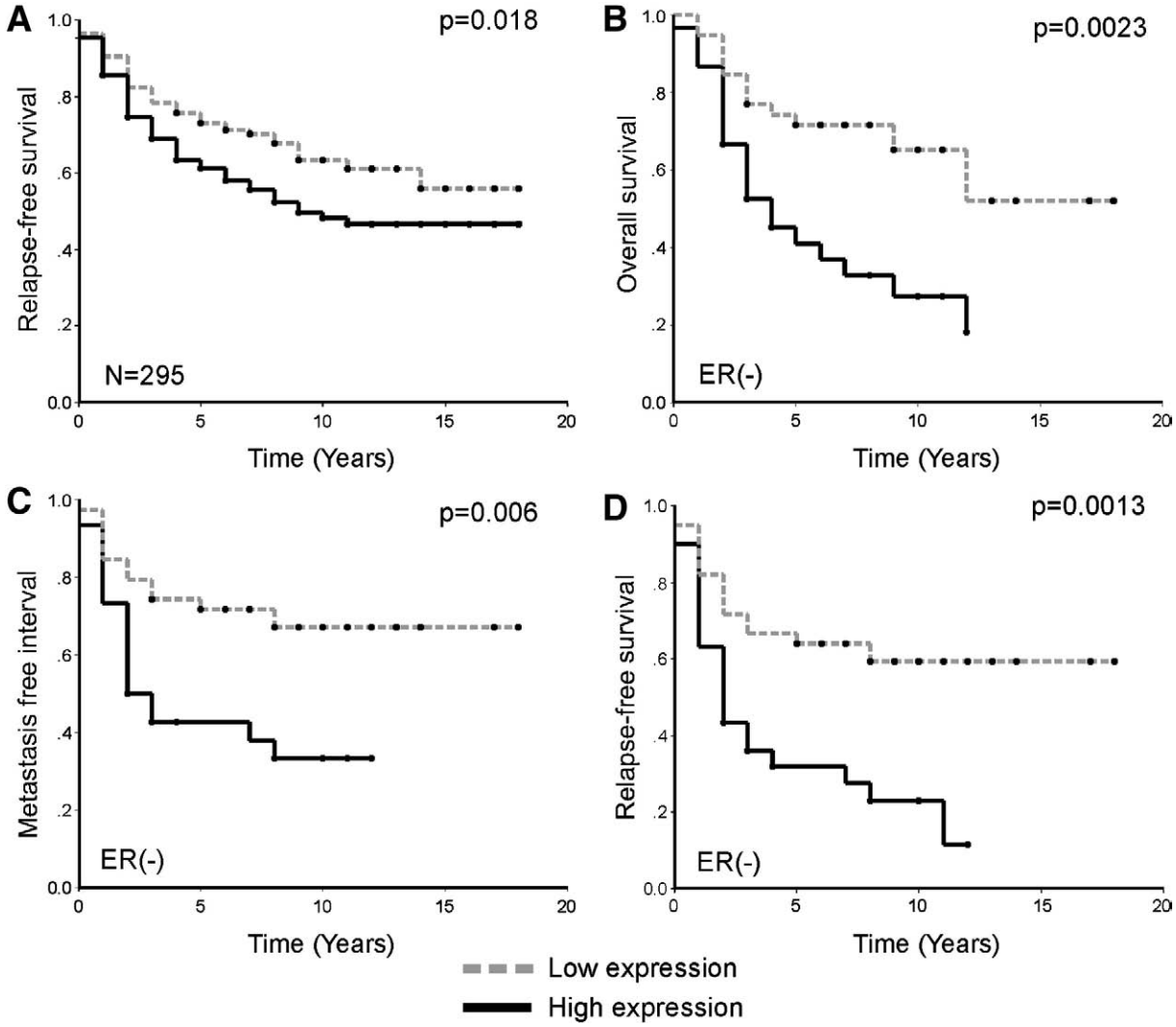

Fig. 7. *RHBDD2* gene expression profile and breast cancer patient follow-up based on a publicly available DNA microarray data set. (A) Relapse-free survival of 295 patients grouped according to low or high *RHBDD2* gene expression levels of the primary invasive breast carcinomas (226 ERα-positive and 69 ERα-negative breast samples). Kaplan–Meier curves of overall survival (B), metastasis-free interval (C), and relapse-free survival (D) of 69 patients with ERα-negative primary invasive breast carcinomas based on high or low expression levels of *RHBDD2* gene. Kaplan–Meier analysis was assessed using the Van de Vijver et al. data set [10].

Unsupervised statistical analysis identified two main breast clusters carcinoma which differed in their lymph node status, suggesting that lymph node status leads to a global distinct expression profile. In this study, we identified a rhomboid-like family member gene known as *RHBDD2* to be up-regulated in lymph node (+) breast carcinomas compared with lymph node (−) counterparts. Real-time RT-PCR analysis of an independent set of breast carcinomas demonstrated statistically significant *RHBDD2* over-expression in the primary breast cancers from patients that recurred within 6 years of follow-up [1].

In the present study, we first determined the human rhomboid-like family member gene expression profile in a set of 46 normal and breast carcinoma SAGE libraries. We found that the *RHBDD2* transcript was readily detectable in almost all primary invasive breast carcinoma and over-expressed compared to normal breast samples. In addition, a second human rhomboid-like gene, *RHBDF1* (*Rhomboid family-1 gene*), was identified as highly expressed in primary invasive breast SAGE libraries. Interestingly, in a recent study Yan et al. showed that RHBDF1 is significantly elevated in invasive ductal carcinomas at both mRNA and protein levels, and also seems important for breast cancer cell growth *in vivo* and *in vitro* [16].

We further explored RHBDD2 protein expression in 213 breast tissue samples by immunohistochemistry. This study confirmed the SAGE observations, showing that RHBDD2 protein is highly expressed in primary invasive breast carcinoma stage III and lymph node metastasis. However, this study also found that RHBDD2 was highly expressed in some DCIS, suggesting that RHBDD2 expression may occur early in the development of cancer. Interestingly, we detected that normal breast tissue adjacent to invasive breast carcinomas showed moderate or strong RHBDD2 expression while normal breast epithelium from mammoplastic specimens showed to be predominantly negative for RHBDD2 expression. This observation of abnormal high RHBDD2 expression in mammary epithelium adjacent to cancer allows us to speculate that expression of this protein could be influenced by the tumor microenvironment. It is possible that autocrine or paracrine stimuli maybe operative in modulating RHBDD2 expression in mammary epithelial cells.

Tumor-specific up-regulation of some genes can be attributed to aberrant DNA amplification, a phenomenon frequently found in many breast tumors [12]. Usually genomic amplification in cancer involves genes that provide some sort of growth advantage or transformation to the cells bearing the amplification. Typically amplification involves growth factors, growth factor receptors, certain cyclins, transcription factors or co-activators, i.e. positive regulators of proliferation and by definition many are dubbed oncogenes. Interestingly, microarray-

based comparative genomic hybridization evidence is available reporting amplification of the chromosomal region 7q11.2 (the same region to which *RHBDD2* maps) in breast cancer cell lines and primary breast carcinomas [12,15]. Moreover, a genome-wide transcriptome map revealed clusters of genes located at 7q11.2 exhibiting non-random increased expression in breast cancer cells [17]. To determine whether RHBDD2 was also amplified and over-expressed in breast tumors, we studied 98 primary invasive breast carcinomas for gene amplification and protein expression by RG-PCR and IHC respectively. *RHBDD2* was amplified in 21% of the analyzed samples. Although, almost all amplified samples showed moderate to strong RHBDD2 protein expression, some breast carcinomas showed over-expression of *RHBDD2* in the absence of gene amplification, suggesting in addition an up-regulation of expression by transcriptional regulatory means. We also determined that *RHBDD2* over-expression in cell lines MCF7 and T47D was likely the result of amplification of chromosome region 7q11.23. In addition, we identified and characterized the expression of two alternative spliced *RHBDD2* mRNA isoforms in these breast cancer cell lines.

Interestingly, in a recent study Yan et al. demonstrated that abrogation of RHBDF1 expression in MDA-MB435 cells and in head and neck squamous cancer cell line 1483 leads to inhibition of cell proliferation. siRNA-mediated *RHBDF1* silencing results in apoptosis in breast cancer cells and inhibition of xenograft tumor growth *in vivo* [16]. More importantly, in a very recently study Zou et al. demonstrated that RHBDF1 participates in the modulation of G protein-coupled receptor-mediated EGFR transactivation. Furthermore, RHBDF1 over-expression in head and neck cancer cells results in facilitated export of TGFα [18]. These data suggest that RHBDF1 plays a critical role in the mechanism responsible for the production of activated EGFR ligand. Similar to the observations of Yan et al. [16] in our studies we found that siRNA-mediated silencing of RHBDD2 expression in MCF7 breast cancer cell line leads to a marked decrease in cell proliferation. Thus, the possibility exists that RHBDD2 may also play a role in signaling pathways related to cell proliferation/survival as shown for RHDBF1; as such it could be a protein of relevance in breast cancer initiation and progression. Interestingly, and supporting this speculation, *in silico* analyses revealed that patients whose tumors expressed high *RHBDD2* had significantly shorter relapse-free survival when compared with those whose tumors had low *RHBDD2* levels. More importantly, we found that high *RHBDD2* expression was significantly associated with shorter overall survival, relapse-free survival, and metastasis-free interval in patients with ERα-negative breast carcinomas. These data are in line with our observation that increased RHBDD2 protein expression is associated with advanced tumor stages. In addition, we further confirmed our initial finding in which we observed a statistically significant over-expression of *RHBDD2* in primary tumors of patients who after follow-up developed recurrent disease [1]. In summary, our findings indicate that RHBDD2 over-expression might play a role in breast cancer tumor progression facilitating the development of more aggressive phenotypes in at least a subset of breast carcinomas. Further studies are required to elucidate the exact role of this protein in breast cancer.

5. Competing interests statement

The authors declare that they have no competing financial interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbadis.2009.07.006.

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