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RESEARCH ARTICLE

Rhomboid family gene expression profiling in breast normal tissue and tumor samples

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Abstract Rhomboid is an evolutionary conserved and functionally diversified group of proteins composed of proteolytically active and inactive members that are involved in the modulation of multiple biological processes such as epidermal growth factor receptor signaling pathway, endoplasmic reticulum-associated degradation, cell death, and proliferation. Recently, several human rhomboid genes have been associated with the development of chronic myeloid leukemia and pituitary, colorectal, ovarian, and breast cancers. In this study, we evaluated the mRNA and protein expression profiles of rhomboid genes in cancer cell lines and breast tissue/tumor samples. In silico analysis of publicly available gene expression datasets showed that different rhomboid genes are specifically expressed according to the breast cancer intrinsic subtypes. Quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis showed a significant *RHBDD2* mRNA overexpression in advanced breast cancer compared with normal tissue samples ($p=0.012$). In addition, we found that *RHBDL2* and *PARL* mRNA expression was associated with a low/intermediate histologic tumor grade ($p=0.024$ and $p=0.015$, respectively). Immunohistochemistry analysis showed a significant increase of RHBDD2 protein expression in association with breast cancer samples negative for progesterone receptor ($p=0.015$). Moreover, protein expression analysis corroborated the quantitative RT-PCR results, indicating that breast primary tumors belonging to patients with a more disseminated disease expressed significantly increased levels of RHBDD2 protein compared with less disseminated tumors ($p=0.01$).

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Introduction

Rhomboid genes encode polytopic intramembrane serine proteases that are conserved throughout evolution [1]. *Rhomboid-1* (*Rho1*) was the first member of the rhomboid family to be discovered and has been described as the major modulator of the epidermal growth factor receptor (EGFR) in *Drosophila melanogaster* [2]. Years later, the first mammalian rhomboid gene was cloned (*RHBDL1*) [3], and since then, a growing list of human rhomboids have been characterized, being functionally grouped into proteases (*RHBDL1/2/3*, *RHBDD1*, and *PARL*) and pseudoproteases (*RHBDF1* also known as *iRhom1*, *RHBDF2* also known as *iRhom2*, *Derlin1/2/3*, *RHBDD2/3*, *UBAC2*, and *TMEM115*) [4].

Rhomboids have been involved in diverse cellular processes including the modulation of EGFR pathway, mitochondrial membrane fusion, endoplasmic reticulum-associated degradation, among others [5–7]. Also, it has been recently demonstrated that other rhomboid genes (*RHBDD1*, *RHBDD3*, and *PARL*) regulate the apoptosis process in human epithelial cancer cells [8–16]. More importantly, rhomboids have been recently linked to human neoplastic diseases like chronic myeloid leukemia and esophageal, ovarian, colorectal, and breast cancers [17–19], modulating cell proliferation and survival by the activation or transactivation (*RHBDL2*, *iRhom1*, *iRhom2*) of the EGFR signaling pathway [20–23]. In this regard, we have demonstrated in previous studies that *RHBDD2* is overexpressed in the advanced stages of breast and colorectal carcinomas [24, 25], suggesting that this rhomboid member could be associated with tumor progression. Despite this, tissue-specific expression pattern and molecular biological functions of each human rhomboid gene in breast cancer have not been determined yet.

Therefore, we consider that it will be important to establish the expression profile of rhomboid genes in normal breast tissue and tumor samples, not only because of their clinical implication but also to get a better understanding of their biological function. Here we present the expression profile of rhomboid family genes, in order to describe their gene expression patterns in the context of histopathological variables. Furthermore, we evaluate RHBDD2 protein expression in an independent set of breast carcinomas, corroborating our previous findings that the advanced stages of breast cancer expressed significantly increased levels of RHBDD2 protein compared with early stage breast carcinomas.

Materials and methods

In silico rhomboid family gene expression profiling in breast cancer cells

To perform a comparative analysis of the human rhomboid family members expressed in breast tissue, we analyzed 409 tissue/tumor samples. To this end, we combined 143 normal breast tissues and 266 primary breast carcinomas derived from two independent studies: GSE10780 and GSE21653, respectively, using inSilicoDb and inSilicoMerging R/Bioconductor packages [26]. These gene expression profiles were developed using the Affymetrix HG U133 Plus2 platform (GPL570). Briefly, the frozen RMA preprocessed expression profiles of these studies were downloaded from the InSilico database and merged using the COMBAT algorithm as batch removal method. Heatmap visualization and statistical analysis of gene expression profiles were done with MultiExperiment Viewer software (http://www.tm4.org/mev.html).

Breast cancer cell lines and human breast tissue samples

Breast cancer cell lines MCF7, T47D, ZR75, and MB-MDA-231 were cultivated to confluence in RPMI 1640 medium (Sigma, USA) supplemented with 10 % (*v*/*v*) heat-inactivated fetal bovine serum (FBS, Gibco, USA), 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C, and subsequently harvested for total RNA and protein purifications.

One hundred twenty-six formalin-fixed paraffin-embedded breast tissue samples were studied: 112 invasive ductal carcinomas and 14 normal samples derived from cosmetic mammoplastic specimens were included. Samples were obtained from institutions related to the Faculty of Medical Sciences, National University of La Plata, La Plata, Argentina. Procedures involving human samples following the World Medical Association Declaration of Helsinki (Finland, 1964) and further modifications were done. Informed consent was obtained from all patients included in this study. This research was approved by the Regional Ethical Committee. Primary tumor samples were obtained from 112 breast cancer patients (age range 39–82 years); clinical and histopathological data were obtained from clinical records. Stage at time of diagnosis was established: stage I/II or stage III; histologic tumor grade (low/intermediate or high grade) and nuclear grade (grade I/II or grade III) were recorded. Staging according to the Union for International Cancer Control's TNM classification system was established.

Quantitative RT-PCR analysis

Total RNA was isolated from 38 breast samples (14 normal tissues, 24 invasive ductal carcinomas) and 4 breast cancer cell lines using RNAzol® (MCR Inc., USA) following the previously described protocol with modifications [27]. Quality control of RNA integrity was made by running out RNA samples onto formaldehyde denaturing 1.2 % agarose gel, and RNA concentration was measured using the NanoDrop spectrophotometer. Following DNAse I digestion, template cDNAs were synthesized using SuperScript™ II First-strand Synthesis System (Life Technologies, USA). Primer pairs span exon–exon junctions were designed using Primer-blast (NCBI-NIH, USA) and Unipro UGENE softwares for *RHBDD1*, *RHBDD2*, *RHBDD3*, *RHBDF1*, *RHBDF2*, *RHBDL1*, *RHBDL2*, *RHBDL3*, and *PARL* genes (see Table 1). *PARL* mRNA expression was evaluated using two independent pair of primers (*PARL* and *PARL(2)*) in order to confirm the results obtained. Experiments were performed in triplicate for each data point. Amplification of 18S rRNA and beta-actin (ACTB) was used as the control for normalization. Thermal profile was programmed as follows: an initial denaturation step of 5′ at 95 °C followed by 40 cycles of 40″ at 95 °C, 30″ at described annealing temperature (see Table 1), 30″ at 72 °C, and one final cycle of 95 °C for 1 min/55 °C for 30 s/96 °C for 30 s. Polymerase chain reaction (PCR) was performed in a 25-μl volume, using Taq DNA polymerase (Lifetechnologies, USA) and EvaGreen dye (Biotium, USA). Quantification of mRNA expression levels were done using Stratagene MX3000PTM Real-Time PCR System based on 2^(−dCt) values. Results were expressed as categorical variable (negative, low/moderate, and high expression levels) based on the distribution of 2^(−dCt) values of each assayed gene. The basic significant level was fixed at $p < 0.05$ and all data were analyzed using SPSS statistic software (SPSS Inc., Chicago, IL, USA).

Immunohistochemistry and Western blot analyses of RHBDD2 protein expression

A total of 88 primary invasive breast carcinomas were analyzed by immunohistochemistry (IHC). Prior to immunostaining, endogenous peroxidase activity was blocked with 3 % H_2O_2 in methanol for 10 min; heat-induced antigenic retrieval was performed with 10 mM sodium citrate buffer (pH 6.0) for

Table 1 List of primers used in the study

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon (bp)	Annealing (°C)
RHBDD1	GCACCCAGGAACTATGACACG	TCTGGTGAGAGATGAAACCCG	137	55
RHBDD2	GGTGTTTGGCATGGTTGTG	CGATGGAATAGCAGTAGGTGA	146	56
RHBDD3	GCACCATGGCTGTCCAAGT	AACCAACAGTGACACGGCAC	135	55
RHBDF1	CAGGCTGGCACCGCATAGCC	GCCGAACTGGGAGCCAGCAG	116	55
RHBDF2	GGCAACCTCGCCAGTGCCAT	GGCCTTCCAGGGCCTCTCCA	135	55
RHBDL1	ACCGTCTCCATCACCGACA	GCACCATCCTCAGCAACTTGT	139	55
RHBDL2	TGTTGGACATGGGATTTGCTC	CACCGTGTAGCCAATGGACA	119	50
RHBDL3	ATTGGGCTTGTCTACGTGGC	GATAGGCCGACGGGTGGAA	250	60
PARL	CGCCATGGATACAGCAGGA	CACTAGCGGCTCCCTGTTCTT	136	52
PARL (2)	TGGACCATCACTTGGTGCAT	GCTGTGAACGTGAACATCGG	114	55
ACTB	TCAAGATCATTGCTCCTCCTGAG	CTCCTGCTTGCTGATCCACA	104	53
RNA18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	151	50

10 min in a microwave oven followed by a 20-min cool down. In order to block nonspecific antibody binding, the slides were incubated with 10 % horse serum in PBS for 30 min. Primary polyclonal RHBDD2 antibody (TA306891, Origene, USA) was diluted 1:150. Immunodetection was performed with DakoCytomation LSAB+System-HRP (Dako, Denmark). Sections were counterstained with hematoxylin (Sigma, USA) and examined with a light microscope. 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 tissues. A reaction was considered positive when more than 5 % of the breast epithelial cells was stained. The staining of the cytoplasm, plasma membrane, and nucleus was evaluated; cells were considered positive when at least one of these components was stained. IHC analysis of estrogen receptor (ER-alpha), progesterone receptor (PR), and HER2/neu were performed using primary monoclonal antibodies (Vector Labs, Burlingame - CA). To evaluate associations between RHBDD2 protein expression (IHC) and qualitative variables, we employed Fisher's exact test. Ordinal-by-ordinal associations were assessed by Kendall's tau *b* test.

For Western blot, 50 μg of total protein was separated by 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, USA). RHBDD2 protein was detected using a commercially anti-RHBDD2 polyclonal antibody (TA306891, Origene, USA) and horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Dako, Denmark, 1:2,000) followed by enhanced chemiluminescence (ECL). Beta-actin protein was detected using monoclonal anti-actin antibody (ICN Biomedicals, USA; 1:1,000) and HRP-conjugated anti-mouse secondary antibody (1:5,000). We used an RHBDD2 HEK293T overexpression lysate as positive control of reaction for RHBDD2 immunodetection.

Results and discussion

In silico rhomboid mRNA profiling in breast cancer

To analyze the rhomboid family member gene expression profiles on a larger breast dataset, two independent, publicly

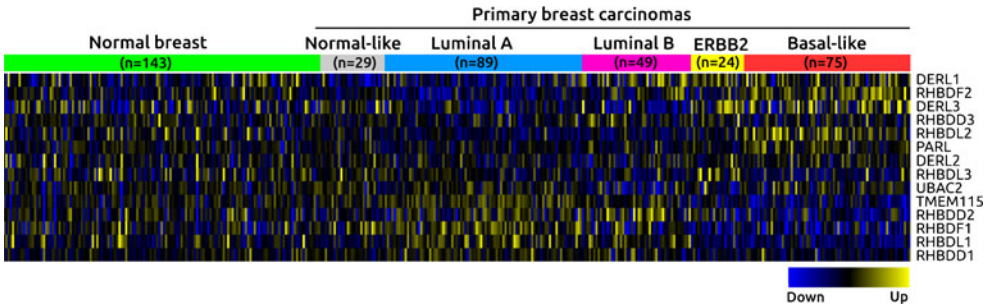


Fig. 1 Heatmap of fourteen rhomboid-like genes in a compiled dataset of 409 normal and primary invasive breast carcinomas classified according genomic intrinsic subtypes. *Color scale* at the *bottom* of the picture is used to represent expression level: low expression is represented by *blue*, and high expression is represented by *yellow*

available oligo microarray studies were compiled (Fig. 1). Among the rhomboid family members, *RHBDF2*/*iRhom2* (p =2.9E−9), *RHBDL2* (p =1.8E−7), *PARL* (p =5.4E−7), *DERL3* (p =1.3E−4), and *DERL1* (p =0.002) were identified as overexpressed rhomboid genes in basal-like breast carcinomas compared with normal breast tissues (p <0.05). On the other hand, a significant increase in *RHBDF1*/*iRhom1* (p = 3.67E−4), *TMEM115* (p =3.76E−4), *RHBDD1* (p =0.013), and *RHBDL1* (p =0.014) mRNA expression was detected in luminal A breast carcinomas compared with normal breast tissues. Specifically, *RHBDD2* (p =0.007) overexpression was associated with luminal B breast cancer subtype according to in silico analysis (Fig. 1).

Quantitative RT-PCR analysis of rhomboid genes in breast cancer cell lines

We analyzed the mRNA expression levels of nine rhomboid genes in different breast cancer cell lines. We found that *RHBDF1*/*iRhom1*, *RHBDL1*, *RHBDD1*, *RHBDD2*, and *RHBDD3* were commonly expressed in the luminal tumor-derived cell lines MCF7, T47D, and ZR75 (poorly invasive and epithelioid; Fig. 2a). In this sense, *RHBDD1* gene was previously shown to be expressed in a number of human cancer cell lines including MCF7, displaying anti-apoptotic properties [10]. Regarding *RHBDD2* mRNA expression in breast cancer cell lines, we demonstrated in previous reports that siRNA-mediated silencing of *RHBDD2* expression results in a decrease of MCF7 breast cancer cell proliferation [24]. Moreover, we recently found that stable *RHBDD2* silencing decreases the colony formation/anchorage-independent growth and cell migration in T47D breast cancer cells (unpublished data). RHBDF1, a pseudoprotease member involved in GPCR-mediated transactivation of EGFR via TGF-alpha secretion, was previously found in head and neck and breast cancer cell lines such as MCF7, T47D, MDA-MB231, and MDA-MB-435 cell lines [21, 22]. In this study, we detected *RHBDF1* mRNA expression in the cell lines MCF7, T47D, and MDA-MB231 and the luminal ZR75, which had not been previously characterized relative to the mRNA expression of this gene. *RHBDL2* mRNA expression was detected in the T47D and ZR75 cells and in the basal-like breast cancer cell line MDA-MB-231; this rhomboid protease member was previously identified in the T47D cancer cell line promoting EGF secretion in a metalloprotease-independent manner [20].

Quantitative RT-PCR analysis of rhomboid genes in tissue/tumor samples

Quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis on a panel of breast tissue samples allowed us to detect rhomboid genes in normal and breast cancer epitheliums with specific gene expression patterns (Fig. 2b). Statistical analysis between normal tissues and primary invasive carcinoma samples showed that *RHBDD2* was overexpressed in primary invasive carcinomas compared to normal breast samples (p =0.012; Fig. 3). *RHBDD2* was expressed in breast carcinoma showing moderate to high mRNA expression, while normal epithelium showed negative or low expression levels. In addition, when its expression was analyzed in relation to tumor stage, a statistically significant correlation between high levels of *RHBDD2* and advanced breast carcinoma (p =0.011) was found. In this sense, 94 % of early stage (I and II) breast carcinomas showed low *RHBDD2* mRNA expression, while 60 % of tumor stage III showed high *RHBDD2* mRNA expression (Fig. 3). A nonstatistically significant association was detected between the other rhomboid genes analyzed and tumor stage in this dataset (p >0.05).

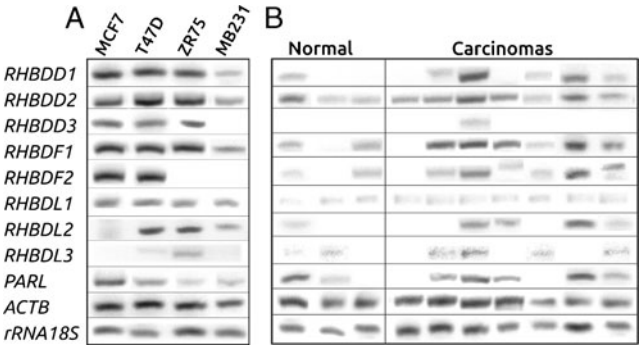
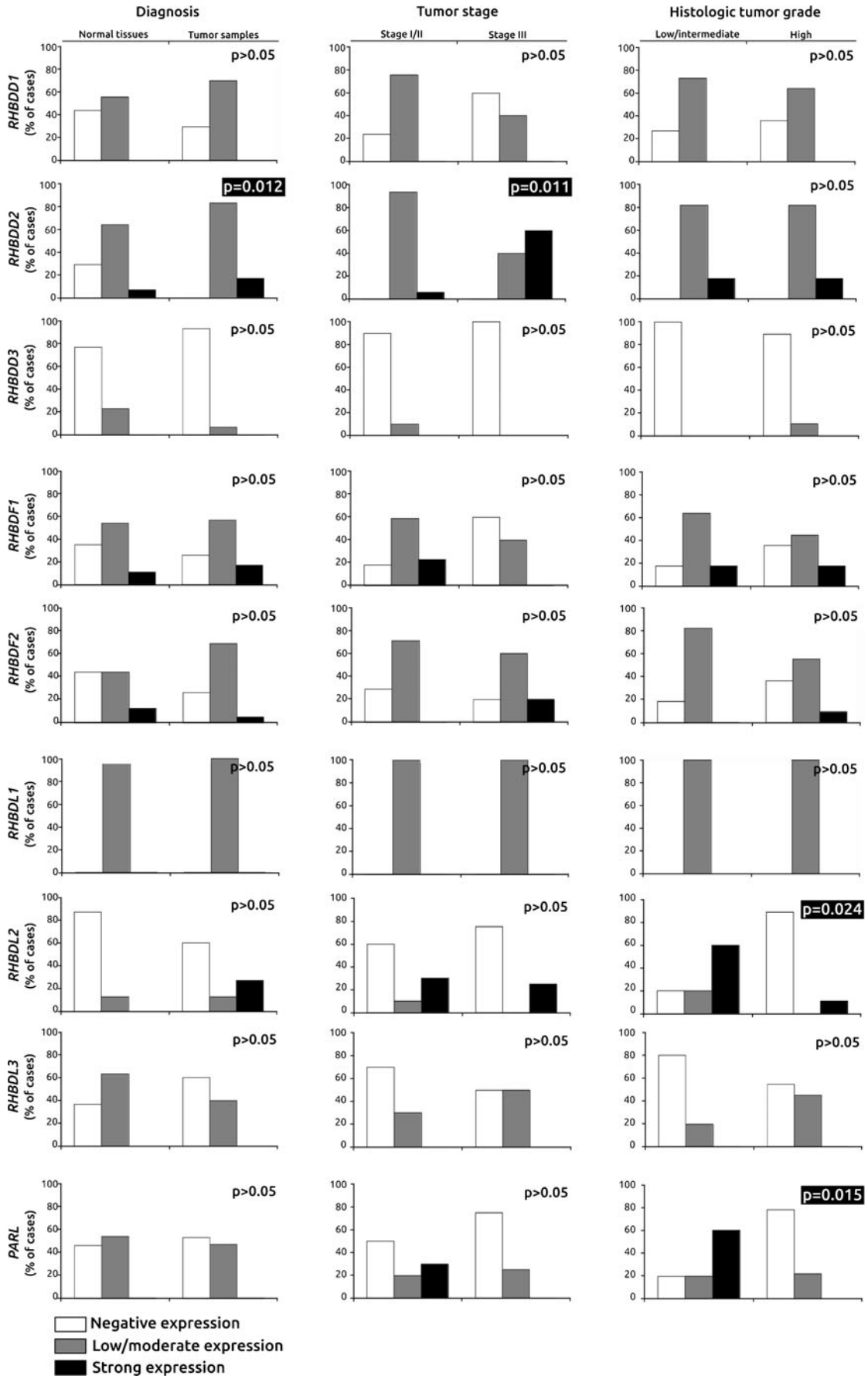


Fig. 2 RT-PCR analysis of nine human rhomboid genes in breast cancer cell lines (**a**) and normal breast tissue/tumor samples (**b**). Electrophoresis were carried out on a 2 % agarose minigel

Furthermore, we found that *RHBDL2* and *PARL* mRNA expression was associated with low/intermediate histologic tumor grade (p =0.024 and p =0.015, respectively). Sixty percent of low/intermediate-grade carcinomas expressed *RHBDL2* and *PARL* mRNAs, while only 11 and 22 % of high-grade tumors showed expression of *RHBDL2* and *PARL* transcripts, respectively (Fig. 3). Interestingly, *RHBDL2* mRNA expression was significantly increased in low-grade tumors compared with normal breast samples (p =0.01). Nonstatistically significant differences were found between low-grade tumors and normal breast samples for *PARL* mRNA expression (p >0.05). RHBDL2 is well known to cleave EGF just outside its transmembrane domain, releasing the ligand that activates the EGFR, thereby promoting cell proliferation [12]. On the other hand, PARL is thought to have an anti-apoptotic activity that could contribute to the survival of malignant cells [28–30].

Fig. 3 Quantitative RT-PCR analysis of rhomboid genes expression ▶ according to the diagnosis (normal vs. cancer), tumor stage (stage I/II vs. stage III), and histologic tumor grade (low/intermediate vs. high grade)


Diagnosis
Normal tissues
Tumor samples
Tumor stage
Stage I/II
Stage III
Histologic tumor grade
Low/intermediate
High
RHBDD1 (% of cases)
RHBDD2 (% of cases)
RHBDD3 (% of cases)
RHBDF1 (% of cases)
RHBDF2 (% of cases)
RHBDL1 (% of cases)
RHBDL2 (% of cases)
RHBDL3 (% of cases)
PARL (% of cases)
p>0.05
p=0.012
p=0.011
p=0.024
p=0.015
Negative expression
Low/moderate expression
Strong expression

RHBDL1 mRNA was expressed in all breast tissue/tumor samples analyzed at low levels, while only one tumor sample expressed *RHBDD3* mRNA. *RHBDD3* also known as *pituitary tumor apoptosis gene* (*PTAG*) is an important pro-apoptotic gene identified in pituitary tumors where it shows loss of expression; besides, most of the primary colorectal tumors fail to express it [8, 9]. Regarding RHBDF2/iRhom2 function, it is involved in TACE maturation which participates in TNF-alpha and EGFR activation; the latter is an important signaling network in cancer development [5, 31, 32]. In addition, missense mutation of *RHBDF2*/*iRhom2* gene affects its protein subcellular localization and decreases the EGFR levels in immortalized tylotic keratinocytes [17].

RHBDD2 protein expression analysis

To further investigate the RHBDD2 protein expression in breast cancer, immunohistochemical and Western blot analyses were performed in 88 tumor sections using a commercial polyclonal RHBDD2 antibody. RHBDD2 immunostaining showed a granulate reaction mostly localized in the perinuclear area and in the plasmatic membrane. Interestingly, we identified a statistically significant increase of RHBDD2 protein expression in breast cancer samples negative for estrogen and progesterone receptors (ER−/PR−) compared with samples positive for both receptors (ER+/PR+) ($p=0.017$). In this sense, RHBDD2 was detected in 80 % of the ER−/PR− breast carcinomas compared to 40 % of the ER+/PR+carcinomas (Fig. 4a). Also, it is important to note that when both receptors were analyzed separately, a nonstatistically significant association was detected for RHBDD2 expression and the ER-alpha status ($p>0.05$), but a statistically significant association was found when considering the PR status, with 83 % of positive cases for RHBDD2 in the PR− samples compared with 44 % of positive cases in the PR+ counterparts ($p=0.015$). The impact of PR status in breast cancer survival has been largely studied, and the survival of patients whose tumors lack PR is significantly shorter than those who are PR+, indicating that RHBDD2 overexpression could be associated with a poor prognosis. In addition, loss of the progesterone receptor identifies luminal B breast cancer subtypes and is thought to represent a more aggressive phenotype that is less dependent on ER signaling [33]. Interestingly, these data are in line with the in silico analysis of a compiled dataset showing that *RHBDD2* mRNA expression was associated with the luminal B breast cancer intrinsic subtype. A nonstatistically significant association was detected between RHBDD2 protein expression and HER2/neu status ($p>0.05$).

Taking into account RHBDD2 expression and tumor stage, a statistically significant association between strong RHBDD2

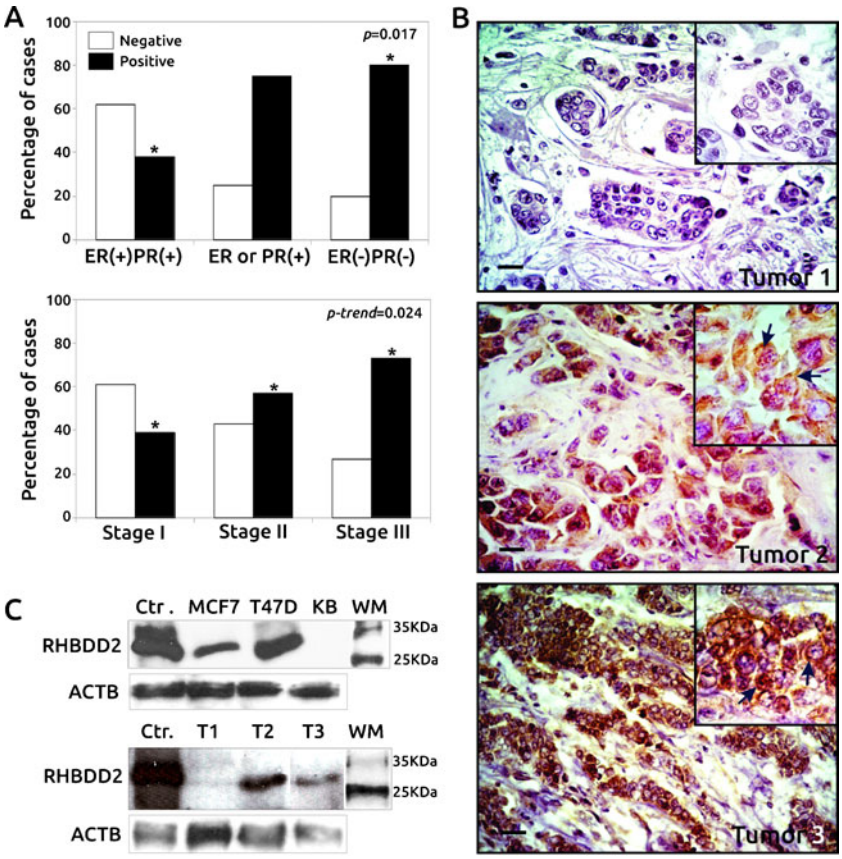


Fig. 4 RHBDD2 protein expression analysis in breast cancer samples. **a** Percentage of negative and positive cases for RHBDD2 expression according to ER/PR status and tumor stages. **b** Immunohistochemical staining for RHBDD2 protein in breast carcinomas showing negative (*Tumor 1*) and positive reactions (*Tumor 2* and *Tumor 3*). The image magnification is ×400, and for the *upper right inset* is ×1,000. *Arrows* indicate brown positive staining localized in the perinuclear area of the cells. Scale bar=20 μm. **c** Western blot analysis of RHBDD2 in a positive control derived from RHBDD2 HEK293T overexpression lysate (*Ctr.*), *MCF7*, *T47D*, *KB* (derived from HeLa) cell lines and the three primary breast carcinomas previously analyzed by IHC (*T1*, *T2*, *T3*). *WM* molecular weight marker

protein expression and advanced tumor stage III was observed ($p = 0.024$). In this sense, 60 % of the negative cases for RHBDD2 expression were primary tumors from early stage (I/II) patients, while 75 % of tumors from stage III patients were positive for RHBDD2 protein expression (Fig. 4a). Immunohistochemical results were subsequently validated by RHBDD2 Western blot analysis (Fig. 4b, c). These data corroborated the *RHBDD2* quantitative RT-PCR analysis, indicating that the breast primary tumors belonging to a more disseminated disease expressed significantly increased levels of RHBDD2 protein than the less disseminated tumor counterparts. Furthermore, these results are in line with the recent observation that increased RHBDD2 mRNA, and protein expression is associated with the advanced stages of colorectal carcinomas [25].

Conclusion

Our results showed that breast cancer cells are capable of expressing multiple rhomboid genes. Although different human rhomboid genes are typically expressed in normal breast tissues, the overexpression of individual rhomboid members, in particular breast cancer subtypes, could lead to alterations needed for tumor progression. In addition, the rhomboid genes RHBDL2 and PARL were found to be significantly associated with breast cancer histologic tumor grade. This study also corroborates and extends our previous findings on RHBDD2 expression, demonstrating that a high proportion of invasive breast carcinomas express significantly increased levels of *RHBDD2* mRNA/protein compared with normal breast samples. More importantly, strong RHBDD2 protein expression was highly associated with the advanced stages of the disease. Further studies will be required focusing on the functional characterization and mechanistic aspects of rhomboid genes in the context of key cellular pathways altered during breast cancer development.

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Conflicts of interest None

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