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RESEARCH ARTICLE

RHBDD2: a 5-fluorouracil responsive gene overexpressed in the advanced stages of colorectal cancer

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Abstract In previous studies, we identified *rhomboid domain containing 2* (*RHBDD2*) gene to be markedly overexpressed in breast cancer patients that developed recurrence of the disease. In this study, we evaluated for the first time *RHBDD2* gene expression in colorectal cancer (CRC). Five public available DNA microarray studies were compiled in a homogeneous dataset of 906 colorectal samples. The statistical analysis of these data showed a significant increase of *RHBDD2* expression in the advanced stages of CRC ($p<0.01$). We validated these findings by immunohistochemistry on 130 colorectal tissue samples; RHBDD2 protein overexpression was also observed in the advanced stages of the disease ($p<0.001$). In addition, we investigated RHBDD2 expression in response to the chemotherapy agent 5-fluorouracile (5FU). We detected a significant increase of RHBDD2 mRNA and protein after 5FU treatment (20–40 μM; $p<0.001$). Overall, these results showed that RHBDD2 overexpression might play a role in colorectal cancer progression.

Keywords Colorectal cancer · *RHBDD2* gene expression · 5FU treatment

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Introduction

Cancer progression requires the alteration in the expression of several genes that allow tumor cells to evade growth control and apoptotic signaling [1]. The identification of these genes has provided new insights into tumor biology, allowing the improvement of cancer therapies [2]. Rhomboid genes encode intramembrane serine proteases that are conserved throughout evolution. Rhomboid-1 from *Drosophila melanogaster* was the first member of this widespread protease family to be identified and is responsible for cleaving Spitz, the primary ligand of the epidermal growth factor receptor (EGFR) in *Drosophila* [3–5]. The human genome contains nine rhomboid genes which can be phylogenetically grouped into three main categories: the protease active rhomboids (e.g., *RHBDL-1/-2/-3*, *RHBDD1*, and *PARL* genes), the inactive rhomboids, denominated iRhoms (e.g., *RHBDF-1/-2* genes) and a distant evolutionary related group for which there is no evidence of their catalytic activity (e.g., *RHBDD-2/-3*).

It is well known that EGFR and its downstream signaling pathways are involved in tumor progression, including colorectal cancer (CRC) [6–8]. In recent years, this information has lead to the study of human rhomboids in cell proliferation, apoptosis, and tumor progression. It has been demonstrated in tumor cells that *RHBDD1* and *RHBDF1* genes regulate the processes of apoptosis and cell proliferation via modulation of EGFR signaling pathway [9–11]. Moreover and similarly to in *Drosophila*, the active rhomboid RHBDL2 has been recently implicated in the cleavage of the extracellular domain of the EGFR in normal and tumor cells, promoting its release as an extracellular ligand, which consequently activates EGFR [12].

In previous studies, we identified a related rhomboid-like family member gene known as *rhomboid domain containing 2* (*RHBDD2*) to be markedly overexpressed in primary

invasive breast carcinomas from patients that developed recurrent disease [13, 14].

In this study, we conducted an in silico analysis of *RHBDD2* expression profile in a compiled oligo-microarray dataset of human CRC samples. Statistical analysis of these data identified a significant *RHBDD2* mRNA up-modulation in the advanced stages of the disease, suggesting that *RHBDD2* overexpression might be associated with malignant progression. We reported the validation of this finding at protein level.

Furthermore, since the pyrimidine 5-fluorouracil (5FU) is one of the most commonly used agents for the treatment of CRC at early and advanced tumor stages, we also investigated *RHBDD2* gene expression in modulating the response of colon cancer cells to 5FU treatment.

Material and methods

In silico *RHBDD2* expression profiling in a compiled CRC dataset

To perform a comparative analysis of *RHBDD2* mRNA expression in colorectal cancer, we combined 866 CRC with 40 normal colon tissue samples obtained from five studies available in a public database (Fig. 1a). To generate a homogeneous dataset, the frozen robust multi-array analysis (fRMA) preprocessed expression matrixes of the studies GSE2109 (n=338 CRC samples), GSE14333 (n=290 CRC samples), and GSE17538 (n=238 CRC samples) were downloaded from the InSilico database (http://insilico.ulb.ac.be/) [15–17]. Normal samples were extracted from two CRC datasets, GSE15960 (n=18, total samples; n=6, normal samples) and GSE20916 (n=145, total samples; n=34 normal samples) obtaining a total of 40 normal tissue samples.

These gene expression profiles were developed by using the Affymetrix HG U133 Plus2 platform (GPL570). *RHBDD2* mRNA expression level was estimated by using the expression values of the Affymetrix probe 222995_s_at. The fRMA preprocessing algorithm allows analyzing independent oligo-microarray studies/batches, and then combines the data for further statistical analysis. This yielded a compiled gene expression data from n=906 colorectal samples (866 CRC samples and 40 normal samples) with clinicopathological data. Samples were grouped according to Dukes' stage classifier system. Univariate analysis of clinicopathological parameters—based on *RHBDD2* gene expression—was determined by *T* test and ANOVA. In addition, we employed the Oncomine (https://www.oncomine.org/) and GeneSigDB (http://compbio.dfci.harvard.edu/genesigdb) resources to query differential expression results for *RHBDD2* mRNA among colon cancer cell lines treated with 5FU.

Human colorectal samples

One-hundred and thirty formalin-fixed paraffin-embedded colorectal tissue samples (105 carcinomas, 15 benign lesions, and 10 normal biopsies) and 10 fresh CRC samples were obtained from the "General San Martín" Hospital of La Plata and the "Dr. Castro Rendón" Hospital of Neuquén, Argentina. CRC samples were classified according to Dukes' stage classification (Table 1). The use of human tissue blocks and clinical records was approved by the appropriate institutional committees.

Colon cancer cell line culture and 5FU treatment assay

The human colon cancer cell line Colo205 was cultured 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 in a humidified atmosphere including 5 % CO2. Colo205 cells were incubated by triplicate with 20, 40, and

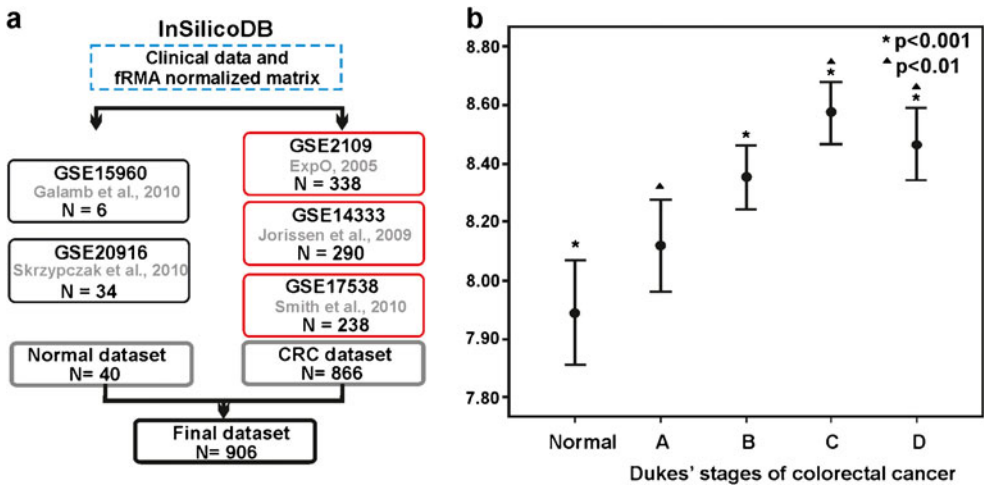

Fig. 1 In silico *RHBDD2* expression profile based on oligo-microarray datasets of CRC samples. **a** Strategy followed to obtain the compiled dataset. **b** A positive association between *RHBDD2* expression and tumor progression is observed, with a significant increase in stage *C*. The *asterisk* indicates a p value of <0.001 between normal and the stages B, C and D. The *triangle* indicates a p value of <0.01 between stage A and stages C and D

Table 1 Distribution of colorectal tissue samples according to the histopathological diagnosis

Histopathology	*N*
Normal	10
Benign	15
CRC (Dukes' stage)	20 (A)
	41 (B)
	37 (C)
	7 (D)
Total	130

80 μM of 5FU (Microsules, Argentina) during 72 h. *RHBDD2* mRNA/protein levels were measured after 5FU treatment assay.

To evaluate protein expression, total protein was obtained from TRIZOL. For Western blot, 50 μg of total protein was separated by 12.5 % 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 antirabbit secondary antibody (Dako, Denmark, 1:2,000) followed by chemiluminescence autoradiography. Beta-actin protein was detected using monoclonal anti-actin antibody (ICN Biomedicals, USA; 1:1,000) and HRP-conjugated antimouse secondary antibody (1:5,000).

RT-qPCR analysis of *RHBDD2* mRNA

RHBDD2 mRNA levels were measured for 10 fresh CRC sample tissues and the Colo205 cell line. Expression of each sample was normalized with mRNA from beta-actin as housekeeping gene. Total RNA was isolated using TRIZOL (Invitrogen, USA). cDNAs was synthesized using High Capacity Reverse Transcription Kit (Applied Biosystems, USA) The following primers were designed and used: forward 5′-GGTGTTTGGCATGGTTGTG-3′, reverse 5′-CGATGGAATAGCAGTAGGTGAG-3′. Thermal profile was programmed as follows: an initial denaturation step of 2′ at 94 °C followed by 40 cycles of 40″ at 94 °C, 45″ at 57 °C and 40″ at 72 °C. We employed the ANOVA test to analyze quantitative real-time PCR data based on $2^{-\Delta\Delta Ct}$ values. The basic significance level was fixed at $p<0.05$ and all data were analyzed using SPSS statistic software (SPSS Inc., Chicago, IL, USA).

RHBDD2 protein expression by immunohistochemistry, immunocytochemistry, and electron microscopy

For immunohistochemistry (IHC), 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 pH6.0 for 10 min at 100 °C. Primary polyclonal RHBDD2 antibody was used at 1:250 dilution as was previously described (GenScript Corp., NJ, USA) [14]. Briefly, immunodetection 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 (+++). A similar protocol, without antigen retrieval, was applied for immunocytochemistry (ICC) detection of RHBDD2.

Following the ICC protocol, RHBDD2 was identified in the cell line Colo205 by immunogold labeling. Grids were stained with uracil and lead citrates. Samples were observed in a transmission electron microscopy JEM1200EXII (JEOL). 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.

Results

RHBDD2 mRNA profiling and protein expression analyses in colorectal cancer

To analyze the *RHBDD2* gene expression profile in association with clinicopathologic parameters on larger colorectal dataset, five independent publicly available oligo-microarray studies were compiled ($n=906$; Fig. 1a). A significant increase in *RHBDD2* expression is observed in the stages B, C, and D compared with normal tissue ($p<0.001$, Fig. 1b). The ANOVA analysis also showed that the positive association between *RHBDD2* mRNA expression and Dukes' stages is highly significant in the stage C ($p<0.01$). We also evaluated the correlation of *RHBDD2* gene expression and the other clinical parameters (grade, anatomical site, adjuvant therapy) provided in the datasets and no significant differences were detected ($p>0.05$).

To further validate the relevance of *RHBDD2* expression at protein level, we analyzed 130 colorectal samples by IHC (Table 1). A statistically significant increase in RHBDD2 protein expression from normal to advanced colorectal cancer tissues was identified ($p<0.01$). In a first approach, we grouped colorectal samples into two groups: the normal/benign colorectal tissues and the CRC samples (Table 2). Although RHBDD2 protein expression was observed in 72 % of normal/benign colorectal samples, all of them showed a weak to moderate intensity of reaction, while from the 74 % of positive cases found in CRC group, the majority showed a moderate (21 %) to strong (35 %) intensity of reaction (Table 2). Moreover, the pattern of expression was clearly different in the two groups. In normal mucosa and benign lesions, the reaction was predominantly restricted to the cytoplasm of columnar cells, being mainly localized in

Table 2 IHC results of RHBDD2 expression in colorectal tissues. p value<0.01

	Intensity of reaction; cases/total cases (%)				
	Negative	Low	Moderate	Strong	Total
Normal/benign	7/25 (28)	7/25 (28)	11/25 (44)	0	25
Carcinoma	27/105 (26)	19/105 (18)	22/105 (21)	37/105 (35)	105

the perinuclear space (Fig. 2a). On the other hand, CRC samples showed RHBDD2 protein expression in the entire cytoplasm and in the membrane (Fig. 2b).

In a second approach, to validate the findings obtained with the oligo-microarray CRC datasets, we firstly analyzed *RHBDD2* mRNA expression in a subset of 10 CRC samples and the Colo205 cell line. Variation in *RHBDD2* expression was observed in the different samples, with the higher expression in samples of stages C and in the Colo205 cell line, which was originally obtained from a stage D tumor (Fig. 3a). We then performed the IHC analysis of CRC samples according to Dukes' stages (n=105). Due to the few numbers of samples of stage D, we grouped them with those of stage C, defining the groups C–D. Interestingly, we found a significant positive association between the intensity of reaction and the staging (p<0.001); with 86 % (38/44) of the C–D group samples showing a strong intensity of reaction compared with the 25 % (5/20) and 60 % (25/41) of the stages A and B, respectively (Fig. 3b). Overall, these results are in agreement with those observed at transcriptional level in the compiled dataset of 906 samples, indicating that the advanced stages of CRC (C–D Dukes' stages) expressed significantly increased levels of RHBDD2 protein compared with A or B stages.

Analysis of *RHBDD2* expression in the colon cancer cell line Colo205 and its response to 5FU treatment

Previously, we determined whether these cells express RHBDD2. By ICC, cells were incubated with RHBDD2 antibody (dilution of 1:200) and a positive reaction was observed in the cytoplasm of Colo205 cells (Fig. 4a). This result was then validated by electron microscopy, with RHBDD2 protein mainly detected in the endoplasmic reticulum (Fig. 4b). Having determined the expression of RHBDD2 in the Colo205 cells, we followed with the 5FU assay. The results of each concentration point were evaluated by RT-qPCR and WB (Fig. 4c, d). A dramatic increase in *RHBDD2* mRNA expression was observed after 72 h of 5FU–20 μM treatment (p<0.001). Interestingly, although *RHBDD2* expression still increased with higher concentrations of 5FU (40 μM), the increase reached a plateau at 80 μM (Fig. 4c). To validate this result at protein level, each concentration point was evaluated by western blotting. A positive control of RHBDD2 protein was included (Fig. 4d). As noted at the transcriptional level, a significant increase of RHBDD2 protein was evidenced in 5FU-treated cells. Difference was also more evident between control and 20 μM than between 40 and 80 μM, which might suggest that concentrations of 5FU greater than 20 μM, would affect dramatically transcription, inhibiting consequently protein synthesis.

Discussion

In the present study, we provide the first evidence of RHBDD2 gene/protein expression in CRC progression. We firstly evaluated the *RHBDD2* gene expression profile in a dataset of 906 colorectal samples. A significant increase in *RHBDD2* expression is observed in stages B, C, and D compared with normal tissue. We did not find significant differences between normal and stage A, which may suggest that *RHBDD2* is upregulated during tumor progression and it would not be an early event. Interestingly, we also identified a positive association between *RHBDD2* mRNA

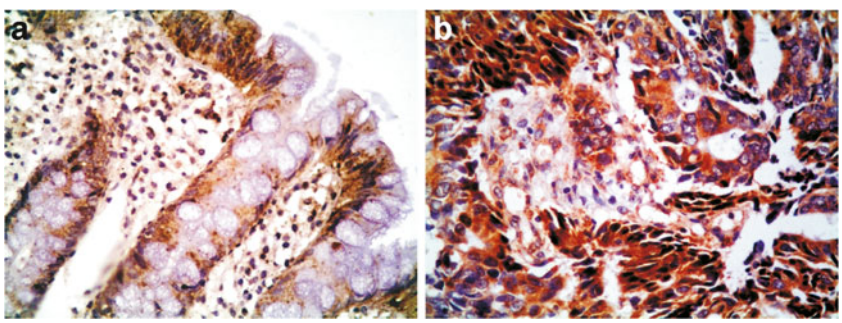

Fig. 2 Comparison of RHBDD2 protein expression between normal and CRC samples. Although normal mucosa showed RHBDD2 expression (**a**), an increase in the intensity of reaction was usually found in colorectal carcinomas (**b**). Microphotographs are ×40

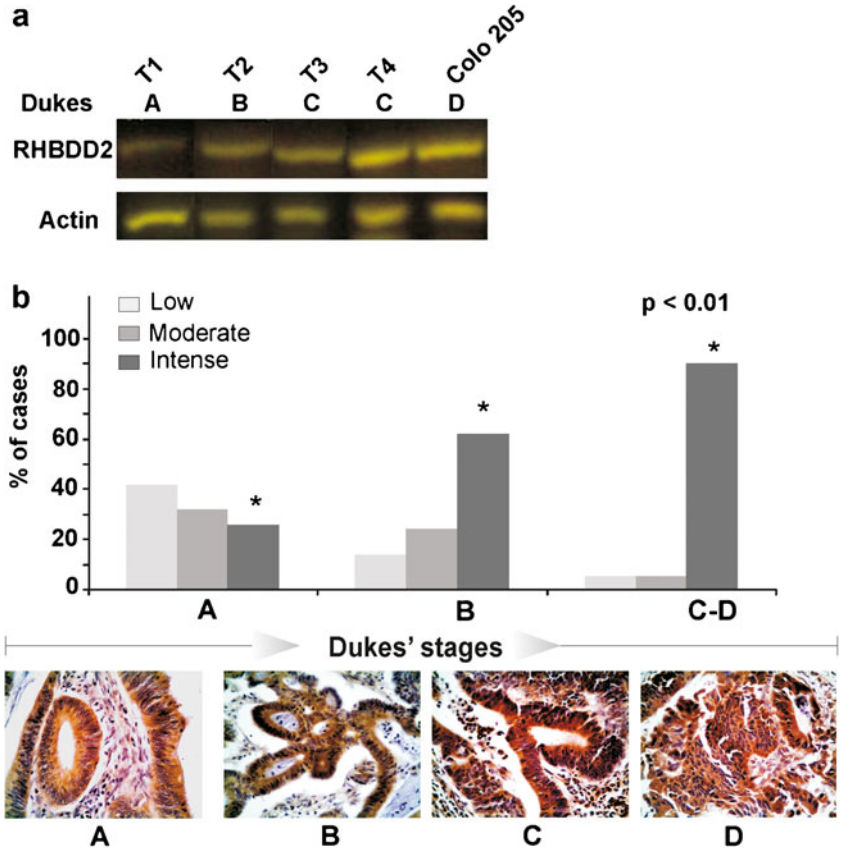

Fig. 3 RHBDD2 mRNA/protein expression in CRC samples according Dukes' stages. **a** RT-qPCR analysis of *RHBDD2* mRNA in a set of CRC samples and the cell line Colo205. **b** IHC analysis of RHBDD2 protein expression in 105 CRC samples. Samples were clustered into three groups according to Dukes' stages: **a**, **b** and **c–d**. A positive significant association between intensity of reaction and Dukes' stages was found ($p<0.01$). The *bottom panel* shows microphotographs of CRC tissue sections of the IHC results obtained in the different stages. An increase in the intensity of reaction (*brown staining*) is clearly associated with the advanced stages of the disease. All microphotographs are ×40

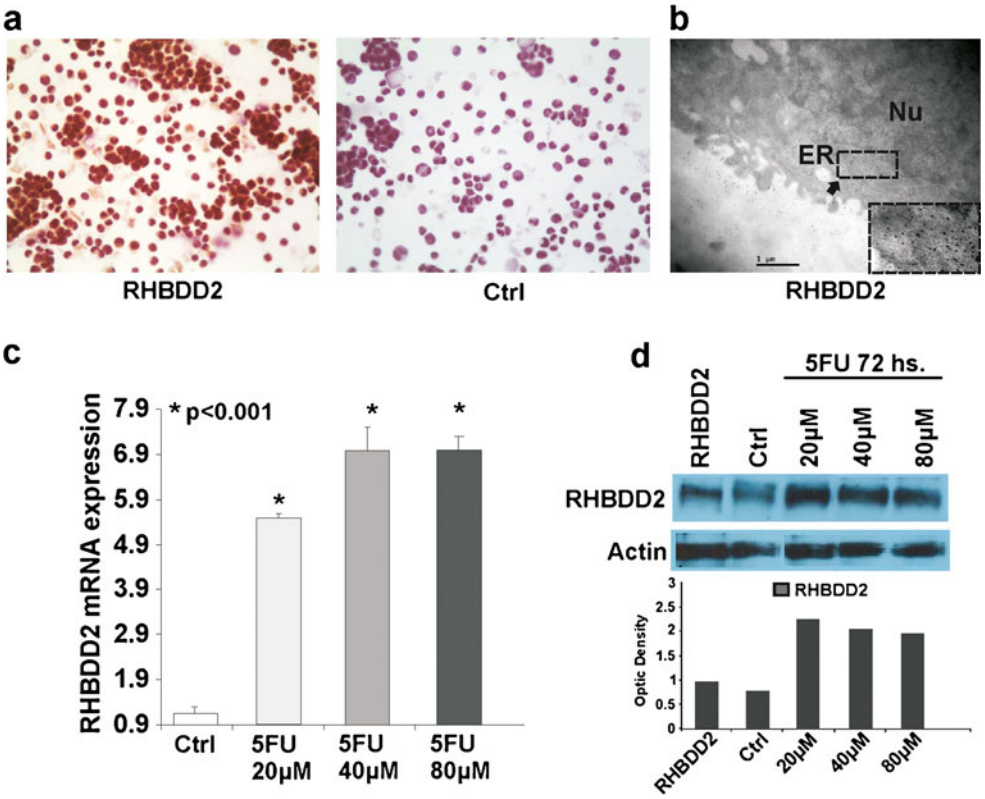

Fig. 4 RHBDD2 expression analysis in the Colo205 cell line. **a** A strong reaction is observed in the cytoplasm of Colo205 cells incubated with RHBDD2 antibody (*left*) compared with the negative control (*right*). **b** RHBDD2 protein is localized in the endoplasmic reticulum of the Colo205 cells. **c** *RHBDD2* mRNA expression in Colo205 cells after being exposed to different concentrations of 5FU. *Asterisk* indicates a p value of <0.001 in the 5FU-treated cells compared with control. **d** Western blotting of the 5FU assay

expression and the different stages, being statistically significant in the stage C, according to Dukes' classification. Dukes' C means the cancer has spread to at least one lymph node in the area close to the bowel. These data are in line with our previous observation that increased RHBDD2 protein expression is associated with advanced breast cancer stages [14].

Next, we validated RHBDD2 overexpression at protein level by IHC analysis. Although normal and benign lesions showed a high percentage of positive cases of RHBDD2 expression, a significant increase in the intensity of reaction was observed in the CRC samples. Moreover, the highest intensity was observed in the stages C and D, which is in agreement with the in silico mRNA profiling, indicating that a high proportion of colorectal carcinomas overexpressed RHBDD2 protein in the advanced stages. Importantly, the pattern of protein expression also varied, being mainly restricted to the perinuclear region of columnar cells in normal and benign samples, while covering all the cytoplasm in tumor cells. In this sense, we demonstrated by immunodetection and electron microscopy in the Colo205 cell line that RHBDD2 protein is mainly localized in the endoplasmic reticulum.

Finally, we evaluated RHBDD2 response to the chemotherapy agent 5FU. In the last decade, a large number of studies have been conducted to identify gene expression signatures that may be useful predictors of response to chemotherapy treatments. In this sense, Mariadason et al. employed cDNA microarray technology to predict the 5FU response based on the gene expression profile of 30 colon cancer cell lines [18]. They identified a gene expression signature of 420 transcripts that correlated with 5FU induced apoptosis (GeneSigDB ID: 14695196). Interestingly, *RHBDD2* transcript (GenBank Accesion #AA453994) was identified among the 50 genes that best correlated with the cellular response to 5FU treatment (see Electronic supplementary material (ESM) Fig. 1a). Moreover, *RHBDD2* mRNA was detected as being expressed in 80 % (24 out of 30) of the CRC cell lines according to the Mariadason's dataset; this is in agreement with our RHBDD2 IHC observations in CRC tissues.

In a later study, Boyer et al. used an oligo-microarray platform to analyze the transcriptomic changes of the HCT116 colon cancer cell line following exposure to the 5FU chemotherapy agent [19]. Interestingly, expression profiling analysis of Boyer's dataset allows us to identify a statistically significant increase of *RHBDD2* mRNA expression in the HCT116 cell line after 5FU treatment (ESM Fig. 1b). Notably, from this study we also observed that *RHBDD2* mRNA is constitutively up-regulated in the 5FU-resistant daughter HCT116 cell line (see ESM Fig. 1b), which would suggest that RHBDD2 overexpression might be associated with a mechanism of cell resistance to 5FU treatment.

To further investigate the effect of 5FU on *RHBDD2* expression, we performed an in vitro assay with the colon cancer cell line Colo205 exposed to different concentrations of 5FU (20–80 μM) during 72 h. After the treatment, we identified a highly significant upmodulation of RHBDD2 mRNA/protein, compared with the untreated cells. These data are in line with the mentioned studies, suggesting that *RHBDD2* expression could be modulated by the chemotherapy agent 5FU in CRC cells. Whether RHBDD2 expression is induced as an adaptive cellular response to the 5FU cytotoxic effects, or has a direct role in modulating the 5FU cytotoxicity, has to be elucidated. Several reports have established an association of different Rhomboid member's family and the process of apoptosis. For instance, it was determined that the overexpression or knockdown of *RHBDD1* in HEK 293 T cells can reduce or enhance BIK-mediated apoptosis, respectively [9]. Similarly, silencing of *RHBDF1* causes apoptosis of epithelial cancer cells [10]. Therefore, the overexpression of *RHBDD2* gene in response to 5FU-induced apoptosis would indicate a possible association with the modulation of the programmed cell death. Since the biological function of RHBDD2 remain to be determined, the high levels of protein expression detected in the advanced stages of colorectal and breast cancers would suggest a role in the development and progression of these tumor types [14]. Future studies will examine the relevance of this gene as a predictive biomarker as well therapeutic target to enhance 5FU drug efficacy

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Conflicts of interest None.

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