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Original Article

Immunohistochemical expression of RPRM is associated with low expression of proliferation marker Ki67 in patients with breast cancer

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Abstract: Reprimo (*RPRM*) is a potential p53-dependent tumor suppressor gene, which plays an important role in cell cycle arrest at G_2/M checkpoint. The aim of this study was to characterize RPRM protein expression in breast cancer tissues and its relation with clinic-pathologic features and proliferation marker protein Ki67. RPRM protein expression was examined by immunohistochemistry in tissue microarray containing 275-breast cancer and 16 normal breast tissues. These cases were classified as negative or positive expression for RPRM expression level with clinic-pathologic variables. The Kaplan-Meier curve was used to estimate survival over time. Positive expression of RPRM was observed in 68.4% (188/275) of tumors and 100% of breast normal tissues (16/16). RPRM expression has a significant relationship with age (P = 0.000). Moreover, positive RPRM expression was significant associated with low expression of proliferation marker protein Ki67; however, survival analysis did not show significant differences. These results suggest that RPRM is not a good prognosis marker but likely had an important role modulating negatively cell proliferation in breast cancer tissues.

Keywords: Immunohistochemistry, RPRM, breast cancer, Ki67

Introduction

Breast cancer (BC) is the second most common cancer in the world and, by far, the most frequent cancer among women, with about 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers), affecting mainly to women from developed countries in Western Europe and North America [1]. BC is the fifth cause of cancer-related death worldwide and, in women from developed countries, constitutes the second cause of cancer-related death, after lung cancer [1].

BC is a hormone related disease, by this reason a variety of important cellular regulators have been identified for this neoplasia, such as: receptors for growth factors, intracellular signaling pathways, regulators of apoptosis and nuclear proteins associated with cell cycle control and deregulation [2]. In fact, BC is classified in four different molecular subtypes according to immunohistochemical receptor status of estrogen (ER), progesterone (PR), epidermal growth factor 2 (Her2/neu) and proliferation marker Ki67: Luminal A (ER positive, PR positive, Her2/neu negative and Ki67 negative), Luminal B (ER positive, PR positive, Her2/neu positive or negative and Ki67 positive), Her2/neu positive (ER negative, PR negative, Her2/neu positive) and triple negative (TNBC) (ER negative, PR negative, Her2/neu negative) [3, 4]. Molecular stratification is useful for evaluating patient prognosis and outcome. Survival analyses show significant differences in outcome for patients belonging to the various subtypes, emphasizing the clinical relevance of stratification by such molecular profiling [5].

However, besides the genes involved in these molecular subtypes of BC, the presence of mutated *TP53* is still one of the main molecular characteristics. Depending on the cellular context and on the type of stress; p53 induces apoptosis, DNA repair, transient or permanent cell cycle arrest [6, 7]. In the same way, Ohki *et al*. found a p53-mediated downstream gene involved in cell cycle arrest at the G_2 phase when wild type mouse embryonic fibroblasts were exposed to X-ray irradiation; it was called *Reprimo* (*RPRM*) and was proposed as a potential tumor suppressor [8].

The *RPRM* gene encodes a cytoplasmatic protein involved in p53-mediated G2 phase arrest of the cell cycle and can be induced by X-ray-irradiation. Overexpression of RPRM, in HeLa cells by adenovirus infection leads to G2/M arrest through inhibition of Cdc2 activity by dephosphorylation, which subsequently avoids the translocation of the Cdc2-cyclin B1 complex into nucleus [8], suggesting a potential role for *RPRM* as a tumor suppressor gene. In gastric cancer, the aberrant hypermethylation of RPRM is considered as a potential biomarker for early detection [9]. Furthermore, immunohistochemical profile of *RPRM* in gastric cancer has shown that negative expression was significantly correlated with the depth of tumor invasion, lymphatic vessel invasion, and lymph node metastasis. In the same way, Luo et al., found a positive correlation between RPRM and S100A expression, proposing as potential diagnostic marker for gastric adenocarcinoma [10].

Despite these evidences in gastric cancer, there are no immunohistochemical reports about p53-mediated gene RPRM in human clinical breast tumor tissues, where the deregulation of TP53 is a frequent feature. Therefore, the purpose of this study was to evaluate for first time the RPRM expression using a Chilean cohort of 275 patients with BC and correlate these results with clinic-pathological parameters and Ki67 expression.

Material and methods

Patients and tissue samples

This study included formalin-fixed, paraffin-embedded tissues from 275 patients with BC and 16 normal breast samples obtained by mammary reduction. The tissues were retrieved from the Pathology Anatomy and Cytology Unit of Hernán Henríquez Aravena Hospital (Temuco, Chile) and BC tissue were used for tissue microarrays (TMAs) construction. Normal breast tissue samples were used as whole tumor sections. Hematoxylin and Eosin staining was performed on TMA to confirm the presence of a tumor tissue by a medical pathologist. The clinic-pathologic features were obtained from medical records. The BC samples were grouped according to immunohistochemistry profile for ER, PR, Her2/neu and Ki67 in Luminal A (156), Luminal B (30), Her2/neu (10) and TNBC (79). Complete postoperative follow-up was available for all 275 patients witch BC. That data were used for the Kaplan-Meier survival analyses. The Institutional Review Board of the School of Medicine of Pontificia Universidad Católica approved this study and issued a waiver authorizing the use of archival material without informed consent for samples of more than two years old, thereby preserving the anonymity of the patients.

Tissue microarray construction

One 2.0-mm tissue core was taken from representative area of BC samples using a tissue microarrayer (Pathology Devices TMArrayer, Westminster, CA) and mounted on a new recipient block. Four 4.0-μm thick sections were cut consecutively from the recipient block and transferred to poly-L-lysine-coated glass slides.

Immunohistochemical staining

The samples were deparaffinized and dehydrated using a graded series of xilol and ethanol solutions and placed in an antigen retrieval solution (citrate buffer, pH 6.0) for 15 min at 96°C in a TintoRetriever Pressure Cooker PC-2000 (BioSB, Inc. 69 Santa Felicia Dr, Santa Barbara CA 93117, USA). After cooling for 30 min, the tissue sections were quenched with 5% hydrogen peroxide for 20 minutes to block endogenous peroxidase activity. RPRM was detected with rabbit polyclonal antibody using a 1:500 dilution (Catalog N° bs-1885R; Bioss antibodies, Woburn, Massachusetts, USA). Ki67 protein was detected with rabbit monoclonal antibody using a 1:100 Dilution (Catalog N° BSB 5711; Bio SB, Santa Barbara, CA 93117, USA). Specimens were incubated with the primary antibodies overnight at 4°C. Labeling was

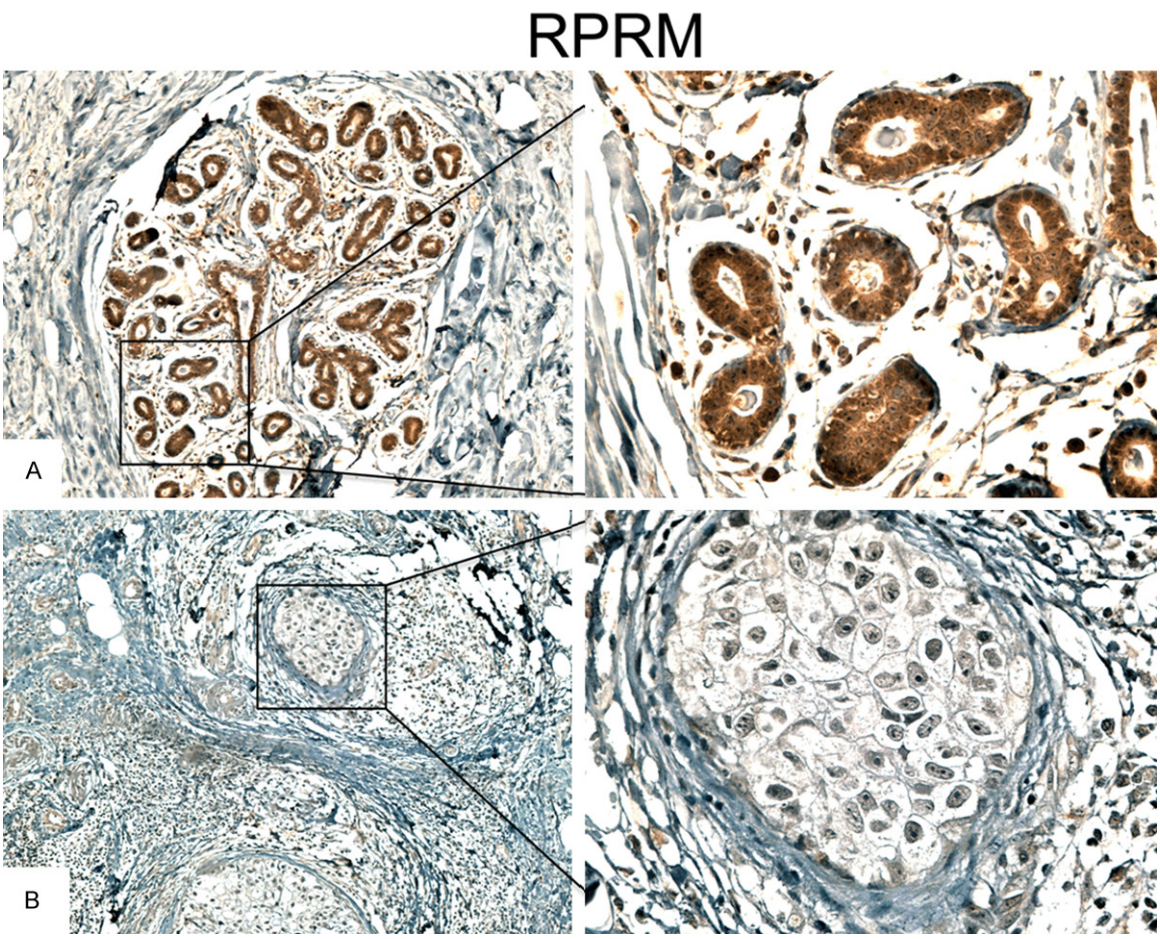

Figure 1. Immunohistochemical staining for RPRM in human breast tissues. A. Representative picture of breast normal tissue showing positive RPRM staining. B. Representative picture of breast cancer tissue showing negative RPRM staining. Magnification at 10× at left and 40× at right.

detected with LSAB+System-HRP (Catalog N° K0690, Dako North America Inc., Carpinteria, CA) according to the manufacturer's protocol. Sections were counterstained with Harris hematoxylin, then dehydrated, cleared, and mounted. Normal breast tissue was included as positive control, and negative controls were prepared with the omission of the primary antibody. The area counted in each section was randomly selected from a representative tumor area.

Evaluation of staining

The RPRM and Ki67 expression was examined by 2 independent and specialized pathologists without any information about clinic-pathologic features or prognosis. The assessments of all samples were conducted blindly by calculating of positive cells in 3 fields under a 400× microscope. The evaluation of staining was performed considering the percentage of positive cells. To RPRM percentage of positive cells ≤ 10% was considered negative (-) and > 10% positive cells was considered positive (+) [10]. To Ki67, the percentage of positive cells ≤ 14% was considered low and > 14% was considered high [11].

Statistical analysis

The analyses were performed using the statistical package SPSS version 20 (SPSS Inc., Chicago, IL). The correlation of RPRM with clinic-pathological variables was assessed using the χ^2 test or Fisher exact probability test (2-sided). Kaplan-Meier survival curves were plotted for patients with positive versus negative RPRM expression and compared using the log-rank test. To determine the relationship between RPRM and Ki67 expression was used Pearson χ^2 test. $P < 0.05$ was considered statistically significant.

Table 1. Relationship between Clinic-pathological parameters and *Reprimo* expression in patients with Breast cancer

	No.	Reprimo (+)	P*	Ki67 (high)	P*
Total	275	188 (68.4%)		217 (78.9%)	
Age (year; mean 60)					
≤ 60	61	29 (47.5%)	0.000	13 (21.3%)	1.000
> 60	214	159 (74.3%)		45 (21.0%)	
Tumor Size					
pT1+pT2	191	133 (69.6%)	0.574	38 (19.9%)	0.521
pT3+pT4	84	55 (65.5%)		29 (23.8)	
Lymph node metastasis					
pN0 (negative)	123	82 (66.7%)	0.604	26 (21.1%)	1.000
pN1-3 (positive)	152	106 (69.7%)		32 (21.1)	
Metastasis†					
pM0 (negative)	261	178 (68.2%)	1.000	53 (20.3%)	0.182
pM1 (positive)	14	10 (71.4%)		5 (35.7%)	
Stage by TNM					
Stage I+II	177	123 (69.5%)	0.591	37 (20.9%)	1.000
Stage III+IV	98	65 (66.3%)		21 (21.4%)	
Histologic gradeΦ					
Low grade	51	35 (68.6%)	0.923	6 (11.8%)	0.000
Intermediate grade	128	89 (69.5%)		19 (14.8%)	
High grade	90	59 (65.6%)		33 (36.7%)	
Molecular Subtype					
Luminal A	156	112 (71.8%)	0.083	0 (0.0%)	0.000‡
Luminal B	30	16 (53.3%)		26 (86.7%)	
Her2/*neu*	10	9 (90%)		3 (30%)	
TNBC	79	51 (64.6%)		29 (36.7%)	
Estrogen receptor					
ER (-)	89	59 (66.3%)	0.678	32 (36.0%)	0.000
ER (+)	186	129 (69.4%)		26 (14.0%)	
Progesterone receptor					
PR (-)	128	89 (69.5%)	0.795	39 (30.5%)	0.001
PR (+)	147	99 (67.3%)		19 (12.9%)	
Her2/*neu*					
Her2/*neu* (-)	231	160 (69.3%)	0.241	51 (22.1%)	1.000
Her2/*neu* (+)	14	12 (85.7%)		3 (21.4%)	

*Fisher's Exact test; †Thirty cases, with missing information were excluded from that analysis. ΦFour cases,with missing information were excluded from that analysis. ‡Ki67 is used in the differentiation of luminal A and B tumors. Luminal tumors with $Ki67^{high}$ are Luminal B tumors by consensus.

Results

A total of 291 cases were analyzed: normal breast samples (16) and BC (275). Within BC tissue we classified as: Luminal A (156), Luminal B (30), Her2/neu (10) and TNBC (79). All of these BC cases are of ductal type. When present, RPRM expression was detected predominantly as cytoplasmic staining in breast epithelial cells. No staining was found in negative control slides. Positive expression was detected in all 16 (100%) normal breast tissues. Examples of staining intensity are illustrated in **Figure 1**. In BC cases, only 31.65% (87/275) cases showed absence of RPRM staining; these were classified as RPRM negative tumors. Within the 156 BC cases of Luminal A type analyzed, 28.2% (44/156) had negative RPRM expression, whereas 71.8% (112/156) showed positive RPRM levels. In Luminal B, negative and positive expression levels of RPRM were 46.7% (14/30) and 53.3% (16/30), respectively. The Her2/neu group presented only a 10% (1/10) of negative expression of RPRM. For TNBC subtype, RPRM expression was negative in 35.4% (28/79) of cases analyzed. However, there was no significant association between RPRM expression and different molecular subtypes (P = 0.083).

The median age of the patients was 59 years old (range 21 to 88 years old), which was close to 60 years old. Then, patients were grouped according to age: less than or equal to 60 and over 60 years old. RPRM expression was lost in 52.5% (32/61) of patients ≤ 60 years old, whereas in patients > 60 years old was 25.7% (55/214). This difference was statistically significant (P = 0.000), demonstrating that patients ≤ 60 years old presented frequently loss of RPRM expression.

The relationship between RPRM expression and each clinic-pathologic factor was analyzed for BC cases. Nevertheless, no significant cor-

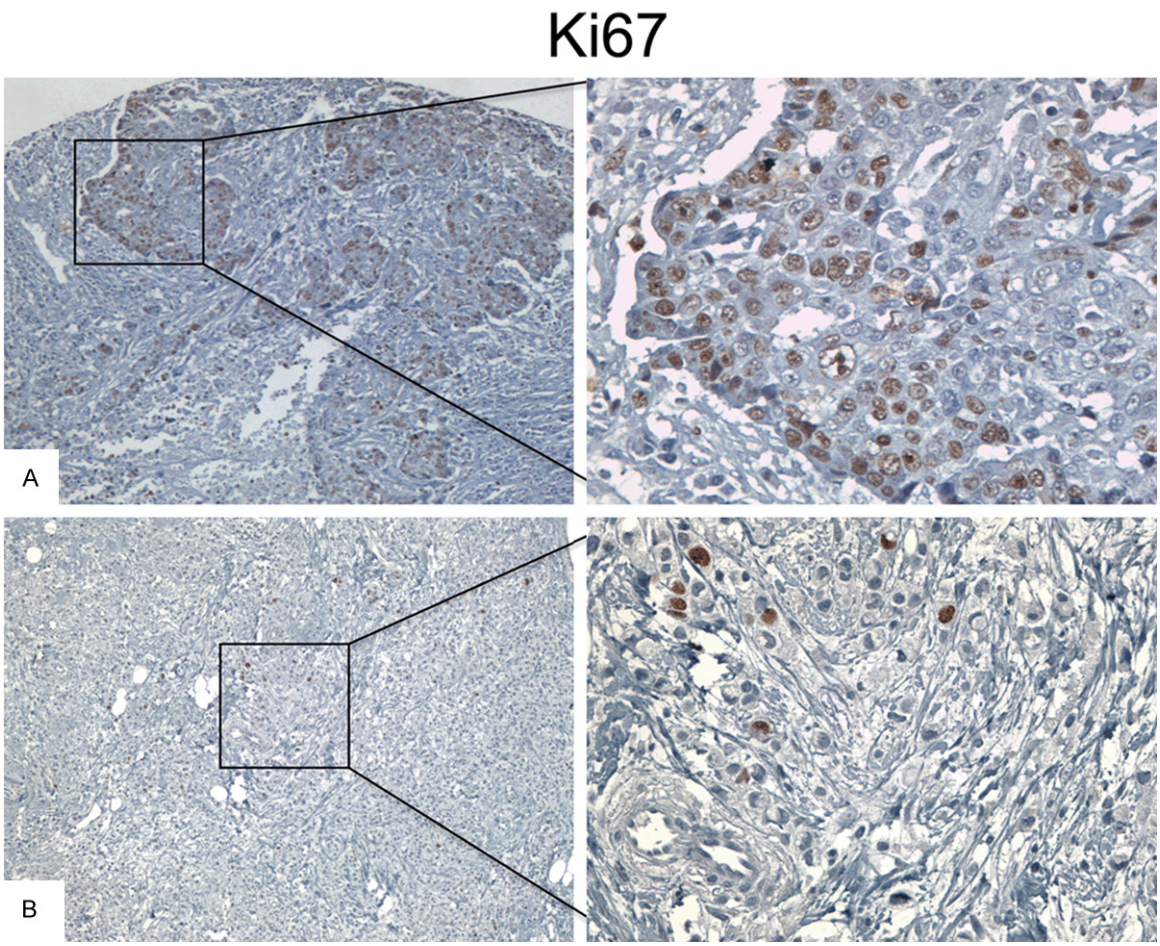

Figure 2. Immunohistochemical staining for Ki67 in human breast tissues. A. Representative picture of breast cancer tissue showing high Ki67 staining. B. Representative picture of breast cancer tissue showing low Ki67 staining. Magnification at 10× at left and 40× at right.

Table 2. Correlation between *Reprimo* and *Ki67* expression in breast cancer

	Reprimo		
	+	-	*P**
Ki67			
High	33 (56.9%)	25 (43.1%)	0.040
Low	155 (71.4%)	62 (28.6%)	

*Fisher's Exact test.

relation was found between level of RPRM and tumor size, lymph node metastasis, metastasis to distant organ, histologic grade, TNM stage, expression of estrogen receptor (ER), progesterone receptor (PR) or Her2/neu (**Table 1**).

To determine the correlation in the co-expression of RPRM and proliferation marker Ki67, samples were divided into different groups. Examples of staining intensity for Ki67 are illustrated in **Figure 2**. The percentages and number of cases with both RPRM and Ki67 positive and high, respectively (RPRM+, $Ki67^{high}$), both negative/low (RPRM-, $Ki6^{low}$), only RPRM positive (RPRM+, $Ki67^{low}$), and Ki67 high (RPRM-, $Ki67^{high}$) were 56.9% (33/58), 28.6% (62/217), 71.4% (155/217) and 43.1% (25/58), respectively. These results showed that there is a significant inverse correlation between expression of RPRM and Ki67 (P = 0.040) (**Table 2**).

Clinical outcome was also analyzed in these 275 patients with BC. The observation time ranged from 1 to 150 months, with a median time of 106 months. The relationship between RPRM expression and patient survival was examined by Kaplan-Meier analysis. The Kaplan-Meier showed no association between

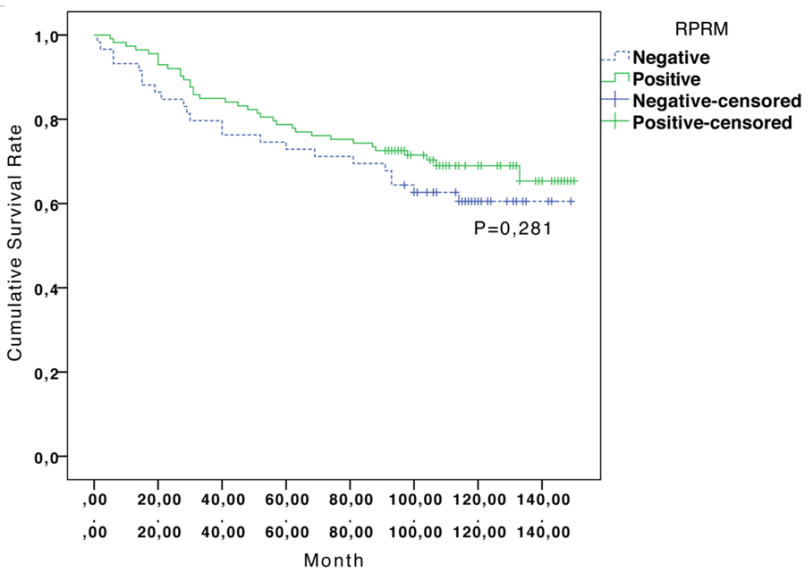

Figure 3. Kaplan-Meier analysis of 275 patients with Breast Cancer. The solid line indicates patients whose tumors are positive for RPRM and the dotted line indicates those tumors negative for RPRM (P = 0.281).

negative RPRM expression and survival in BC patients (P = 0.281) (**Figure 3**).

Discussion

Tumor suppressor genes play an important role in BC carcinogenesis, acting as regulators in processes such as cell cycle, apoptosis, growth signals, cell replication and DNA stability. RPRM is a potential p53-dependent tumor suppressor, located at 2q23 and encodes a highly glycosylated protein, located predominantly in the cytoplasm. The overexpression of RPRM leads to arrest at the G_2-phase of the cell cycle, regulating the activity of CDC2-Cyclin B1 complex in HeLa cells [8]. Several reports indicate that RPRM expression is lost in human malignances including pancreatic cancer, gastric cancer, among others, mainly by aberrant methylation of RPRM promoter [12-14]. In esophageal cancer, RPRM methylation is a frequent finding in patients non-responsive to chemotherapy and poor outcome than those without methylation [15]. Furthermore, aberrant methylation of RPRM in pancreatic cancer is a common event and is correlated with genetic instability and unfavorable outcome after surgical resection [13], all of those evidences suggest to RPRM as a potential tumor suppressor gene. In this matter, specific methylation of RPRM was proposed as a potential biomarker for early detection of gastric cancer [9]. Likewise, Ooki *et al*., indicated that clinical assessment of RPRM promoter methylation may serve not only as a predictive marker for chemotherapy consisting of cisplatin and the fluoropyrimidine class, but also as a marker for tumor aggressiveness [16]. It is suggested that the epigenetic mechanism involving DNA methylation is responsible for the silencing of tumor associated genes in a variety of human cancers [10]. However, it is frequent that hypermethylation affects protein expression as occur with relevant proteins like RASSF1A, CXCL12 [17], IL-8 [18], MGMT [19], p16 [20]. In case of RPRM, it is important to note that its expression is dependent of p53, by this reason is probably that the loss of RPRM protein expression is mainly regulated by p53 instead of DNA methylation. Nevertheless, more studies are necessary in order to establish association among DNA methylation, protein expression of RPRM and p53 status in BC.

On the other hand, in addition to our study of DNA methylation in RPRM gene and its protein expression, we compared the immunohistochemical profile of RPRM and Ki67. Interestingly, we found a significant inverse correlation between both proteins (P = 0.040). Our results showed that RPRM-positive cells are correlated with low Ki67 protein expression, indicating that these cells are not in a proliferative phase, due to RPRM effect on cell growth as a potential tumor suppressor. These results provide the first immunohistochemical evidence that suggests RPRM expression is involving probably in cell proliferation as a negative regulator of this process in human clinical BC tissues. Similar correlation has been reported among others tumor suppressor genes (*ANX7, Maspin*) and Ki67 [21-23]. Ki67 is a proliferative antigen represents an important marker of cell proliferation, a higher index of Ki67 seeming to correlate with tumor aggressiveness and poor survival rate [24]. In

BC, immunohistochemical assessment of the proportion of stained cells for the nuclear antigen Ki67 has become the most widely used method for comparing proliferation between tumor samples [25]. This approach may help to suggest RPRM as a potential prognosis marker in BC, however, a univariate analysis using Kaplan-Meier method showed that those cases positive for RPRM did not have a better survival rate compared to those with negative expression (P = 0.281). In the same way, two immunohistochemistry reports of RPRM suggest an important tumor suppressing activity associated with the aggressive features of gastric adenocarcinoma (tumor invasion, lymphatic vessel invasion lymph node metastasis and invasive stage) [10, 26]. However, in our study we found no statistically significant associations between RPRM expression and patient clinic-pathological features. These finding indicate that RPRM is not associated with the same clinic-pathological parameter in different tumoral types; probably because its effects are exerted in a tissue-specific manner as was proposed by Xu et al. [27].

In conclusion, our findings suggest that *RPRM* could play a role as a tumor suppressor gene modulating cell proliferation in breast tumors. Nevertheless, it is necessary additional studies to determine whether *RPRM* will be considered as a *driver* or merely *passenger* gene in breast tumorigenic process.

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Disclosure of conflict of interest

None.

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