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RLIP76 expression as a prognostic marker of breast cancer

C.-Z. WANG[1], P. YUAN[1], B. XU[2], L. YUAN[3], H.-Z. YANG[1], X. LIU[4]

[1]Breast Cancer Center, the Affiliated Cancer Hospital of Zhengzhou University, Zhengzhou, Henan, P. R. China
[2]Central Laboratory, the Affiliated Cancer Hospital of Zhengzhou University, Zhengzhou, Henan, P. R. China
[3]Department of Surgery, the Affiliated Cancer Hospital of Zhengzhou University, Zhengzhou, Henan, P. R. China
[4]Department of Radiotherapy, the Affiliated Cancer Hospital of Zhengzhou University, Zhengzhou, Henan, P. R. China

Abstract. – OBJECTIVE: RLIP (Ral-interacting protein)-76/RalBP11 (Ral-binding protein-1), a multifunctional protein and stress-inducible non-ABC transporter, have been proven to serve as a critical role in cancer development and progression; however, little is known about the pathological role of RLIP76 in breast cancer patients. The study aimed to determine the correlation between RLIP76 expression in breast cancer patient and clinical outcomes.

PATIENTS AND METHODS: Using RT-PCR and Western blot, messenger RNA (mRNA) and protein expression of RLIP76 were determined in breast cancer and adjacent normal mammary tissues. The relationship of RLIP76 expression with clinical characteristics of 245 breast cancer patients was analyzed by immunohistochemistry.

RESULTS: In the present study, our results indicated that RLIP76 mRNA and protein were highly expressed in the breast cancer tissues while compared with adjacent normal mammary tissues and the correlation with RLIP76 protein expression was significantly associated with age (the non-ABC transporter, stage and the expression were significantly associated–T2 vs. T3–T4, $p < 0.01$), lymph node metastasis (N0-N1 vs. N2–N3, $p < 0.01$), and PR (positive vs. negative, $p < 0.01$) in breast cancer patients; furthermore, we also found that RLIP76 protein overexpression was an unfavorable prognostic factor in the patients suffered from breast cancer.

CONCLUSIONS: RLIP76 overexpression serves as an unfavorable prognostic biomarker in breast cancer patients.

Key Words:
RLIP76, Breast cancer, Prognosis.

Introduction

Breast cancer, as the most common malignancy in women and the second leading cause of cancer-related mortality in females, makes breast cancer a severe threat to women worldwide[1,2]. Approximately 40,000 women died of breast cancer in 2014, only second to the lung cancer. On the basis of the data reported by National Central Cancer Registry of China, Beijing, the breast cancer is also the most common malignancy in women in China and the incidence of breast cancer continuously grows by 3% annually[3]. Although the advanced systematic therapies including surgery, radiotherapy, and chemotherapy have been introduced for breast cancer treatment, the 5-year overall survival rate still remains unsatisfactory. The five-year survival rates vary significantly depending on the stage to which a tumor has progressed at the time of diagnosis. The localized breast cancer has 99% survival rate, whereas that rate falls to 24% in cancers metastasizing to the distant organs. Numerous researches have established the association between biomarkers and malignant statuses and prognosis[4-6]; however, more studies are needed to identify novel biomarkers that can effectively predict unfavorable prognosis and serve as novel therapeutic targets for breast cancer treatment.

RLIP (Ral-interacting protein)-76/RalBP11 (Ral-binding protein-1), a multifunctional protein and stress-inducible non-ABC transporter, is involved in a variety of cellular functions, such as cell proliferation, metastasis and ligand-dependent receptor endocytosis[7]. Accumulating pieces of evidence have showed that RLIP76 is overexpressed in most cancer cell lines and many human cancers[7-12]. Early study has identified RLIP76 as an effector of Ral, a GTPase activated during Ras signaling activation[13-15]. Sehrawat et al showed that RLIP76 has the activity of dinitrophenyl-S-glutathione (DNP-SG)-ATPase, which mediates ATP-dependent efflux of glutathione conjugates (GS-E) of elec-

Corresponding Author: Wang Chengzheng, MD; e-mail: angengwcz@163.com

trophilic compounds, and other xenobiotics including chemotherapeutic agents[16,17]. Moreover, *in vivo* studies using knockout mouse models showed that RLIP76-/- mice were highly resistant to chemical carcinogenesis and even resistant to the growth of subcutaneously implanted cancer cells[7,12,18-21]. In the context of breast cancer, a previous work has reported lower protein expression and specific activity of RLIP76 in breast cancer cell lines while compared with the lung cancer cell lines[22]. However, the clinical significance of RLIP76 in breast cancer remains unclear.

Here, we showed that RLIP76 expression was significantly increased in the breast cancer samples and positively correlated with the malignant status of breast cancer patients. Moreover, our results indicated that RLIP76 was an independent factor for poor prognosis in breast cancer patients. In the future, more studies are needed to verify the role of RLIP76 as a reliable clinical predictor for the outcome in breast cancer patients.

Patients and Methods

Sample Collection

This work included 245 patients, who had undergone breast cancer surgeries between March 2002 and December 2003 in the Affiliated Cancer Hospital of Zhengzhou, University, Zhengzhou, China. The study protocol was approved by the Institutional Review Board of our hospital. The inclusion criteria include: (1) pathologically confirmed breast cancer, (2) availability of paraffin-embedded specimens of the primary tumor and relatively complete follow-up data. Among 292 consecutive patients who had undergone radical mastectomy, 37 were excluded because of unavailability of primary tumor specimens and 10 cases were eliminated due to lack of follow-up data. Eventually, 245 patients were included. The fresh specimens were snap frozen in liquid nitrogen for real-time PCR and western blot. None of these patients had received tumor-specific therapy before diagnosis. The systematic treatments were performed according to NCCN guideline.

Real-time PCR (RT-PCR)

The expressions of *RLIP76* in breast cancer tissues and paired adjacent normal mammary tissues were determined. Total RNA was isolated by using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RT-PCR was carried out by means of a MX3000P instrument (Stratagene, Santa Clara, CA, USA) and SYBR Premix ExTaq kit (Takara, Otsu, Shigu, Japan) to detect the level of *RLIP76* with *β-actin* as control. The PCR primers for RLIP76 or β-actin were synthesized as published previously[23]. The relative expression of *RLIP76* was calculated and normalized by the $2^{-\Delta\Delta Ct}$ method relative to *β-actin*. The experiments were performed independently in triplicate.

Western Blot

Proteins were extracted from the cancer tissue and then subjected to western blot analysis following standard protocol. Briefly, after blocking with 5% non-fat milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) for 1h at room temperature, the membranes were blotted with primary antibody against RLIP76 (Abcam, Hongkong, 1:1,000 dilution). HRP-conjugated anti-rabbit IgG antibody was used as the secondary antibody (1:2000; Cell Signaling Technology, Denvers, MA, USA). Signals were detected by the enhanced chemiluminescence reagents (Pierce Biotechnology, Waltham, MA, USA).

Immunohistochemistry and Expression Analysis

The immunohistochemistry and expression analysis were conducted as previously described[24]. The paraffin sections from clinical specimens were deparaffinized in xylene and rehydrated in a descending ethanol series (100, 95, 90, 80, 70% ethanol) and double distilled water following standard protocols. The heat-induced antigen retrieval was performed in citrate buffer and boiled for 10 min. After the antigen retrieval, the sections were treated with 3% hydrogen peroxide and 1% bovine serum albumin to block the endogenous peroxidase activity and nonspecific binding. The sections were incubated in non-immune serum for 30 min and then incubated overnight at 4°C in RLIP76 primary monoclonal antibody (Abcam, Hongkong, 1:500 dilution). After washed in Tris-buffered saline with Tween 20 (TBST, Glostrup, Denmark), the immuno-labeled sections were incubated with biotin-conjugated secondary antibody for 20 min at room temperature, then with peroxidase-conjugated complex (Dako, Carpinteria, CA, USA) for 20 min and finally visualized with 3,3-diaminobenzidine and counterstained with hematoxylin. In order to ensure the specificity of the immunostaining, the negative controls were prepared by replacing the primary antibody with non-immune serum. The staining intensity in

the cytoplasma was graded using a scale from 0 to 3 (0 for no immunostaining, 1 for a light brown color, 2 for a medium brown color and 3 for dark brown staining). The percentage of positively stained cells was scored as follows: 0, no staining; 1, < 25% of the entire malignant cell population stained, 2, 25-75% of the entire malignant cell population stained and 3, > 75% of the entire malignant cell population stained. Combining intensity and percentage staining resulted in the final staining score (0-6). Final staining scores of 0-3 and 4-6 were respectively considered to be of low and high expression. Five random fields (1 field = 0.159 mm^2 at ×100 magnification) in each sample of the tissue sections stained immunohistochemically for RLIP76 were reviewed and scored separately by two pathologists blinded to the clinical parameters. Any disagreement was arbitrated by the third pathologist.

Statistical Analysis

The statistical analyses were performed by means of the SPSS 13.0 statistical software package (SPSS Inc., Chicago, IL, USA). The difference of RLIP76 messenger RNA (mRNA) expression between the breast cancer tissues and the paired adjacent normal mammary tissues was detected by the Wilcoxon signed rank test. The Pearson chi-square test was used to analyze the association between RLIP76 protein expression and clinicopathologic parameter. The patients were divided into two groups, i.e., high- and low-expression group in terms of the optimal cut-off values of above three variables and were subjected to univariate and multivariate survival analysis, respectively. LOG-RANK univariate analysis was performed to compare the association between RLIP76 expression and DFS, DDFS and OS. *p* values less than 0.05 were considered significant.

Results

Patient Characteristics

The clinical and histopathological data of the enrolled subjects are summarized in Table I.

Table I. Clinicopathological features of patients

Clinicopathological features		N (%)
Age (year)	≤ 48	149 (60.8)
	> 48	96 (39.2)
Menstrual status	Premenopausal	166 (67.8)
	Postmenopausal	79 (32.2)
Pathological type	Invasive ductal carcinoma	220 (89.8)
	Invasive lobular carcinoma	6 (2.4)
	Medullary carcinoma	4 (1.6)
	Mucinous carcinoma	5 (2.0)
	Invasive eczematous carcinoma of nipple	7 (2.9)
	Invasive ductal carcinoma and invasive lobular carcinoma	3 (1.2)
Tumor size	T1	83 (33.9)
	T2	127 (19.3)
	T3	26 (10.6)
	T4	9 (3.7)
Lymph node metastasis	N0	110 (44.9)
	N1	64 (26.1)
	N2	28 (11.4)
	N3	43 (17.6)
Clinical stage	I	68 (27.8)
	IIA	85 (34.7)
	IIB	52 (21.2)
	IIIA	26 (10.6)
	IIIB	10 (4.0)
	IIIC	4 (1.6)
ER	Positive	168 (68.6)
	Negative	77 (31.4)
PR	Positive	153 (62.4)
	Negative	92 (37.6)
HER-2	Positive	49 (20)
	Negative	196 (80)

Table II. Correlations between RLIP76 protein expression and clinicopathological characteristics in patients.

Characteristics	N	Low expression (Final score 0-3)	High expression (Final score 4-6)	*p*
Age				< 0.01
≤ 48	149	110	39	
> 48	96	27	69	
Clinical stage				< 0.01
I-II	205	125	80	
III-IV	40	12	28	
Tumor size				< 0.01
T1-T2	210	105	105	
T3-T4	35	32	3	
Lymph node metastasis				< 0.01
N0-N1	174	121	50	
N2-N3	71	16	55	
ER				> 0.05
Positive	168	93	75	
Negative	77	44	33	
PR				< 0.01
Positive	153	112	41	
Negative	92	25	67	
HER				
Positive	49	25	24	> 0.05
Negative	196	112	84	

Pathological types included the invasive ductal carcinoma, the invasive lobular carcinoma, the medullary carcinoma, and the mucinous carcinoma, the invasive eczematous carcinoma of nipple, the invasive ductal carcinoma and the invasive lobular carcinoma. The invasive ductal carcinoma is the most common pathological type, which takes up approximately 90% cases.

No patient received surgical castration, neoadjuvant chemotherapy and targeted therapy. The median follow-up time was 119 months (range: 9-145 months). The DFS, DDFS and OS rates were 72.6%, 75.4% and 78.4% respectively.

Increased Expression of RLIP76 in Breast Cancer

In order to identify the role of RLIP76 in breast cancer, we performed quantitative RT-PCR to measure the mRNA expression of *RLIP76* in 50 pairs of breast cancer tissues and adjacent normal mammary tissues. Compared with adjacent normal mammary tissues, the breast cancer tissues showed significant higher mRNA expression levels of *RLIP76* ($p < 0.01$, Figure 1A); furthermore, we detected the protein expression of RLIP76 in 50 pairs of breast cancer tissues and adjacent normal mammary tissues by western blot and found that RLIP76 protein was overexpressed in breast cancer tissues (Figure 1A).

Correlation between RLIP76 Protein Expression and Clinicopathological Characteristics in Breast Cancer Patients

We detected the levels of RLIP76 protein expression in 245 archived paraffin-embedded breast cancer samples using immunohistochemical staining and analyzed the correlation between the protein expression of RLIP76 and clinicopathological characteristics of breast cancer. As summarized in Table II, RLIP76 protein expression was significantly associated with age (≤ 48 vs. > 48, $p < 0.01$), clinical stage (I-II vs. III-IV, $p < 0.01$), tumor size (T1-T2 vs. T3-T4, $p < 0.01$), lymph node metastasis (N0-N1 vs. N2-N3, $p < 0.01$), and PR (positive vs. negative, $p < 0.01$). However, RLIP76 expression was not associated significantly with ER (positive vs. negative, $p > 0.05$) and HER2 (positive vs. negative, $p > 0.05$).

Correlation Between RLIP76 Expression and DFS, DDFS or OS

Log-Rank univariate analysis showed that higher density of RLIP76 expression in tumor tissue was related with longer OS ($p = 0.047$); however, the difference was not statistically significant. In contrast, higher RLIP76 expression in tumor tissue was significantly related with shorter DFS ($p = 0.026$) and DDFS ($p = 0.032$), as shown in Figure 2.

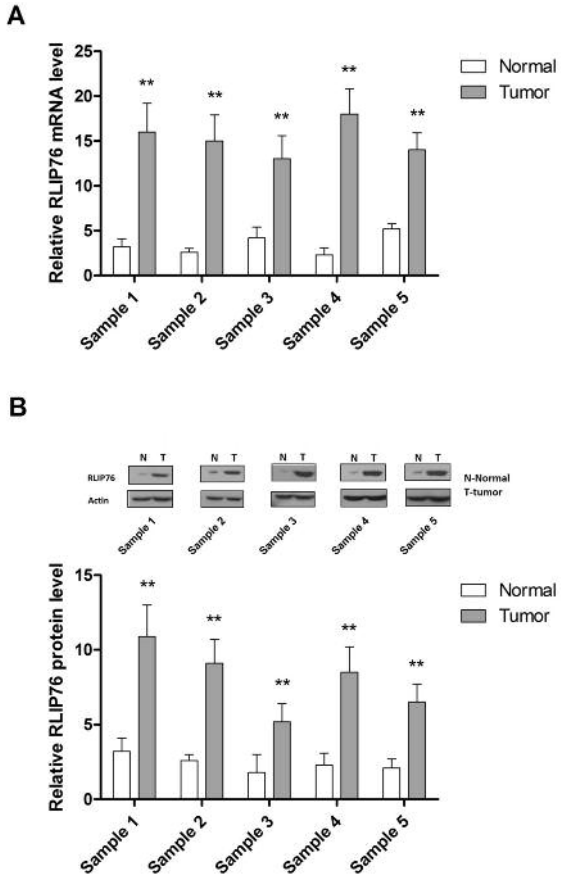

Figure 1. mRNA and protein expression of RLIP 76 in tumor and normal tissue (5 pairs representative sample from all tested samples). $^{**}p < 0.05$ vs. normal.

Discussion

It has been well documented that RLIP76 is a multifunctional protein that transports glutathione-electrophile conjugates as well as chemotherapy drugs across the plasma lemma[8,16,25-29]. It is also indispensable for diverse cellular functions, such as mitosis[30,31], apoptosis[32,33] and endocytosis[34]. Singhal et al[20] reported that RLIP76 could exert suppressive effect on tumor growth via Ral or Ras-R signaling pathway or by regulating the expression of heat shock proteins. Leake et al[19] demonstrated that RLIP76 plays a role in regulating P13/Akt signaling pathway, which is important in signal transduction from upstream growth factor receptors and cell proliferation. The role of RLIP76 in cell cycle progression has also been reported. It was also reported that inhibition of RLIP76 expression arrested glioma cells at the G1 phase[9]. Recently, Yao et al[23] also found that the reduced RLIP76 expression by shRNA resulted in cell cycle arrest at G1 phase in leukemia cells. The anti-apoptotic effect of RLIP76 has also been found in a number of human cancer cell lines, such as glioma and leukemia cell lines[9,23]. In addition, Wang et al[24] proposed another anti-proliferative mechanism that RLIP76 may suppress apoptosis and promote the proliferation of glioma cells by direct adenosine triphosphate-dependent xenobiotic transport and by activating the Rac1-JNK signaling pathway. Furthermore, the role of RLIP76 in the metastasis process has been well studied in a variety of *in vitro* and *in vivo* models. Suppressed cell invasion has been reported in colon cancer and glioma cells with RLIP76 knockdown[9,11]. Lee et al[35] suggested a role of RLIP76 in tumor cell induction of angiogenesis by demonstrate that RLIP76 regulates tumor cell transactivation of endothelial cells via control of VEGF expression and secretion. RLIP76 depletion has also been found to inhibit tumor neovascularization in mouse model of melanoma[36]. A recent work further reported that the interaction between ARNO (a guanine nucleotide exchange factor for Arf6) and RLIP76 N-terminus regulates cell spreading and motility via PI3K and Arf6, independent of RLIP76 control of Rac[37]. Taken together, these studies suggested that RLIP76 should act as an effector to promote tumor progression and metastasis.

A number of researches[7,20,24] documented increased expression of RLIP76 in several types of human cancers, such as glioma, melanoma, lung cancer and colon cancer. However, little is known about the pathological role of RLIP76 in breast cancer patients. In the present study, our results indicated that *RLIP76* mRNA and protein were highly expressed in breast cancer tissues compared with adjacent normal mammary tissues and the RLIP76 protein expression was significantly associated with age (≤ 48 vs. > 48, $p < 0.01$), clinical stage (I-II vs. III-IV, $p < 0.01$), tumor size (T1–T2 vs. T3-T4, $p < 0.01$), lymph node metastasis (N0-N1 vs. N2-N3, $p < 0.01$), and PR (positive vs. negative, $p < 0.01$) in breast cancer patients. Furthermore, we've also found that RLIP76 protein overexpression was an unfavorable prognostic factor in breast cancer patients. Similarly, Wang et al[24] also showed that RLIP76 messenger RNA and protein expression are positively correlated with glioma grade and that higher RLIP76 expression was correlated with shorter

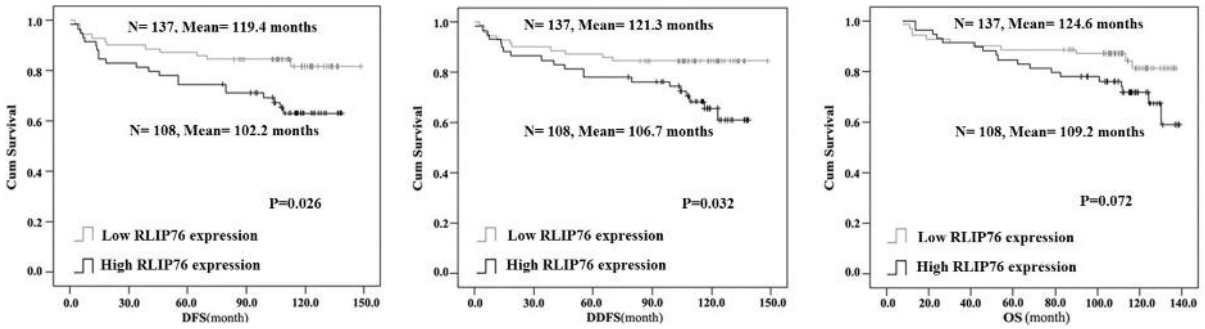

Figure 2. Prognostic value of RLIP76 expression in patients with breast cancer.

patient survival. These studies consistently implied that RLIP76 over-expression should serve as a poor prognostic biomarker for breast cancer patients.

Conclusions

Our report showed that RLIP76 expression was significantly increased in breast cancer samples and positively correlated with the malignant status of breast cancer patients. Moreover, our results indicated that high RLIP76 expression was associated with the poor prognosis of breast cancer patients. In the future, more studies are needed to verify the role of RLIP76 as a reliable clinical predictor of outcome for breast cancer patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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