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Original Contribution

Prolactin receptor expression as a novel prognostic biomarker for triple negative breast cancer patients

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ABSTRACT

Prolactin receptor (PRLR) is a novel emerging prognostic biomarker in different cancers, especially in breast cancer. However, there is limited information about the association of PRLR expression and triple-negative breast cancers (TNBC) prognosis. In this study, 80 TNBC patients were evaluated for PRLR expression by immunohistochemistry. The correlation of PRLR expression with clinicopathological features, patient recurrence, and survival was investigated. PRLR expression was considered positive if >10% of tumor cells were stained. The Fisher's exact test was used to analyze PRLR expression relation with the clinicopathological parameters. Survival distribution was estimated by the Kaplan-Meier method. Positive immunoreactivity for PRLR was observed in 50 out of 80 (62%) specimens. Although expression of PRLR was associated with TNBC patients' stage, no-correlation was observed between its expression and tumor size, grade, lymph node status, and Ki-67 expression. In addition, patients with positive expression of PRLR exhibited lower recurrence ($P = 0.0027$) and higher overall survival ($P = 0.0285$) in comparison with negative expression group. In multivariate analyses, positive expression of PRLR was an independent prognostic marker for lower recurrence ($P < 0.001$) and higher overall survival ($P < 0.001$). Therefore, PRLR plays a crucial role in TNBC and has to be considered as an independent prognostic biomarker for TNBC patients.

1. Introduction

Breast cancer is the second leading cause of cancer-related deaths among women worldwide [1]. Recently, the overall breast cancer-related mortality has decreased due to early diagnosis and application of various treatments. One of the most determinative factors for selecting appropriate treatments is an adequate characterization of the breast tumor. Identifying tumors with poor prognosis can ensure adequate therapeutic approach selection and subsequently improves treatment efficacy.

The most malignant type of breast tumors is triple negative breast cancers (TNBCs) which are characterized by the lack of expression of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2. Approximately one-third of all breast cancers are TNBCs [2,3]. These high-risk group of breast cancers is associated with poor prognostic features including significantly higher nuclear grade, increased incidence of visceral metastases, and shorter recurrence-free interval in comparison with non-TNBC [4,5]. Reasons for this unfavorable prognosis include the heterogeneity and aggressive nature of the tumor and the absence of well-defined molecular targets that could form the basis for targeted therapy [6]. 20 to 30% of patients with TNBC achieve a pathological complete response to neoadjuvant chemotherapy and it is strongly associated with prolonged overall survival and event-free survival [7-9]. These observations have caused many efforts for molecular profiling and sub-classifying TNBC patients into different prognostic groups to find candidate patients for more aggressive therapeutic approaches. Recently, many biomarkers have been investigated by different studies for this purpose. One of the most controversial biomarkers is the prolactin receptor (PRLR). The endocrine hormone prolactin (PRL) is a growth factor required for the

Abbreviations: PRL, prolactin; PRLR, prolactin receptor; TNBC, triple negative breast cancer; PBS, Phosphate buffer solution; DAB, Diaminobanzidine; HRP, horseradish peroxidase

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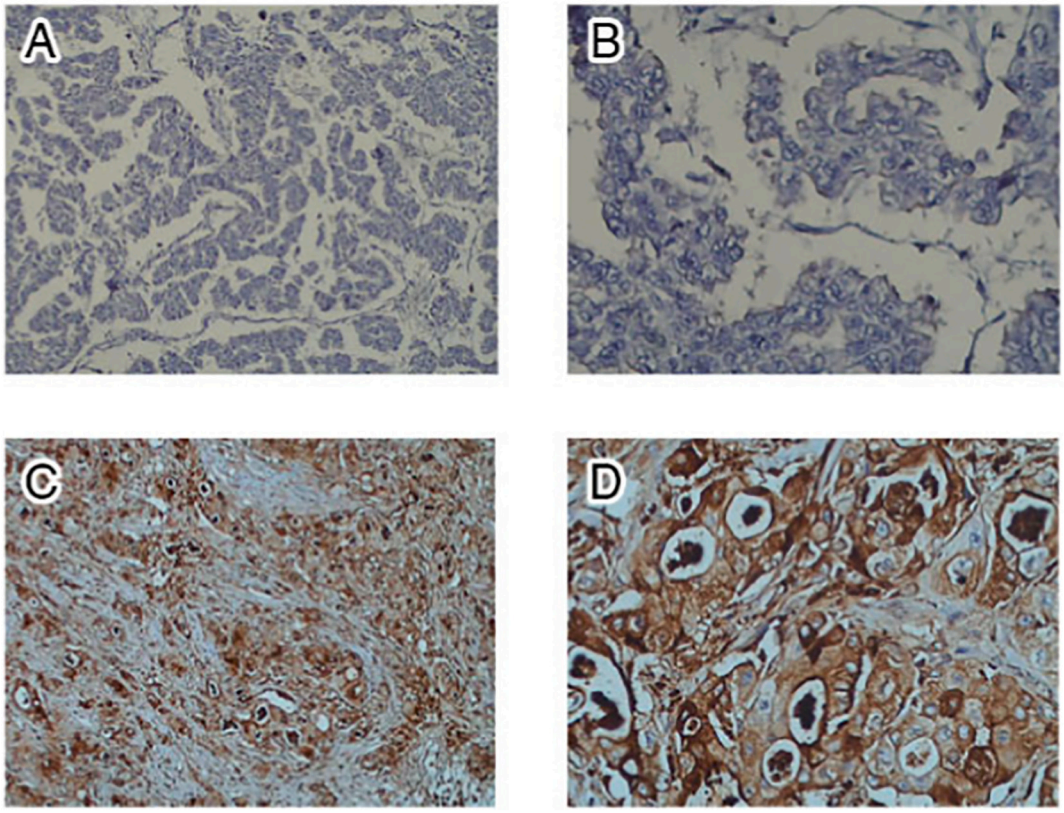

Fig. 1. PRLP expression in TNBC specimens at different magnifications (10× and 40×). A) Negative expression of PRLR (10×). B) Negative expression of PRLR (40×). C) Positive expression of PRLR (10×). D) Positive expression of PRLR (40×).

proliferation and terminal differentiation of the human breast through PRLR activation, a member of the growth factor receptor family [10,11]. PRL is necessary for the preservation and proliferation of ductal cells and activation of the necessary genes for lactation [12-16]. On the other hand, many studies have questioned the pro-oncogenic effect of PRLR and introduced the PRLR pathway as a tumor suppressor agent. Indeed, this pathway can suppress epithelial-mesenchymal-transition process and the invasive properties of breast cancer cells. Moreover, PRL and PRLR expression were decreased in the breast tumor tissues in comparison with normal tissue.

To the best of our knowledge, some studies have mentioned PRLR as a prognostic biomarker for breast cancer [17]. However, PRLR prognostic efficacy in TNBC patients is not potentially investigated. Here we examined PRLR expression in TNBC patients in relation to classic clinical and pathological parameters (tumor size, grade, stage, lymph nodes status, and Ki-67) to investigate the efficacy of PRLR expression as a prognostic biomarker.

2. Materials and methods

2.1. Patient selection

To evaluate the predictive and prognostic value of PRLR expression as a hormonal marker in TNBC patients, a larger cohort containing 80 TNBC specimens was studied using paraffin-embedded tumor tissue specimens archived at the several pathology centers in Isfahan province, Iran. This retrospective study was conducted in the Oncology Department and Histopathological Department of Isfahan University of Medical Sciences. All TNBC patient's primary tumors specimens from January 2013 to December 2017 were involved. The patients who received preoperative chemotherapy or diagnosed with stage IV of disease were excluded. All samples were reviewed by two board-certified pathologists separately and if there was any discrepancy between them or with clinical data, the sample was excluded from the study. We analyzed several clinical (age, menopausal status, type of surgery, adjuvant chemotherapy) and pathological (tumor size, grade, stage, lymph nodes status, and Ki-67) parameters. We used mouse monoclonal anti-prolactin receptor (B6.2 + PRLR742) (# ab199015, Abcam, USA), HRP Polymer, HRP Linker (DBS, USA) and DAB plus chromogen (Thermo Fisher Scientific, USA).

2.2. Immunohistochemistry

The specimens were fixed, paraffin-embedded and dissected into 3–5 mm sections. They were deparaffinized with 40 min incubation at 60 °C and subsequent immersion in xylene. Then, the rehydrated was done in the decreasing ethanol solutions and incubated in 0.3% hydrogen peroxide to inhibit activation of endogenous peroxidases. Subsequently, TNBC specimens' slides were washed with phosphate buffer saline (PBS; pH = 7.4) and heated in an 830 W microwave oven for at least 15 min in sodium citrate buffer (10 mM, pH 6.0) for antigen retrieval. The TNBC specimen slides were incubated with monoclonal anti-prolactin receptor overnight at 4 °C and for the negative control, the primary antibody was replaced with PBS. HRP Polymer and DAB plus chromogen were utilized for detection. Rabbit anti-mouse horseradish peroxidase-conjugated secondary antibody was incubated for 40 min at room temperature. The color was developed using DAB as a chromogen. Slides were extensively washed with PBS after each step.

2.3. Immunostaining scoring

Immunoreactivity was independently assessed by two board-certified pathologists, who were blinded to clinicopathological data, using a semiquantitative scoring system. Discrepancies were resolved by simultaneous re-examination on the slides by both investigators using a double-headed microscope. A semiquantitative method for PRL

receptor (PRLR) expression scoring was utilized. Membranous and/or granular cytoplasmic staining was considered positive, and immunoreactivity was semi-quantitatively categorized as follows: A score of 0 was used for undetectable PRLR expression, +1 for <10% of tumor cells, +2 for 10% to 50% of tumor cells, and +3 for >50% of tumor cells. The staining was considered positive only if there was membranous and/or granular cytoplasmic staining in malignant cells (Fig. 1). For analyzing the prognostic value of PRLR expression, we defined the 0 and +1 as the negative PRLR expression group and summarized tumors with 2+ and 3+ PRLR expression to a positive PRLR expression group [18].

2.4. Statistical analysis

All statistical analyses were performed using JMP version 11.0 software. The Fisher's exact test was used to analyze PRLR expression relation with each clinicopathological parameters. A P-value < 0.05 was considered significant. The overall survival of the patients was calculated using the Kaplan–Meier method and compared by the log-rank test. Then univariate factors with $P < 0.10$ were analyzed using a multivariate analysis to test independence.

3. Results

Eighty TNBC specimens were analyzed in this study. All specimens were female and their ages ranged from 27 to 88 years (Mean: 46 years, Median: 49 years). Thirty-six (45%) patients had post-menopausal status. Quadrantectomy and radiotherapy were used for the treatment of sixty-five (81%) of the patients, the others (n: 15, proportion: 19%) experienced radical mastectomy. Approximately all the patients (98.1%) received systemic adjuvant chemotherapy. Also, sixty-three (79%) of the patients had grade III tumors and tumors larger than 2 cm was observed in sixty-four (80%) of the patients. Twenty (25%) of the patients were diagnosed with stage III and forty-eight 60% of the patients were free of axillary lymph node involvement (Table 1).

3.1. Association of PRLR expression with clinicopathological parameters in TNBC patients

The patients were divided into two groups according to PRLR expression and the patients' characterizations are summarized in Table 2. Fifty (62%) patients exhibited positive immunostaining for PRL according to the utilized scoring and thirty (38%) patients were included in the negative group. The relation between PRLR expression and clinicopathological parameters was investigated. As illustrated in Table 2, patients with positive expression of PRLR had a lower recurrence rate than patients with negative expression ($P = 0.0027$). In addition, the positive expression of this receptor was inversely correlated with patients' death ($P = 0.0022$). No significant differences ($P > 0.05$) in the age of diagnosis, size of the tumor, nodal status, grade, and Ki-67 percentage were detected between these two groups (Table 2).

Table 1
Clinicopathological characteristics of the TNBC patients.

Clinicopathological parameters		Patient number (n = 80)	Proportion (%)
Age	≤55	55	69
	>55	25	31
Menopausal status	Pre-	44	55
	Post-	36	45
Type of surgery	Quadrantectomy	65	81
	Radical mastectomy	15	19
Tumor size	T1	16	20
	T2	56	70
	T3	7	9
	T4	1	1
Nodal status	N0	48	60
	N1	15	19
	N2–3	17	21
Grade	G1	0	0
	G2	17	21
	G3	63	79
Stage	I	14	17.5
	II	46	57.5
	III	20	25
Recurrence	No	70	87.5
	Yes	10	12.5
Death	No	72	90
	Yes	8	10

Table 2
Correlations between PRLR expression and clinicopathological parameters of the TNBC patients.

Clinicopathological parameters		PRLR immunoreactivity		P-value
		Positive No. of patients (%)	Negative No. of patients (%)	
Age	≤55 years	34 (62)	21 (38)	0.9908
	>55 years	16 (64)	9 (36)	
Tumor size	T1	9 (56)	7 (44)	0.5798
	T2–T4	41 (64)	23 (36)	
Nodal status	N0	34 (71)	14 (29)	0.1526
	N1–N2	16 (50)	16 (50)	
Grade	I–II	11 (65)	6 (35)	0.7758
	III	39 (62)	24 (38)	
Stage	I–II	39 (65)	21 (35)	0.0379
	III	11 (55)	9 (45)	
Ki-67%	≤30%	15 (68)	7 (32)	0.6107
	>30%	33 (59)	23 (41)	
Recurrence	Yes	3 (30)	7 (70)	0.0027
	No	47 (67)	23 (33)	
Death	Yes	2 (25)	6 (75)	0.0022
	No	48 (67)	24 (33)	

3.2. Predictive value of PRLR expression for recurrence and survival of TNBC patients

The efficacy of the PRLR expression as a predictive marker for TNBC patients' survival was investigated. The patients were followed-up for 18 months. 10 patients have developed recurrence and 8 of them died due to the breast cancer. In PRLR-positive patients, the recurrence rate was 6% (3/50), which was 23% (7/30) in PRLR-negative patients. During this period, the cancer-associated mortality rate in PRLR-positive patients was 4% (2/50), which was 30% (6/30) in the PRLR-negative patients. Therefore, the results exhibited significant ($P = 0.0285$) correlation of PRLR expression with the TNBC patients' overall survivals (Fig. 2). Among the investigated clinicopathological parameters, just tumors' stage exhibited significant association with PRLR expression. This fact is well known that prognosis is correlated with stage and according to our observations (Table 2), positive expression of PRLR was associated with low stage. Therefore, it may be possible that the association of PRLR expression with good prognosis is just due to the correlation of low stage and PRLR expression. Therefore, a multivariate analysis was performed to evaluate whether PRLR expression is an independent prognostic marker. In multivariate analyses, positive PRLR expression was independently associated with lower recurrence rate (odd ratio, OR: 2.44; $P < 0.001$) and higher over survival (hazard ratio, HR, 0.72; $P < 0.001$).

4. Discussion

PRLR expression has been detected in human breast cancer cell lines [19,20], breast tumor biopsies [21,22], and also in a variety of benign

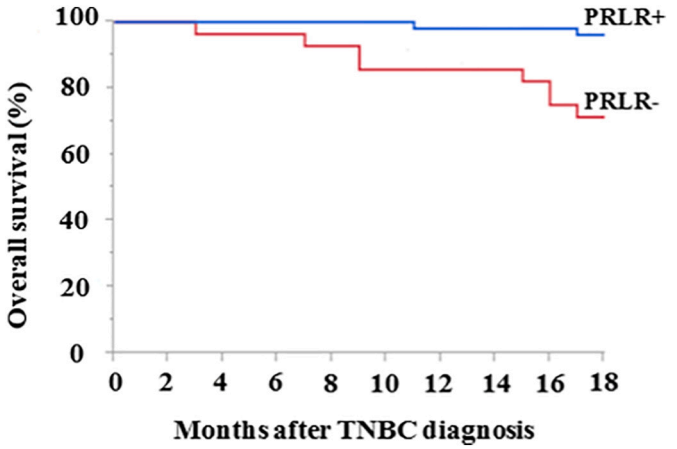

Fig. 2. The overall survival TNBC patients in the PRLR positive and negative groups. (Median follow-up: 18 months, Log-Rank = 0.0011, $P = 0.0285$).

breast lesions, including duct ectasia [23,24], fibrocystic change [25], and granulomatous mastitis [26]. Detection of PRLR by hormone binding or immunocytochemistry exhibited the presence of PRLR in 20–80% of the breast tumor samples [27,28]. The prognostic effect of PRLR expression is controversial according to different studies. Many studies have demonstrated PRLR activation can promote cancer cell proliferation, motility, survival, and angiogenesis [29-32]. PRL was known as a hormone with a significant effect on the pathogenesis and progression in preclinical studies [33]. In addition, PRL and PRLR were recently implicated in breast cancer metastatic spread, However, the efficacy of clinical trials on breast cancer patients for inhibition of pituitary secretion of PRL with pharmacological agents wasn't satisfying [34,35].

While many studies have highlighted a role for PRL in promoting tumorigenesis, other studies have identified PRLR as a potential suppressor of breast carcinogenesis. Therefore, the prognostic relevance of PRLR in breast carcinoma was investigated in animal models. Interestingly, it has exhibited an inhibitory influence on tumor development, depending on the time animals are exposed to elevated PRL levels [36]. Recent epidemiological data suggest that lactation in humans may exert a protective effect on breast cancer [37]. Indeed, they have previously shown that PRL, through PRLR/Jak2 signaling suppresses epithelial-mesenchymal-transition and reduces the invasive properties of breast cancer cells [38]. Furthermore, using both mammary epithelial cells and human breast cancer cells they showed that PRL blocks growth factor-induced mammary cell proliferation and viability of breast cancer cells [39]. More recently they also found that the expression of PRLR in human breast cancer is associated with favorable prognosis and better patient outcome [18,40]. In support of these findings, some studies have exhibited down-regulation of PRLR expression in breast cancer patients and breast cancer cell lines [41,42]. Moreover, expression/activation of the PRL effector molecule Stat5a was found to associate positively with increased levels of histologic differentiation of breast cancer tissues and to distinguish breast cancer patients with favorable prognosis and response to endocrine therapy [43]. loss of expression was also found to be associated with tumor progression and unfavorable clinical outcomes [44]. Together these findings provide compelling evidence regarding the role of PRL pathway in maintaining tissue differentiation and as a suppressor of breast carcinogenesis. This unexpected suppressive role of PRLR in breast cancer is still emerging and needs to be further elaborated.

TNBC tumor cells are thought to originate from a progenitor mammary stem cell population and loss of cellular differentiation is a common feature of TNBC tumors. Therefore, elucidating the role of mammary differentiation pathways like PRLR in TNBC biology might provide many helpful data. Many studies have announced the PRLR pathway as a differentiation pathway according to tissue microarrays and gene profiling databases. *In vitro* and *in vivo* evidence have indicated that restoration and activation of the PRL differentiation program in TNBC results in reversal of the highly proliferative, invasive, mesenchymal and tumorigenic phenotype through induction of cell differentiation [45,46]. Therefore, investigated the role of PRL differentiation pathway in the prognosis of TNBC as a poorly differentiated cancer may be helpful.

In this study, we compared PRLR expression status along with various clinical and pathologic parameters of TNBC patient. 62% of TNBC patients were positive for PRLR expression. Our results revealed that PRLR expression was significantly associated with malignancy stage ($P < 0.05$). Therefore, patients with negative expression of PRLR exhibited higher malignancy stages. In addition, a significant association was observed between TNBC patients' recurrence and overall survival with PRLR expression. According to our data, recurrence was significantly lower in PRLR-positive cases in comparison to PRLR-negative patients. In addition, patients with positive expression of PRLR exhibited better overall survival in comparison to the other group. But no significant relationship was observed between the expression of this receptor and other factors such as the age of the patients (0.9898), grade of malignancy (0.7778), lymph nodes (0.1526), Tumor size (0.5798) and Ki-67 expression (0.6107). All these findings support the role of the prolactin receptor as an independent indicator of TNBC and this could be a new pathway in the development of new treatments for TNBC.

5. Conclusion

PRLR is a novel emerging prognostic biomarker in breast cancer. However, there is limited information about the association of PRLR expression and TNBCs prognosis. Previous work described PRL and its receptor to play a permissive role in the development of mammary tumors and metastasis. However recent studies have not only questioned this role of PRL but highlighted that it can act as a suppressor of breast tumorigenesis. In this study, correlation analysis of PRLR expression by immunohistochemistry and clinicopathological characterizations of the patients exhibited a significant association between higher PRLR expression and patients' overall survival and recurrence in TNBC patients. Together, our results highlight PRLR as an independent indicator of better prognosis in TNBC breast cancer.

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