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ORIGINAL ARTICLE

Breast Cancer Invasion and Metastasis by mPRα Through the PI3K/Akt Signaling Pathway

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Abstract Invasive breast cancer is the most common type of malignancy in women worldwide. However, the mechanism responsible for breast cancer metastasis is still unclear and needs further illustration. It has been proven that matrix metallopeptidase 9 (MMP-9) promotes metastasis of the cancer cells. However, the interaction between mPRα and MMP-9 has not been studied. Therefore, in the present research, the effect of MMP-9 on the malignant progression of invasive breast cancer promoted by membrane progesterone receptorα (mPRα) was investigated. The results showed that the protein expression of mPRα, p-Akt and MMP-9 increased in the cancerous tissues compared to that of the noncancerous breast tissue. Furthermore, a positive correlation was found between mPRα and C-erbB-2, as well as the number of involved local lymph nodes. On the other hand, a negative correlation was observed between mPRα and estrogen receptors (ER) along with progesterone receptors (PR). Similarly, a positive association was found between MMP-9 and the number of involved local lymph nodes. Besides, the high expression of MMP-9 also had a positive correlation with the tumor size. However, the high level of MMP-9 had a negative correlation with ER and PR. In addition, there was a positive correlation between mPRα and p-Akt together with MMP-9. The results confirm that mPRα was a major marker of harmful prognosis and it promoted the expression of MMP-9 during invasion to the local lymph nodes through the pathway of PI3K/Akt. The present study provided a novel therapeutic strategy to inhibit breast cancer growth by preventing mPRα signaling pathway.

Keywords mPRα · p-Akt · MMP-9 · Invasive breast cancer · Metastasis

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Introduction

Worldwide, invasive carcinoma of breast is one of the chief causes of malignant tumor mortality in women. In spite of significant advances in chemical treatment of breast cancer, mortality arises due to local invasion and/or distant metastasis [1]. During metastasis, the tumor cells overcome the cell matrix adhesion to invade the surrounding circumstances or distant organs. The mechanism of tumor invasion and metastasis can be understood by the expression of MMP-9, which can be helpful for their targeted inhibition.

Generally, steroids such as estrogen and progesterone which can activate their nuclear steroid receptors via ligand-activated transcription factors are also helpful in regulation of cell proliferation and development of metastasis [2, 3]. Membrane progesterone receptor (mPR) is one such new potential effector, especially in PR-negative cancer cells.

Although three subtypes of mPRs, including mPRα, mPRβ and mPRγ, have been described in breast tissues and cell lines, mPRα is the predominant subtype in both malignant breast tissues as well as in breast cancer cell lines [4]. It has been reported that mPR agonists could activate the Akt signal pathway when incorporated with MAPK p42/44 in the

apoptotic signal pathway [5]. But the mechanism of mPRα in the development of cancer progression remains unclear. In this study, we tried to identify the expression of several markers, including mPRα, ER, PR, C-erbB-2, Ki-67 index, p-Akt and MMP-9, in order to assess the potential importance of mPRα in breast cancer development and growth by immunohistochemistry.

Patients

In total, 107 invasive breast cancer samples were studied at the Department of Pathology, Qilu Hospital of Shandong University from 2012 to 2014. The median age of the patients was 51 year-old. All the patients accepted the curative operation with axillary lymph node dissection including at least 15 lymph nodes samples. Twenty normal mammary gland tissues were used as controls.

Tissue Microarray

Core tissue samples (2 mm in diameter) were taken from each paraffin-embedded invasive breast carcinomas and the corresponding normal mammary tissues, which were arranged in a new tissue array block. Each tissue array block contained 21 cases and each case was in duplicate, allowing 13 array blocks to contain the total of 107cases. An adequate case was assured as the tumor occupied more than 10 % of the core area.

Immunohistochemistry

Immunohistochemical assays were performed on the tissue fixed with formaldehyde, blocked with 3 % H_2O_2 for 5 min at 20 °C and permeabilized with PBS-Tween; antigen retrieval was done by heat mediation in a citrate buffer (pH 6.0). The samples were incubated with a primary antibody to mPRα (ab75508, dilution 1:500, Abcam USA) and p-Akt (dilution 1:200, CST, USA) overnight at 4 °C or to ER (ZSGB-Bio, Beijing, China), PR (ZSGB-Bio, Beijing, China), C-erbB-2 (ZSGB-Bio, Beijing, China), and MMP-9 (Maixin. Bio., Fuzhou, China) for 2 h at 37 °C. Following washes with PBS-Tween, the slides were reacted with 2-step plus Poly-HRP Anti- Mouse/Rabbit IgG Detection System (PV-9000, ZSGB-Bio, Beijing, China) according to the manufacturer's recommendations. All the slides were visualized with DAB (PV-9000, ZSGB-Bio). When staining for mPRα, p-Akt and MMP-9 were done, the cancer cells with membranous and cytoplasmic dark brown or brownish staining were considered positive for expression of mPRα, while cytoplastic staining were judged positive for p-Akt and MMP-9. The nuclei staining were judged positive for ER and PR and were scored semi-quantitatively for intensity (0 = no expression; 1 = weak; 2 = moderate; 3 = strong) and for the percentage of positive cells (0 <10 %; 1 = 10–40 %; 2 = 40–70 %; and 4 ≥ 70 %). The sum of the staining-intensity and staining-extent scores exceeding 4 was regarded as positive expression. The sum of the proportion and average intensity scores of positive cancer cells were calculated on a scale ranging from 0 to 8. A cutoff point ≥ 4 was used to judge positive, while less than 4 was judged negative. C-erbB-2 was carried out according to the College of American Pathologists (CAP). A 3+ scoring system was considered to be over-expression [6]. The procedures were in accordance with the ethical standards of the responsible committee of the Shan Dong University on human experimentation. A normal breast epithelium was used as positive control. All the slides were reviewed and scored independently by two pathologists without any knowledge of the patient's clinical parameters.

Statistical Analysis

Data analysis was performed with SPSS software version 18.0. The correlations between the immunohistochemical expression and the clinical variables were evaluated by the *chi*--square test and the *Fisher's* exact test. *P*-value less than 0.05 was considered statistically significant.

Results

Correlation Between mPRα Expression and Clinicopathologic Factors

We aimed to validate the expression of mPRα with the clinic pathologic factors on human invasive breast cancer. We studied the expression of ER, PR and C-erbB-2 at the protein level and assessed the relation between mPRα and ER, PR, C-erbB-2, number of the involved lymph nodes, age of the patient, size of the tumor as well as the pathological grade of the breast cancer. As shown in Table 1, 54 samples expressed elevated levels of mPRα, only 14 showed positive ER, 9 showed positive PR and 19 of C-erbB-2 positive. In contrast, among the low level of mPRα expression, 28 showed negative ER, 32 were PR negative and 44 were C-erbB-2 negative. A statistical analysis showed a positive relativity between mPRα and C-erbB-2 (p = 0.032) as well as Ki-67 (p = 0.035), but a negative relevance between mPRα and ER (p = 0.025) along with PR (p = 0.008). In addition, the axillary node metastasis was divided into low levels (LN ≤ 3) and high levels (LN ≥ 4) [7]. A positive relationship was found between the over-expression of mPRα and the high levels of lymph node metastasis (p = 0.005). Furthermore, no correlation was identified between the levels of mPRα expression and the patients' age, the tumor size and the pathological grade Fig. 1.

Table 1 The relationship of membranous/cytoplasmic staining of mPRα with certain immunohistochemical and histopathological features in invasive breast carcinoma

		n	mPRα		*P-value*
			positive	negative	
ER	positive	38	14	24	0.025*
	negative	69	41	28	
PR	positive	30	9	21	0.008**
	negative	77	45	32	
C-erbB-2	positive	28	19	9	0.032*
	negative	79	35	44	
Ki-67	≥15 %	82	46	36	0.035*
	<15 %	25	8	17	
LN	≥4	36	25	11	0.005**
	≤3	71	29	42	
Age	≤35	6	3	3	
	35–50	42	21	21	1.000
	≥50	59	30	29	0.9331
Size	≤2 cm	25	9	16	0.098
	>2 cm	16	45	37	
Grade	I and II	72	40	32	0.1311
	III	35	14	21	

ER estrogen receptor, *PR* progesterone receptor, *C-erbB-2* human epidermal growth factor receptor 2, *LN* lymph node.

**P-value<0.05*; ***P-value<0.01*

Correlation Between MMP-9 Expression and Clinicopathologic Factors

Similar to mPRα, we analyzed the expression of MMP-9 with the clinic pathologic factors. Colorectal carcinoma was used as a positive control of MMP-9 [8]. As shown in Table 2, in the 59 samples over-expressing MMP-9, only 25 were ER positive and 21 PR positive. In contrast, among the low level of MMP-9 expression, 18 were ER negative and 20 PR negative. A statistical analysis showed a negative correlation between MMP-9 and ER ($p = 0.038$) along with PR ($p = 0.019$). In addition, a negative correlation was found between the expression of MMP-9 and the number of lymph nodes involved ($p = 0.011$). Furthermore, no correlation was identified between the level of MMP-9 expression and that of C-erbB-2 and Ki-67, as well as the patients' age, tumor size and pathological grade.

Correlation Between Expression of mPRα, p-Akt, and MMP-9 in Invasive Breast Cancer

Both mPRα and MMP-9 expression are absent or decreased remarkedly in the normal human breast tissue. From among the 107 patients, 54 were mPRα positive (50.48 %), 45 were p-Akt positive (42.06 %) and 59 were MMP-9 positive (55.14 %). As shown in Fig 2a, among the 54 samples of mPRα positive, p-Akt positive cases were 41. In the 53 samples of mPRα negative, p-Akt negative cases were 37. At the same time, showing in Fig 2b, among the 54 samples of mPRα positive cases, the number of MMP-9 positive cases was 43. Statistically, a positive correlation was observed between the expression of mPRα and p-Akt, as well as expression of mPRα and MMP-9 ($n = 107$, $p < 0.0001$, respectively).

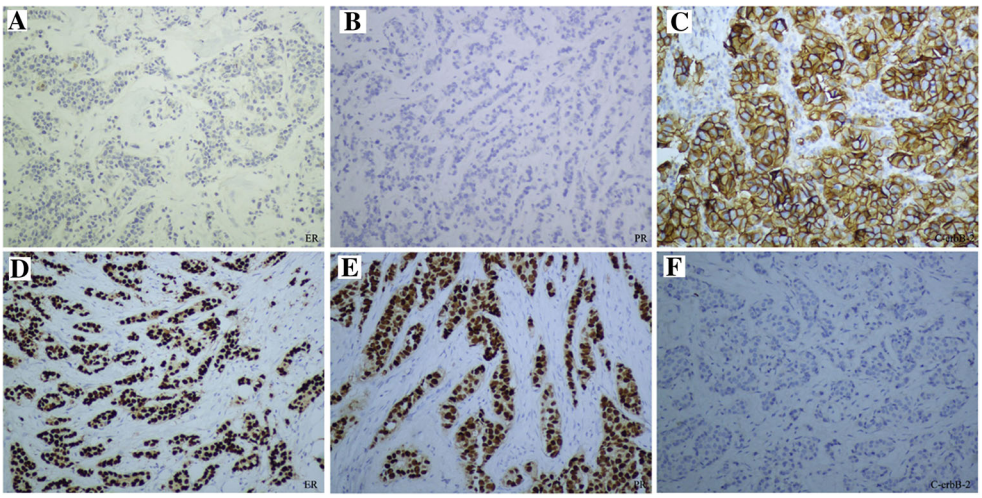

Fig. 1 Immunochemistry staining by anti-ER, anti-PR and anti-C-erbB-2 monoclonal antibody to identify the expression of ER, PR and C-erbB-2 in invasive breast cancer tissue, respectively. ER: estrogen receptor; PR: progesterone receptor; C-erbB-2: human epidermal growth factor receptor 2. ER and PR expression was demonstrated by brown-staining in the nuclear, while C-erbB-2 was shown as brown-staining in the cell membrane. The negative staining patterns are presented to enable a comparison. **a, b** representative image of negative staining for ER and PR, respectively (200×); **d, e** are nuclear staining that was positive for PR and ERα in control sections, respectively (200×); **c** membrane staining was positive for C-erbB-2 (200×); **f** is expression of C-erbB-2 in control section (200×)

Table 2 The relationship of cytoplasmic staining of MMP-9 with certain immunohistochemical and histopathological features in invasive breast carcinoma

		n	MMP-9		*P-value*
			positive	negative	
ER	positive	55	25	30	0.038*
	negative	52	34	18	
PR	positive	49	21	28	0.019*
	negative	58	38	20	
C-erbB-2	positive	28	19	9	0.115
	negative	79	40	39	
Ki-67	≥15 %	82	49	33	0.082
	<15 %	25	10	15	
LN	≥4	36	26	10	0.011*
	≤3	71	33	38	
Age	≤35	6	2	4	
	35–50	42	24	18	0.274
	≥50	59	33	26	0.904
Size	≤2 cm	25	8	17	0.008**
	>2 cm	82	51	31	
Grade	I and II	72	38	34	0.481
	III	35	21	14	

ER estrogen receptor, *PR* progesterone receptor, *C-erbB-2* human epidermal growth factor receptor 2, *LN* lymph node.

**P-value<0.05*; ***P-value<0.01*

Discussion

Although the micronized progesterone does not increase cell proliferation in the breast tissue in postmenopausal women, it is well established that a hormone replacement therapy with progesterone and estrogen is usually associated with an increasing risk of invasive breast cancer [9, 10]. Moreover, in menopausal women, the reproductive factors also suggest an involvement of endogenous progesterone in the carcinogenesis of the breast cancer [11]. But the exact mechanism is controversial.

The novel mPR is a potential mediator in the progress of breast cancer. It was first cloned in fish ovaries [12]. Three isoforms of mPR identified in humans were mPRα, mPRβ, and mPRγ [13].

The mPRs are seven transmembrane proteins located at the plasma membranes of cells and belong to the adipoQ receptor (PAQR) family [14, 15]. The over-expression of mPRs in both PR-positive and PR-negative breast cancer tissues suggest that the mPRs may be involved in the progress of breast cancer, both in the presence and absence of PR. Our results show that the level of mPRα is high in the C-erbB-2 over-expressed group, whereas, the level of mPRα is low in both ER and PR over expressed groups. In the present study, the mPRα exhibited a high level in the higher lymph node metastasis group. The most salient finding in this study was the association between high mPRα expression and the poor prognosis of breast cancer patients. But the mechanism is still not clear.

MMP-9 is a key component in the MMP family and plays a crucial role in the degradation of extracellular matrix (ECM), which plays an important role in the progress of tumorgenesis. The epithelial expression of MMP-9 is also significantly related to the prognosis of the invasive carcinoma of the breast, which has been reported to be associated with the degeneration of ECM resulting in invasion [16]. It was reported that there was a significant correlation between MMP-9 expression and lymph node metastases, the advanced tumor stage

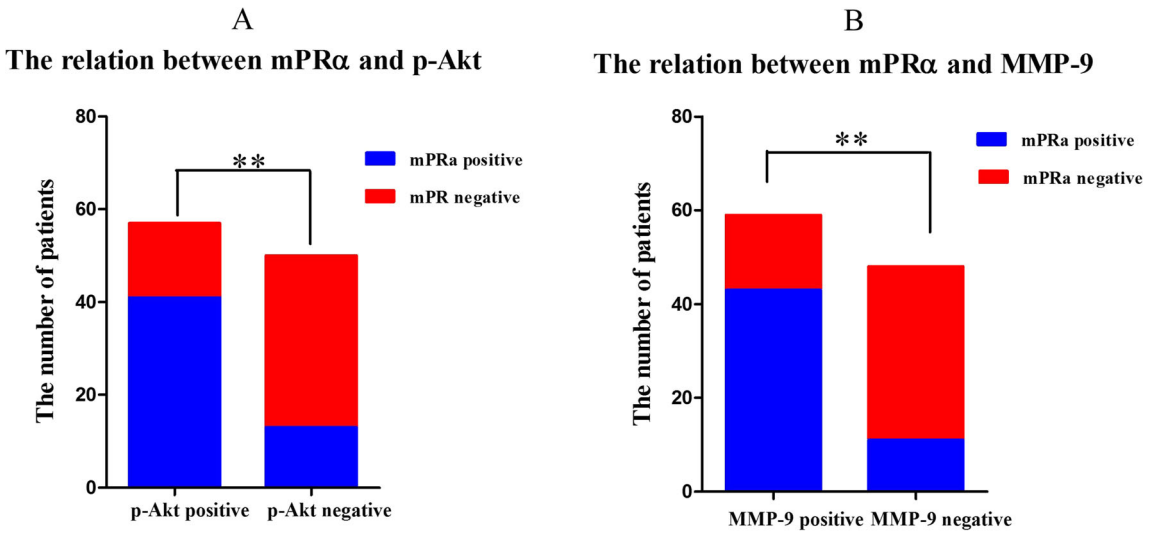

Fig. 2 **a** Correlation between mPRα and p-Akt. *Chi*-square test was performed to analyze correlation. The over-expression of mPRα is positively associated with the high levesl of p-Akt expression. ***P-value<0.001*. **b** Correlation between mPRα and MMP-9. *Chi*-square test was performed to analyze correlation. The over-expression of mPRα is positively associated with the high levels of MMP-9 expression. ***P-value<0.001*

as well as estrogen receptor negative and progesterone receptor negative hormonal status [17]. Herein, middle to strong cytoplastic labeling was observed in the breast carcinoma cells. Thus, we analyzed the effect of mPRα on the expression of MMP-9 and assessed the correlation between the two proteins using immunohistochemistry in invasive breast cancer. It was found that there was a positive correlation between the high levels of mPRα and MMP-9. These findings indicated that the mPRα could promote the expression of MMP-9, but the mechanism is not clear.

Many studies reveal that activation of PI3K/Akt signaling pathway can promote the malignant behavior of many kinds of cancers including breast carcinomas [18, 19]. Akt with its three reported isoforms including Akt1, Akt2 and Akt3, is the central member of this signaling pathway [20]. After phosphorylation by PDK1, Akt was activated as phospho-Akt (p-Akt) to stimulate downstream targets in the nucleus or cytoplasm. Previous study showed that in rat C6 glioma cells, antisense Akt2 constructs could reduce the secretion of MMP-2 and MMP-9 and inhibited the migration and invasion in vitro [21]. Herein, we found that the protein of p-Akt level had a positive correlation with that of mPRα and MMP-9. Our results showed that mPRα exhibited a stimulation on the expression of MMP-9 via the PI3K/Akt signaling pathway activation in breast cancer.

Taken together, the pattern of mPRα expression in invasive carcinomas of breast, as evidenced by immunohistochemistry, is highly heterogeneous. Our results demonstrated that both mPRα and MMP-9's over-expression serve as bad prognostic markers. The findings need to be verified by further studies. In addition, the migratory effect of mPRα on breast carcinoma cells led to efficient invasion when promoted by the secretion of MMP-9 from intrinsically highly invasive cells via PI3K/Akt signaling pathway in breast carcinoma cases. On the other hand, for invasive breast carcinoma patients with high levels of mPRα or p-Akt, the signaling pathway blockers may represent a potential therapeutic strategy. To the best of our knowledge, this is the first study to investigate the relationship between the immunohistochemical expression of mPRα and MMP-9 in breast cancer.

Authors' Contributions Zhishuang Li and ChunyanZhang carried out the tissue microarray. Limin Sun and Yan Wang carried out the immunohistochemistry. Xiaojuan Wu, Peng Su and Xiaowang participated in the design of the study and performed the statistical analysis. Rong Ma and Peng Gao conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards The procedures in this study were in accordance with the ethical standards of the responsible committee of the Shandong University on human experimentation.

Conflict of Interest The experiments abide by the current laws of China and there were no conflict of interest among the authors.

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