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Original Article

Significance of TLR4/MyD88 expression in breast cancer

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Abstract: Objective: To investigate the expression of TLR4/MyD88 in breast cancer, and explore the relationship between their expression and breast cancer tumor growth and invasion. Methods: We examined the protein expression of TLR4 and MyD88 in 60 cases of histologically confirmed breast cancer. The relationship of their protein expressions with clinical features including age at diagnosis, tumor size and stage, lymph node metastasis and distant metastasis were analyzed. Results: The IHC results showed that TLR4 and MyD88 were expressed in 63.3% (38/60) and 58.3% (35/60) of malignant breast tumors respectively. TLR4 expression in breast cancer were significantly higher than in fibroadenoma (n = 4, 20.0%) and adjacent normal tissues (n = 2, 10.0%) ($P < 0.001$). MyD88 expression in breast cancer were also significantly higher than in fibroadenoma (n = 4, 20.0%) and adjacent normal tissue (n = 3, 15.0%) ($P < 0.001$). The gene expressions of TLR4 and MyD88 were significantly higher in breast cancer than in fibroadenoma and adjacent normal tissues ($P < 0.05$). The protein expressions of TLR4 and MyD88 were also significantly associated with poor clinical features ($P < 0.05$). Conclusion: TLR4 and MyD88 expression might be associated with breast cancer growth and regional and distant metastases.

Keywords: Breast cancer, MyD88, TLR4

Introduction

Breast cancer is a heterogeneous malignant and life-threatening disease amongst females. Its incidence rate is increasing worldwide. According to the latest report of "GLOBOCAN 2008" by American Cancer Society [1], breast cancer is the most frequent cancer among women with an estimated 1.38 million new cases and 458,400 cases died of the disease. Most of breast cancer patients have prolonged disease-free survival and overall survival or even been cured after receiving systemic therapy. Nevertheless, there are still some patients died of the disease due to development of chemoresistance, lack of therapeutic targets such as estrogen and HER-2 receptors in tumors. It is therefore important to study pathogenesis and biologic behavior of the malignant disease to help develop new treatment strategy and subsequently prolong survival and improve quality of life of patients.

Toll-like receptor 4 (TLR4) and myeloid differentiation factor 88 (MyD88) specific binding plays an important biological function in pathogenesis by mediating tumor invasion and migration, escaping from immunosurveillence, promoting tumor proliferation, inhibiting apoptosis and developing chemoresistance in colorectal cancer [2], ovarian cancer [3], and prostate cancer [4]. In breast cancer, lipopolysaccharide acting on downstream signaling molecules TLR4 and MyD88 could regulate the growth rate of tumor cells by reducing the expression of TLR4 or MyD88 molecules [5]. Downregulation of MyD88 expression or formation of MyD88 homodimerization with inhibitory peptitde could effectively reduce lung metastasis in breast cancer mouse model as well as decreased CCL2 and CCL5 expression. Yang et al [6] found that the expression of TLR4 was the highest among other toll-like receptors TLR1-TRL10 in human breast cancer cell line MDA-MB-231 and a dramatic reduction of breast cancer cell viability and subsequently decreased IL-6 and IL-8 levels were observed after knockdown of TLR4 gene in the cell line. At present, the expression and significance of TLR4 and MyD88 in human breast cancer and its mechanism of

action are not widely studied. In this study, we aimed to explore the potential prognostic values of TLR4 and MyD88, and their involvement in malignant biological behavior in breast cancer.

Materials and methods

Patients and human tissues

A total 60 cases of histologically confirmed breast cancer with 20 cases of matched normal adjacent tissues and 20 cases of fibroadenoma were obtained from The First Affiliated Hospital of Fujian Medical University, China between January 2008 and September 2012. Female patients diagnosed primary breast cancer at AJCC stage I to III and underwent surgical resection of tumor were selected. Patients who were given neoadjuvant therapy and proven distant metastasis at presentation were excluded. The study was approved by local ethics committee and informed consents were obtained for collection of tissue samples.

Immunohistochemistry

An immunohistochemical streptavidin-peroxidase (SP) method was used in this study. Mouse anti-human monoclonal antibodies TLR4 and MyD88 were purchased from Zhongshan Jinqiao Biotechnology Co., Ltd. (Beijing, China). All specimens were routinely fixed with 10% formalin and embedded in paraffin, and sectioned at a thickness of 2.5 μm. The slides were incubated at 65°C overnight and the tissue sections were deparaffinized and hydrated. The antigens were recovered by natural cooling to room temperature. After rinsed with PBS, the specimen was treated with 3% hydrogen peroxide and incubated at room temperature for 10 mins. After rinsing samples with PBS for three times three minutes each, the slides were incubated for 1 hour with primary antibodies (anti-TLR4 at 1:300 dilution and anti-MyD88 at 1:200 dilution). The slides were then rinsed with PBS for three times three minutes each followed by incubation with primary reagent for another 30 minutes. Further rinsed with PBS for three times three minutes each, the slides were incubated with secondary reagent for another 20 minutes. The DAB staining agent was added to samples stained for 3 minutes after rinsed with PBS for three times three minutes each and the sections were counterstained with hematoxylin, dehydrated, transparent and mounted for storage. Known TLR4-positive sample obtained from mouse spleen and MyD88-positive sample obtained from papillary carcinoma of lung were used as positive control and the use of PBS without primary antibody was used as negative control.

RNA preparation and RT-PCR

RNA was isolated using Trizol (Beijing ComWin Biotech Co., Ltd., China) as per the instruction. The purity and concentration of the total extracted RNA was determined using UV spectrophotometer (Nano Photometer, Germany) and the absorbance ratio of each sample at OD 260/280 was between 1.8 and 2.0. We applied Prime Premier 5.0 software to design the sequences of primers for TLR4, MyD88 and actin as internal control. The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd as follow: TLR4, 5'-GACCTGTCCCTGAACCCTATGA-3' (upstream), 5'-CTTCTAAACCAGCCAGACCTTGA-3' (downstream), amplified fragment size of 139 bp; MyD88, 5'-CGGTCTCCTCCACATCCTCCCTTCC-3' (upstream), 5'-CTGCCAGTGGGGTCCGCTTGTGTCT-3' (downstream), amplified fragment size of 181 bp; actin, 5'-ACTTAGTTGCGTTACACCCTT-3' (upstream), 5'-GTCACCTTCACCGTTCCA-3' (downstream), amplified fragment size of 156 bp. The following conditions were adopted for the PCR amplification: predenaturation for 3 minutes at 94°C, denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C and extension for 1 minute at 72°C. These steps were repeated for 35 cycles followed by a step of extension for 5 minutes at 72°C. The PCR products were separated by 1.5% agarose gel eletrophoresis, stained with GoldView nucleic acid dye and visualized by a gel HR camera (EC3 300, USA). The experiment was repeated for three times.

Immunohistochemistry analysis

Double immunostaining was used to determine the positive expression of TLR4 and MyD88. TLR4 and MyD88 protein expressions were located in the cancer cells or normal epithelial cells in the cytoplasm, where TLR4 protein expression was also observed occasionally in the nucleus. Immunostaining scoring was performed based on the number of positively stained cells and the immunostatining intensity after tissue sectioning and immunostaining.

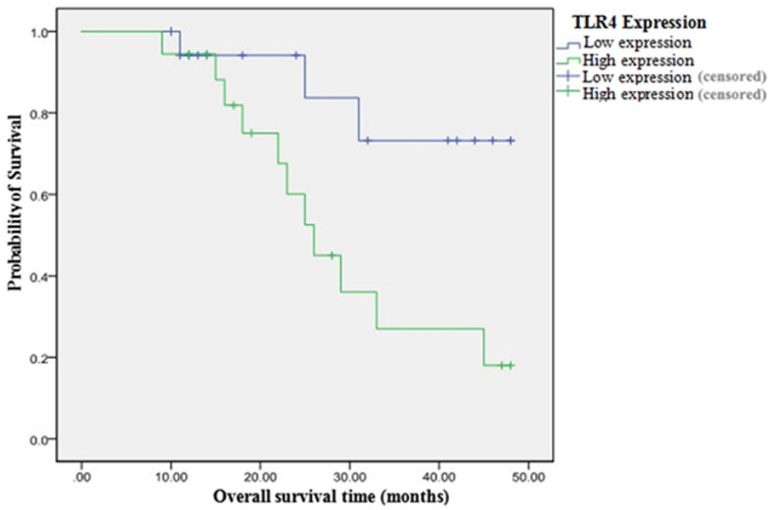

Figure 1. Overall survival of 60 breast cancer patients according to TLR4 expression.

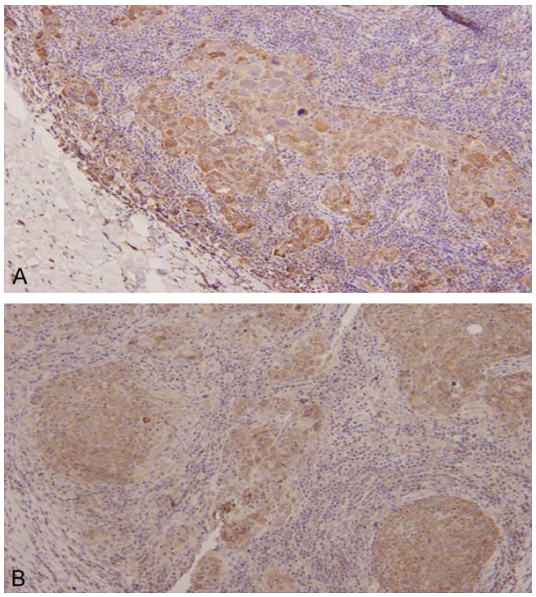

Figure 2. Examples of Positive Immunostaining for TLR4 and MyD88 in breast cancer. A. TLR4 positive staining for tumor cell, 200×; B. MyD88 positive staining for tumor cell, 200×.

The number of positively stained cells was evaluated using a numeric score ranging from 0 to 4, representing the percentage of positively stained cells as follows: 0, less than 5%; 1, 5% to less than 25%; 2, 25% to less than 50%; 3, 50% to less than 75%; 4, greater than 75%. The immunostaining intensity was evaluated using a numeric score ranging from 0 to 3, reflecting the intensity as follows: 0, no staining; 1, weak staining (light yellow); 2, moderate staining (yellow); and 3, intense staining (brownish yellow). The total score was obtained by multiplying the score of percentage of positively stained cells and the intensity score. The total score of less than 6 represented low level expression and that of greater or equal to 6 represented high level of expression.

Statistical analysis

All clinicopathological data, treatment history of individual patients, and results of survival follow-up were compiled into a statistical database analyzed by IBM SPSS statistics 19.0 (Chinese version). Chi-square test and Fisher's exact test were used to compare the differences in expression of TLR4 and MyD88 and explore the relationship between their expressions and clinicopathological features of breast cancer. *P*-values of less than 0.05 were considered statistically significant.

Results

Clinicopathological characteristics

Of 60 female primary breast cancer patients aged 31-86 years (mean = 51.3 years, median = 50 years), all were diagnosed stage I to III without distant metastasis at presentation. All patients underwent surgery including radical mastectomy (n = 3) and modified radical mastectomy (n = 57) followed by adjuvant therapy. Ninety-five percent of patients did not have family history of malignant disease and 35 (58.3%) patients were premenopausal whereas 25 (41.7%) patients were postmenopausal. Primary tumor sizes classified as T1, T2, T3 and T4 were observed in 31 (26.7%), 66 (56.9%), 16 (13.8%) and 3 (2.6%) patients respectively. According to the AJCC TNM staging for breast cancer (version 7) [7], 41 (68.3%) and 19 (31.7%) patients were diagnosed stage I/II and stage III diseases respectively. Histological grading of tumor was based on Chinese Breast Cancer Diagnostic and Treatment Practices (2011 edition) [8] and 32 (53.3%) and 28 (46.7%) cases were classified as histological grade I/II and grade III respectively.

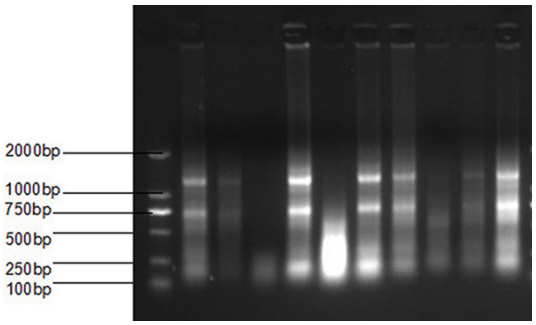

Figure 3. Agarose gel electrophoresis of total RNA extracted from breast cancer, adjacent normal tissue and fibroadenoma. M: DNA Marker: DM2000, 100, 250, 500, 750, 1000 and 2000 bp from the bottom with the brightest band equal to 750 bp. Lanes 1-3: adjacent normal tissue; Lanes 4-7: breast cancer tissue; Lanes 8-10: fibroadenoma.

Survival follow-up

All patients were followed up after surgery until December 2012 with a median follow-up duration of 21 months. No patient defaulted follow-up and the survival data was retrieved at last follow-up visit. Of 60 breast cancer patients, 7 patients had distant metastasis among which lung metastasis was observed in 5 cases and bone metastasis was observed in 2 cases. One patient died of tumor progression. Survival analysis stratified according to the expression of TLR4 has shown that patients with low TLR4 expression have longer survival than that with high TLR expression in tumors (**Figure 1**).

Expression of TLR4 and MyD88 in breast cancer, adjacent normal tissue and fibroadenoma

Protein expression of TLR4 detected by IHC: Positive IHC staining of TLR4 was mainly located at cytoplasm and some at the nuclei showing brownish yellow granules (**Figure 2A**). TLR4 expression was observed in 38 (63.3%), 2 (10.0%) and 4 (20.0%) cases of breast cancer, adjacent normal tissue and fibroadenoma respectively. The expression of TLR4 in malignant tumor was significantly higher than adjacent normal tissues and benign tumor ($P < 0.001$) (**Table 1**).

Protein expression of MyD88 detected by IHC: Positive IHC staining of MyD88 was also located at cytoplasm showing brownish yellow granules (**Figure 2B**). MyD88 expression was observed in 35 (58.3%), 3 (15.0%) and 4 (20.0%) cases of breast cancer, adjacent normal tissue and fibroadenoma respectively. Its expression was also significantly higher in malignant tumor than adjacent normal tissues and benign tumor ($P < 0.001$) (**Table 1**).

Gene expression of TLR4 and MyD88 detected by RT-PCR: The expression of TLR4 and MyD88 genes were comparatively stronger in breast cancer tissues than adjacent normal tissues and benign tumor (**Figure 3**).

Association between TLR4 and MyD88 protein expressions and clinical parameters in breast cancer: High expression of TLR4 was significantly associated with axillary lymph node metastasis ($P = 0.006$), tumor size ($P = 0.017$), tumor staging ($P = 0.013$) and distant metastasis ($P = 0.038$), and high expression of MyD88 was also significantly associated with tumor size ($P = 0.009$), tumor staging ($P = 0.03$), axillary lymph node metastasis ($P = 0.006$) and distant metastasis ($P = 0.004$) (**Table 2**).

Discussion

Toll-like receptors (TLRs) belong to a class of innate immune receptors commonly found in mammals and form a family of TLRs because of their similar homology. TLRs have emerged as a family of pattern recognition receptors (PRRs) able to recognize a variety of pathogen-associated molecular patterns and interact with other families of PRRs which triggers a series of signal transduction leading to the release of inflammatory mediators as part of important innate immunity and ultimately activation of the acquired immune system. To date, there are at least 12 different TLRs which possess different ligands and among which TLR4 is the major receptor mediated by lipopolysaccharide [9].

TLR-4 was first discovered by Medzhitov *et al* [10] on extracellular domain of human cells which were mostly immune cells to identify pathogens, endogenous ligands and drug and involve in innate and adaptive immune responses. In recent years, TLR4 expression was also observed in different human tumors. The first identified effector actively involved in TLR4 activation and subsequent signal transduction was TIR-containing adaptor MyD88.

Our study demonstrated that TLR4 and MyD88 signaling factors were highly active and strongly expressed in breast cancer as seen with high

Table 1. Expression of TLR4 and MyD88 in breast cancer, adjacent normal tissue and fibroadenoma

Group	No. of cases	TLR4		χ^2	*P* value	MyD88		χ^2	*P* value
		Positive	Negative			Positive	Negative		
Breast cancer	60	28	32	89.62	< 0.001	25	35	84.71	< 0.001
Adjacent normal tissue	20	2	18			3	17		
Fibroadenoma	20	4	16			4	16		

Table 2. Expression of TLR4 and MyD88 in breast cancer and its relationship with clinicopathological characteristics

Feature		Total case	TLR4		χ^2	*P* value	Total case	MyD88		χ^2	*P* value
			-	+				-	+		
Age	≤ 50	37	11	12	0.455[b]	0.341	37	22	15	0.050[b]	1.000
	> 50	23	21	16			23	13	10		
Menopausal state	Pre-menopausal	35	18	17	0.122[b]	0.796	35	20	15	0.049[b]	1.000
	Postmenopausal	25	14	11			25	15	10		
Family history of malignancy	Yes	3	1	2	0.508[b]	0.594	3	1	2	0.812[a]	0.565
	No	57	31	26			57	34	23		
Tumor size	T1	18	8	10	9.102[a]	0.017	18	11	9	10.63[a]	0.009
	T2	32	22	10			32	24	8		
	T3	8	1	7			8	2	6		
	T4	2	1	1			2	0	2		
Axilla lymph node status	Yes	37	16	21	12.75[a]	0.001	37	17	20	6.094[a]	0.017
	No	23	16	7			23	18	5		
The number of lymph node metastasis	1-3	15	5	10	1.722[a]	0.445	13	5	8	0.768[a]	0.802
	4-9	18	10	8			21	11	10		
	≥ 10	5	2	3			4	2	2		
Tumor stage	I/II	41	27	14	8.155.[b]	0.006	32	21	12	0.634[b]	0.447
	III	19	5	14			28	15	13		
Histological grade	I/II	23	11	12	0.455[b]	0.341	27	17	10	0.433[a]	0.602
	III	37	21	16			33	18	15		
Distance Metastasis	Yes	7	2	5	4.926[a]	0.037	7	1	6	6.326[b]	0.017
	No	53	30	23			53	34	19		
	Lung	5	2	3	0.058[a]	1.000	5	1	4	3.733[a]	0.143
	Bone	2	1	1			2	2	0		

[a]Fisher's test; [b]χ^2 (Chi-square) test.

protein and gene expressions. However, their expressions were both lower in adjacent normal tissues and benign breast tumors. Despite the unclear causal relationship between the expression of TLR4 and MyD88 and the tumor progression at this stage, we at least recognized a strong association between the signals and tumor growth. In our study, high expression of TLR4 was found in patients with positive axillary lymph node metastasis, which is similar to a study by Ehsan *et al* [11] that high tumoral expression of TLR4 was significantly associated with local metastasis. The possible prognostic indication was also observed with poor survival in patients with high expression of TLR4 expression. However, its correlation with survival was not observed in another study by Petricevic *et al* [12]. Our study provided us with preliminary results on the possible involvement of TLR4 and MyD88 signaling factors in pathogenesis of breast cancer. The upstream signaling factors might activate the downstream signaling factors via TLR4 and MyD88 to activate other signaling pathways. However, further investigation is required to explore the role of these signaling factors in tumor formation, development and progression.

Conclusion

It is now more evident that TLR4 and MyD88 are one of the major signaling factors involved

in carcinogenesis and pathogenesis of various types of cancer as well as breast cancers. TLR4 and MyD88 expressions might be associated with breast tumor growth and progression. Further investigation on their involvement in breast cancer pathogenesis is warranted.

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Disclosure of conflict of interest

None.

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