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# Association of Toll like receptor 9 expression with lymph node metastasis in human breast cancer

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The toll like receptor 9(TLR9) has been suggested to play an important role in the invasion and metastasis of cancer cells in vitro. However, the expression of TLR9 in human cancer specimens has never been characterized. In this study, we investigated the expression of TLR9 in breast cancer specimens and determined the association between its expression and the clinicopathological features observed. We found that TLR9 expression was significantly higher in patients with breast cancers displaying large tumor size (P = 0.040), lymph node metastasis (P <0.001) or advanced pathological stage (P = 0.006). TLR9 expression was also increased in ER negative breast cancer specimens(P=0.043). Logistic regression analysis revealed that pathological stage(P = 0.007) and TLR9 (P = 0.001) expression were independent factors that influenced axillary lymph node metastasis of breast cancer. Patients with positive TLR9 expression had a higher progress free survival compared with the TLR9-negative cohort(P=0.037). Therefore we concluded that Lymph node metastasis more likely occur in breast cancer patients with a postive TLR9 status and its expression might serve as an indicator of poor prognosis in patients with breast cancer.

*Key words: breast cancer, TLR9, lymph node metastasis*

Tumor cell invasion and subsequent dissemination via lymph vessels are the most important steps in the progression of breast cancer [1]. Over the past years, accumulating evidence has demonstrated novel roles for chemokines and their receptors in regulating biology of the malignant tumors, including metastasis [2-4]. Determination of such a predictive molecular marker that is correlated with lymph node metastasis might be important in elucidating the mechanism of metastasis and aid in the personalized theraphy of breast cancer.

Toll-like receptors (TLRs) are evolutionarily well-conserved transmembrane proteins that are present in almost all multicellular organisms and recognize patterns specific for microbial components [5]. Different immune cells express distinct subsets of the TLRs, which likely enables the immune system to tailor its responses against different pathogens. Among resting human immune cells, TLR9 is almost exclusively expressed in B cells and plasmacytoid dendritic cells (pDC). TLR9 can detect invading viral nucleic acids, while avoiding accidental stimulation by CpG motifs within self DNA by specifically recognizing unmethylated CpG oligonucleotides in vertebrates [6]. TLR9 is localized at endoplasmic reticulum, from where it is translocated to the endosomal/lysosomal compartment for ligand recognition [7]. On ligand binding, TLRs and their associated adaptors, such as MyD88 and TRIF, recruit intracellular signaling mediators that activate transcription factors, such as nuclear factor κB(NF-κB). The outcome of TLR activation is an immune reaction characterized by increased production of various proinflammatory cytokines and interleukins [8].

Several previous studies have determined that TLR9 is highly expressed in breast cancer cells and capable of promoting cellular invasion in vitro by increasing matrix metalloproteinase activity [9-11]. However, their expression in a large number of clinical samples of human breast cancer has never been characterized. Therefore, we investigated the expression of TLR9 in breast cancer specimens and the association between TLR9 expression and the clinicopathological features of breast cancer.

## Patients and methods

In this study, we examined 124 patients with stage I-IIIc breast cancers. All those patients received surgery, chemotherapy or radiotherapy in the third people's hospital of Hangzhou during the past five years. Informed consent was obtained from

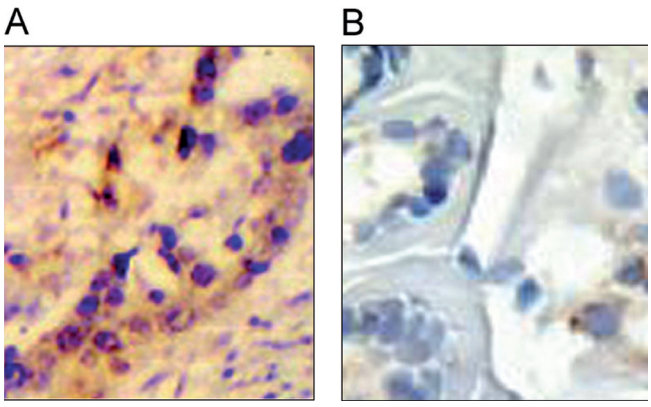


**Fig 1. Representative immunohistochemical staining of TLR9 in breast cancer specimens A, Positive TLR9 staining in an invasive breast cancer specimen. B, Negative TLR9 staining in an invasive breast cancer specimen. Original magnification×400.**

each patient before sample collection. Tumor specimens were obtained at the time of surgery and were immediately fixed in 10% neutral-buffered formalin and embedded in paraffin.

**Table 1. Association between TLR9 expression and clinicopathological factors in breast cancer.**

| Variable | N. | TLR9 expression | | P value[a] |
|---|---|---|---|---|
| | | Positive | Negative | |
| Age(years) | | | | |
| ≤45 | 38 | 27 | 11 | 0.212 |
| >45 | 86 | 51 | 35 | |
| Menses status | | | | |
| Premenopause | 53 | 37 | 16 | 0.169 |
| Postmenopause | 71 | 41 | 30 | |
| Tumor size(cm) | | | | |
| ≤2.0cm | 83 | 47 | 36 | 0.040 |
| >2.0cm | 41 | 31 | 10 | |
| Lymph node metastasis | | | | |
| Negative | 62 | 23 | 39 | <0.001 |
| Positive | 62 | 55 | 7 | |
| 1-3 | 40 | 34 | 6 | 0.213 |
| ≥4 | 22 | 21 | 1 | |
| Histological grade | | | | |
| G1 | 26 | 13 | 13 | 0.126 |
| G2,3 | 98 | 65 | 33 | |
| Pathological stage | | | | |
| Stage I,II | 82 | 48 | 34 | 0.006 |
| Stage III | 42 | 30 | 12 | |
| ER status | | | | |
| Negative | 55 | 40 | 15 | 0.043 |
| Positive | 69 | 38 | 31 | |
| Her-2 status | | | | |
| Negative | 69 | 43 | 26 | 0.880 |
| Positive | 55 | 35 | 20 | |

α, Chi-square test.

ER and Her-2 were considered positive if more than 10% tumor cells were stained. Pathological stage was evaluated after primary surgery according to TNM staging system of AJCC. Histopathological grade adopted the Elston-Ellis system.

Histological cell typing was conducted according to the World Health Organization classifications and the result was: 104 were classified as invasive ductal carcinomas, 16 as infiltrative lobular carcinoma, and 4 as infiltrative papillary carcinomas. The median age at the time of surgery was 46 years (range 27–83 years).

**Immunohistochemistry and staining evaluation.** Sections of 4μm thickness were obtained from several representative areas of each tumor specimen and were mounted on to glass slides for immunostaining according to the labeled streptavidin biotin procedure of the Dako LSAB kit (Dako North American Inc., Carpinteria, CA). Briefly, after the slides were dewaxed in xylene and rehydrated in an alcohol series, antigen retrieval was carried out in a microwave oven in 10 mM citric acid buffer (pH 6.0) for 3×10 min. The sections were then incubated with 0.3% hydrogen peroxide to block endogenous peroxidase activity, followed by incubation with normal horse serum for 5 min at room temperature. Immunostaining was then carried out by incubation with anti-TLR9 rabbit antibody (Cat.2254, cell signaling) at a final dilution of 1:100 for 2 h at room temperature. The sections were subsequently incubated for 20 min with biotinylated anti-rabbit immunoglobulin, followed by incubation with peroxidase-conjugated streptavidin for 20 min and 0.05% 3,39-diaminobenzidine tetrahydrochloride solution (Wako Pure Chemical Industries Ltd., Osaka, Japan) containing hydrogen peroxide for 10 min. Finally, the slides were counterstained with Mayer's hematoxylin and mounted in an aqueous mounting medium. At each step, the slides were washed carefully in phosphate-buffered saline (pH= 7.4).The specimen was considered as positive if the distinct cytoplasmic staining was observed in >10% cancer cells. Microscopic analyses were conducted independently by two of the authors who had no prior knowledge of the clinical data. The final evaluations of a few ambiguous cases were carried out using a conference microscope.

**Statistical analyses.** The association between the variables was tested using the chi-square test or a stepwise logistic regression analysis. Kaplan-Meier survival analysis was used to study the effect of TLR9 status on clinical outcomes of patients. All these analyses were carried out using the SPSS17.0 software. When P<0.05, the difference was considered statistically significant.

## Results

**TLR9 expression in human breast cancer tissues.** Of the 124 tumor samples examined, 78 (63%) were positively stained and 46 (37%) were negatively stained. The representative Immunohistochemistry results of positive and negative TLR9 staining were shown in Fig 1. The association between TLR9 expression and clinicopathological factors was then analyzed (Table 1). We found that TLR9 expression was significantly higher in tumors of larger size (P=0.040), positive axillary lymph node metastasis (P=0.006) and advanced pathological stage (P=0.001). Interestingly, we also noted that ER negative

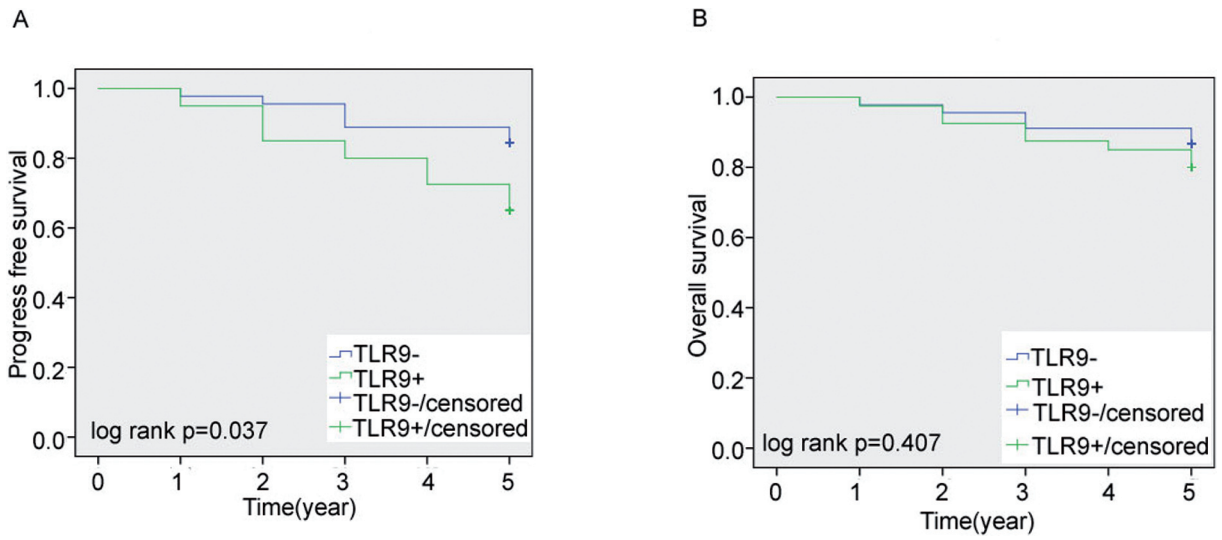


**Fig 2. Kaplan-Meier survival curve for the clinical outcome of breast cancer patients correlated with TLR9 status.**
**A, the survival curve for the association of PFS and TLR9 status. B, the survival curve for the association of OS and TLR9 status.**

tumor tissues express higher TLR9 (P=0.043). No significant association was observed between TLR9 immunoreactivity and age, menses status, histological grade or Her-2 status.

**Risk factors of lymph node metastasis in breast cancer.** The risk factors of lymph node metastasis were determined by stepwise logistic regression analysis. We found that the lymph node metastasis of breast tumors was significantly correlated with tumor size, histological grade, pathological stage, ER status and TLR9 expression in the univariate analysis(Table 2). After adjusting for factors including age, tumor size, histological grade, pathological stage, ER status, Her-2 status and TLR9 expression with multivariate analysis, we observed that only pathological stage(P=0.007) and TLR9 (P=0.001) were two independent risk factors which influenced axillary lymph node metastasis of breast cancer (Table 3).

**Univariate survival analyses.** We also examined the effect of TLR9 on the clinical outcome of breast cancer patients. From the survival curves of 124 patients with breast cancer on the basis of the TLR9 expression status, both the PFS and OS rates of patients exhibiting TLR9 expression were lower than those of patients lacking TLR9 expression, but only the difference of PFS was statistically significant (Fig 2).

**Table 2. Univariate analysis for risk factors of lymph node metastasis in breast cancer**

| Variables | Univariate analysis | | |
|---|---|---|---|
| | Odds ratio | 95% confidence interval | P value |
| Age (years) | | | |
| ≤45 *vs* >45 | 0.594 | 0.261-1.351 | 0.214 |
| Tumor size(cm) | | | |
| ≤2.0 *vs* >2.0 | 2.374 | 1.031-5.470 | 0.042 |
| Histological grade | | | |
| G1 *vs* G2, 3 | 2.842 | 1.339-6.031 | 0.002 |
| Pathological stage | | | |
| Stage I,II *vs* Stage III[a] | 5.125 | 1.822-14.419 | <0.001 |
| ER status | | | |
| Negative *vs* positive | 0.760 | 0.215-0.983 | 0.045 |
| Her-2 status | | | |
| Negative *vs* positive | 2.058 | 1.508-4.205 | 0.006 |
| TLR9 expression | | | |
| Negative *vs* positive | 7.480 | 2.530-19.440 | <0.001 |

a, the stage III breast cancer of T0-4N2-3M0.

## Discussion

Infection and the resulting inflammation are important regulators of tumor development [12]. For example, chemokines and cytokines derived from the immune and inflammatory cells can dramatically affect cancer cell behavior by shaping the host microenvironment. Since TLR9 was recognized as the receptor for unmethylated CpG DNA of most bacteria and DNA viruses, many attention has been given to explore its anti-tumor effect. However, there were also some reports concerning the direct effect of TLR9 ligation on tumor behavior such as invasion and metastasis [5, 10, 13-15]. Our study

**Table 3. Multivariate analysis for risk factors of lymph node metastasis in breast cancer**

| Variables | Multivariate analysis | | |
|---|---|---|---|
| | Odds ratio 95% | confidence interval | P value |
| Pathological stage | | | |
| Stage I,II *vs* Stage III[a] | 3.120 | 1.220-9.450 | 0.007 |
| TLR9 expression | | | |
| Positive *vs* negative | 5.870 | 2.120-16.340 | 0.001 |

a, the stage III breast cancer of $T_{0\text{-}4}N_{2\text{-}3}M_{0.}$

is the first to investigate the expression of TLR9 in large breast cancer specimens and correlate TLR9 with clinicopathological features of breast cancer. We demonstrated that TLR9 expression was significantly higher in breast tumors of larger size, positive lymph node metastasis and advanced pathological stage, while TLR9 expression seems not associated with patients' age, menses status and Her-2 staining. The stepwise logistic regression revealed that TLR9 expression as well as advanced pathological stage was significantly correlated with the trend of breast cancer to metastasis. These data suggests that TLR9 may induce the invasion and metastasis of breast cancer as shown in some previouse in vitro studies, which may involve an up-regulation of matrix metalloproteinase-13 activity [10, 13,14].

Interestingly, we also found that TLR9 expression was significantly higher in ER negative breast cancer compared with ER positive one in this study, which is consistent with the report by Jukkola-Vuorinen [16]. Given that TLR9 ligation could inhibit ERα-induced transactivation by activating NF-κB of breast cancer cells [11], we speculate that TLR9 may play a role in the transformation of breast cancer from hormone-dependent to hormone-independent stage. As a very essential signaling event in TLR9-mediated signal pathway, NF-κB activation has been reported to have many reciprocal interactions with ER [17-24]. Further work needs to be done in order to clarify the effect of TLR9 on ER expression and even the possible involvement of TLR9 in the drug resistence of some breast cancer to the selective estrogen receptor modulators(SERM).

Finally, we also studied the difference of clinical outcome of the breast cancer patients based on the TLR9 expression. We found that patients with TLR9 positive breast cancer had a significantly longer progress free survival time than the TLR9 negative cohort. However, we didn't observed a statistically significant difference concerning overall survival (OS) between TLR9 positive and negative breast cancer patients, which might be correlated with the limited interval from surgery and observation end point, but we did note that the longer we follow up, the wider was the difference between the two group of patients. In a recent study about the association of TLR9 and estrogen receptor expression, Jukkola-Vuorinen reported that distant metastasis-free survival was higher in the lower TLR9-expressing part of the cohort than in the higher TLR9-expressing part of breast cancer patients, which, together with our data suggested that TLR9 may be correlated with a poor prognosis by promoting metastasis of breast cancer(16). We will continue to follow those patients and analyze prospectively the association between clinical outcome of patients and TLR9 expression. At the same time, a follow up of PEC-CT scaning the lymph node metastasis may help to precisely confirm this role of TLR9 in vivo.

Since TLR9 was recognized as an important receptor in innate immunity, more and more people have dedicated themselves to explore its translational research to enhance anti-tumor immunity [25]. Commonly referred to as CpG oligodeoxynucleotides (ODN), TLR9 agonists directly induce the activation and maturation of plasmacytoid dendritic cells and enhance differentiation of B cells into antibody-secreting plasma cells. Preclinical and early clinical data support the use of TLR9 agonists as vaccine adjuvants, where they can enhance both the humoral and cellular responses to diverse antigens. Phase I and II clinical trials have indicated that these agents have antitumor activity as single agents and enhance the development of antitumor T cell responses when used as therapeutic vaccine adjuvants(26-30). In another phase II study of TLR9 agonist oligodeoxynucleotide in combination with first-line taxane plus platinum chemotherapy in the treatment of advanced-stage non-small-cell lung cancer, the author concluded that addition of PF-3512676, a TLR9 agonist, to taxane plus platinum chemotherapy for first-line treatment of NSCLC improves objective response and may improve even survival of patients (31). It may be only a result of the chemosensitization effect of TLR9 agonist by upregulating antitumor activity of immunocells. But it may also be a final result complicated by a promotive effect of TLR9 in the metastasis and invation of lung cancer.

Doubtlessly, more work is necessary to clarify the exact biological effect of TLR9 ligation in vivo and before that, there should be a caution during applying TLR9 agonist as therapeutic vaccine adjuvants especially concering breast cancer.

In summary, this is the first report to provide clinical evidence that TLR9 expression may be associated with lymph node metastasis of breast cancer. Although follow up study unfinished, we believe that TLR9 is an independent indicator of poor prognosis by promoting tumor metastasis.

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