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Toll-Like Receptor-9 Expression Is Inversely Correlated with Estrogen Receptor Status in Breast Cancer

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Key Words
Breast cancer · Estrogen receptor · Toll-like receptor 9

Abstract
Toll-like receptor 9 (TLR9) recognizes microbial and vertebrate DNA. We previously demonstrated TLR9 expression in human breast cancer cell lines and showed that TLR9 ligands stimulate their in vitro invasion. The aim of this study was to characterize TLR9 expression in clinical breast cancer specimens. Immunohistochemical staining intensity was compared with known baseline prognostic factors and distant metastasis-free survival. TLR9 expression was detected in 98% of the tumors studied (n = 141). The mean TLR9 staining intensity was higher in ER– than in the highly ER+ breast cancers (p = 0.039). High-grade tumors had significantly increased TLR9 expression (p = 0.027) compared with lower-grade tumors. The highest TLR9 expression was detected in the mucinous and the lowest in the tubular breast cancers (p = 0.034). Distant metastasis-free survival was higher in the lower TLR9-expressing half of the cohort than in the higher TLR9-expressing group (p = 0.118). TLR9 expression did not correlate with menopausal, PgR or Her2 status, patient age, tumor proliferative or invasive characteristics.

Introduction

Toll-like receptors (TLRs) are evolutionarily well-conserved transmembrane proteins which recognize both microbe- and host-derived molecular patterns [1, 2]. At least 11 mammalian TLRs are known so far and each recognizes a different ligand. For example, TLR4 is the receptor for bacterial lipopolysaccharide, TLR5 recognizes flagellin, and members of the TLR9 subfamily (TLRs 7–9) are receptors for microbial RNA and DNA [1, 2]. Also, mammalian RNA and DNA have been shown to stimulate TLR7 and TLR9, in which context they have been linked to the development of autoimmune diseases [3]. TLR1, TLR2 and TLR4 are expressed on the cell surface whereas TLR3 and the TLR9 subfamily members are intracellular [4–7]. After ligand binding, TLRs and their adapter proteins, such as MyD88 and TRAM, recruit intracellular signaling mediators which in turn activate transcription factors, such as NF-κB. TLR activation results in an immune reaction, which is characterized by increased production of inflammatory mediators, such as cytokines and interleukins [2].

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In addition to the cells of the immune system, recent studies have also demonstrated TLR9 expression in various normal epithelial cells and in cancer cells, including breast, brain, gastric, lung and prostate cancer cells [6, 8–17]. We previously demonstrated that treatment of TLR9-expressing breast, brain and prostate cancer cells with synthetic TLR9 ligands stimulates their invasion in vitro. We further characterized this effect in breast and prostate cancer cells and showed it to be mediated via MMP-13 activation [8, 16]. No such effect on invasion was seen by TLR9 ligands in cancer cells that do not express TLR9. Furthermore, the TLR9-ligand-induced invasion was blocked by chloroquine, an inhibitor of endosomal acidification and thereby an inhibitor of TLR9 signalling, suggesting that the TLR9-ligand-induced invasion is indeed TLR9 mediated [8, 16]. Similar effects were also recently detected in human lung cancer cells [18]. Interestingly, Toll, the Drosophila homologue of mammalian TLRs, regulates not only anti-fungal immunity but also dorsoventral patterning during embryogenesis [19, 20]. On the other hand, cellular migration during embryogenesis and metastasizing cancer cells typically utilize similar molecular pathways [21]. Taken together, these findings suggest that both the pro-inflammatory and migratory/invasive effects of TLRs are evolutionarily well conserved.

Nothing is known about the possible role of TLR9-mediated invasion in the pathophysiology of breast or any other cancer. We did, however, detect TLR9 expression in protein lysates of clinical breast cancer specimens and also in clinical prostate cancer samples with immunohistochemistry [8, 16]. The aim of this study was to further define the expression of TLR9 in breast cancer. We also sought to determine if there is a correlation between baseline TLR9 expression and other baseline biological and prognostic parameters in a cohort of breast cancer patients at the time of primary surgery.

Materials and Methods

Breast Cancer Specimens and Patient Information

The breast cancer specimens (n = 141) used in this study were obtained from breast cancer patients at the time of primary surgical resection. These patients were being treated for their condition at the Department of Oncology, University Hospital of Oulu, Finland, during the years 1990–2004. All patients were Caucasian and they were treated and followed according to the prevailing, standard therapy. Information about the patients as well as the histopathological diagnosis and other biological parameters of the tumors were obtained from medical records. Estrogen (ER) and progesterone receptor (PgR), and Her2 expression status (which were also obtained from the patient records) were classified as follows: + = 20–40% cells stained positive with a given antibody; ++ = 40–60% cells stained positive with a given antibody; +++ = >60% of cells stained positive with a given antibody, and – = <20% of cells stained with the antibody in question. The study was approved by local ethic committees and by the Finnish Authority of Medicolegal Affairs.

Immunohistochemistry

Four-micrometer sections were prepared from archived, formalin-fixed, paraffin-embedded tissue samples. Immunohistochemical labeling was performed on the LabVision Autostainer™ (LabVision, Fremont, Calif., USA) using the Envision™ Detection System (K500711; Dako Denmark A/S, Glostrup, Denmark). The following antibodies were used according to the manufacturer's recommendations: anti-TLR9 (Img-305A, clone 26C593.2, diluted 1:200; Imgenex, San Diego, Calif., USA), anti-CD3 (RB-360-A, diluted 1:200; NeoMarkers, Fremont, Calif., USA), anti-CD20 (M 0755, diluted 1:1,000; Dako). The positive tissue control consisted of archived, formalin-fixed human tonsillar tissue, and omission of the primary antibody and the use of isotype-matched IgG (Imgenex) instead served as the negative staining control.

Scoring of TLR9 Staining

The TLR9-stained specimens were viewed under a microscope using a ×40 objective. The staining intensity of each sample was scored as previously described in detail [22, 23]. One investigator, who was blinded to the associated information about the specimens, performed the scoring of all specimens. Because TLR9 staining was seen only in the epithelial cancer cells and not in the stroma (apart from the occasional infiltrating lymphocytes), only epithelial cells were scored. Also, due to the homogeneous staining pattern within the individual specimens, four representative microscopic fields were evaluated for each sample. TLR9 staining intensity in the viewed breast cancer cells was arbitrarily assigned to range from 0 to 4, where 0 represents no staining and 4 stands for the highest-intensity TLR9 staining. The percentage of cells (numbers 0.0–1.0 for 0–100%) representing each intensity value (0–4) was calculated per field. The four scores thus obtained from the representative microscopic fields were added together to obtain a total TLR9 score for each individual breast cancer specimen.

Statistical Analysis

Tumor characteristics of the population are presented as means (SD) and proportions. The relationship between TRL9 level and tumor characteristics was compared by the generalized linear models with the Tukey adjustment for pairwise mean comparisons. Some variables were categorized for analysis (age <50 and ≥ 50 years) and tumor size was categorized into tertiles. For survival analysis, the event was defined as distant metastasis-free survival or development of contralateral breast cancer. Time in months was calculated from the date of diagnosis to the date of the event. The relationship between TLR9 and distant recurrence was analyzed by the Kaplan-Meier method and TLR9 was divided into two categories at the approximate median (<8.0 and ≥ 8.0). Cox proportional hazards models were used for the multivariate survival analysis. For all analyses, $p < 0.05$ was deemed statistically significant and SAS version 9.1® was used.

Table 1. Baseline tumor characteristics and tumor recurrence

Characteristics	n (%)
Tumor ER status	
Negative	19 (14.1)
+	13 (9.6)
++	11 (8.2)
+++	92 (68.1)
Tumor PgR status	
Negative	34 (25.4)
+	22 (16.4)
++	19 (14.2)
+++	59 (44.0)
Tumor Her2 status	
Negative	115 (84.6)
+	15 (11.0)
++	4 (2.9)
+++	2 (1.5)
Recurrence	
None	109 (77.4)
Local	6 (4.3)
Regional	2 (1.4)
Distant	24 (17.0)

Results

TLR9 Is Frequently Expressed in Breast Cancer

We investigated the level of TLR9 expression in 141 archived, paraffin-embedded breast cancer specimens which were initially obtained during the primary surgery. Tumor baseline and recurrence information is provided in table 1. We first validated the TLR9 antibody by characterizing its behavior in archived, paraffin-embedded tissue sections of human lymphatic tissue from tonsils. Unlike the CD3 and CD20 antibodies, which recognize T and B lymphocytes, respectively, primarily in their typical tissue areas within the tonsils, the TLR9 antibody recognized lymphocytes in both germinal centers and in the interfollicular zones, suggesting that TLR9 is expressed in both B and T lymphocytes, in agreement with a previous study in the same tissue [26]. As expected, TLR9 staining was intracellular and TLR9-positive lymphocytes were also detected within capillaries [1, 26]. Omission of the primary antibody resulted in no signal. Taken together, these validations suggested that TLR9 staining was specific (fig. 1). Previously, the same TLR9 antibody has also been verified using in situ hybridization in human lung cancer specimens [15]. In the breast cancer specimens, TLR9 staining was localized to the epithelial cancer cells and except for the occasional tumor-infiltrating lymphocytes, no TLR9 staining was detected in breast cancer stroma. The TLR9 staining in the breast cancer specimens was also confirmed to be specific in comparison with an IgG-negative control staining done on a subsequent section of the same tissue block for each individual sample (fig. 2).

TLR9 Expression Is Inversely Correlated with ER Expression in Breast Cancer Specimens

Of the 141 breast cancer specimens studied, TLR9 expression was detected in ~98%. Only 5 breast cancer samples were negative for TLR9 expression. These TLR9-negative breast cancers were not the result of a staining artifact since lymphocytes within the same sections were positive for TLR9 (data not shown). We scored the TLR9 staining intensities in the various breast cancer specimens using previously published methods [22, 27, 28]. The mean TLR9 staining score ranged from 0 to 16, with 0 referring to a negative TLR9 staining result and 16 representing the highest staining intensity. Representative images of breast cancers with high, intermediate and low TLR9 staining scores are shown in figure 2. We compared the baseline tumor TLR9 staining intensity with other, standard predictive biological parameters of the same tumors. This other baseline information, e.g. steroid receptor status, was obtained from patient records. There was a statistically significant ($p < 0.05$), inverse correlation between TLR9 and ER expression levels. The mean TLR9 expression level was low in tumors with high (3+) ER expression and high in ER-negative tumors. Of the 5 TLR9-negative samples detected in this cohort, ER expression status was known for 3 and they all fell into the high ER expression (3+) group. Interestingly, the intermediate ER expression level group (ER1–2+) also had an intermediate mean TLR9 expression level between the two extremes, supporting an inverse relationship between TLR9 and ER expression levels. There were no differences in the mean TLR9 expression levels when the tumors were stratified according to the PgR or Her2 expression status. The mean TLR9 expression level was slightly higher in triple-negative tumors (n = 10) compared with a combination of all tumors with positive (ER, PgR, Her2) or double-positive (ER and PgR) expression (n = 99). However, these differences did not reach statistical significance (fig. 3.)

TLR9 Expression Is Associated with Tumor Histology and Grade

When the tumors were stratified according to their histological subtype, the highest mean TLR9 expression

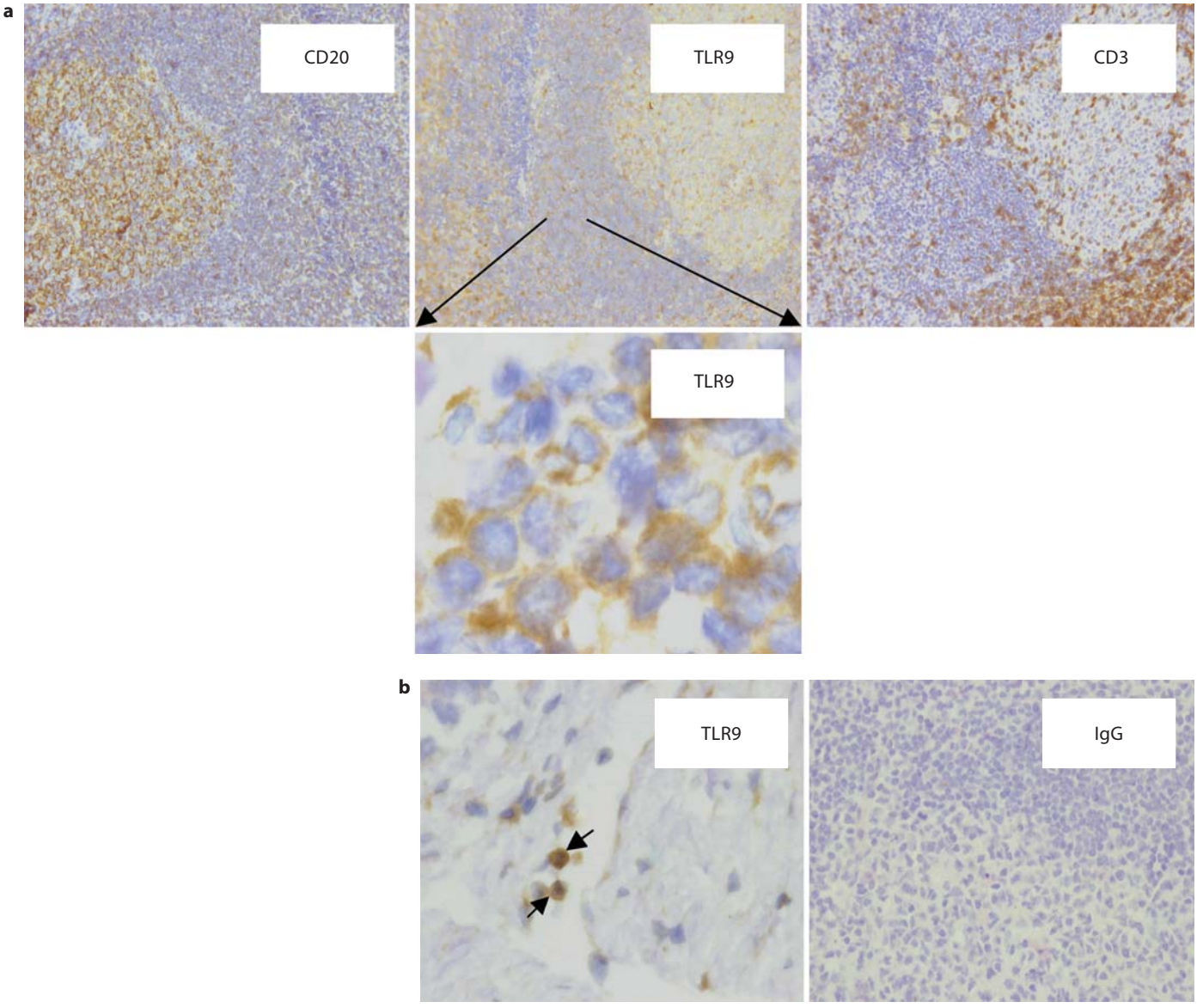


Fig. 1. a Characterization of the anti-TLR9 antibody was done using tissue sections of paraffin-embedded human tonsil. Anti-CD20 and -CD3 antibodies, which detect B and T lymphocytes, respectively, were used as references to compare TLR9 staining localization within the tissue (×100 objective). Accumulation of CD20+ cells was seen in the B-cell follicles, whereas CD3+ cells were mostly seen in the interfollicular T-cell zones. TLR9 staining was detected in lymphocytes in both the follicles and in the interfollicular areas. The TLR9 staining pattern was intracellular, as detected in lymphocytes in the germ center (×1,000, arrows). **b** Intracellular TLR9 staining also detected lymphocytes within a capillary (small arrows), whereas no staining was detected if IgG was used instead of the primary anti-TLR9 antibody.

level was detected in the mucinous breast cancers and the lowest in the tubular breast cancers ($p < 0.05$, mucinous vs. the tubular type). The mean TLR9 expression levels were within a similar range in ductal and lobular subtypes. Tumors with the highest grade (3) had the highest TLR9 mean expression level compared with tumors that had a lower grade (1 or 2, $p < 0.05$ for grade 2 vs. grade 3). There were no statistically significant differences in the TLR9 expression levels in tumors that were stratified according to the patient's age and menopausal status or tumor proliferative activity (Ki67 staining intensity). Although there were no statistically significant differences

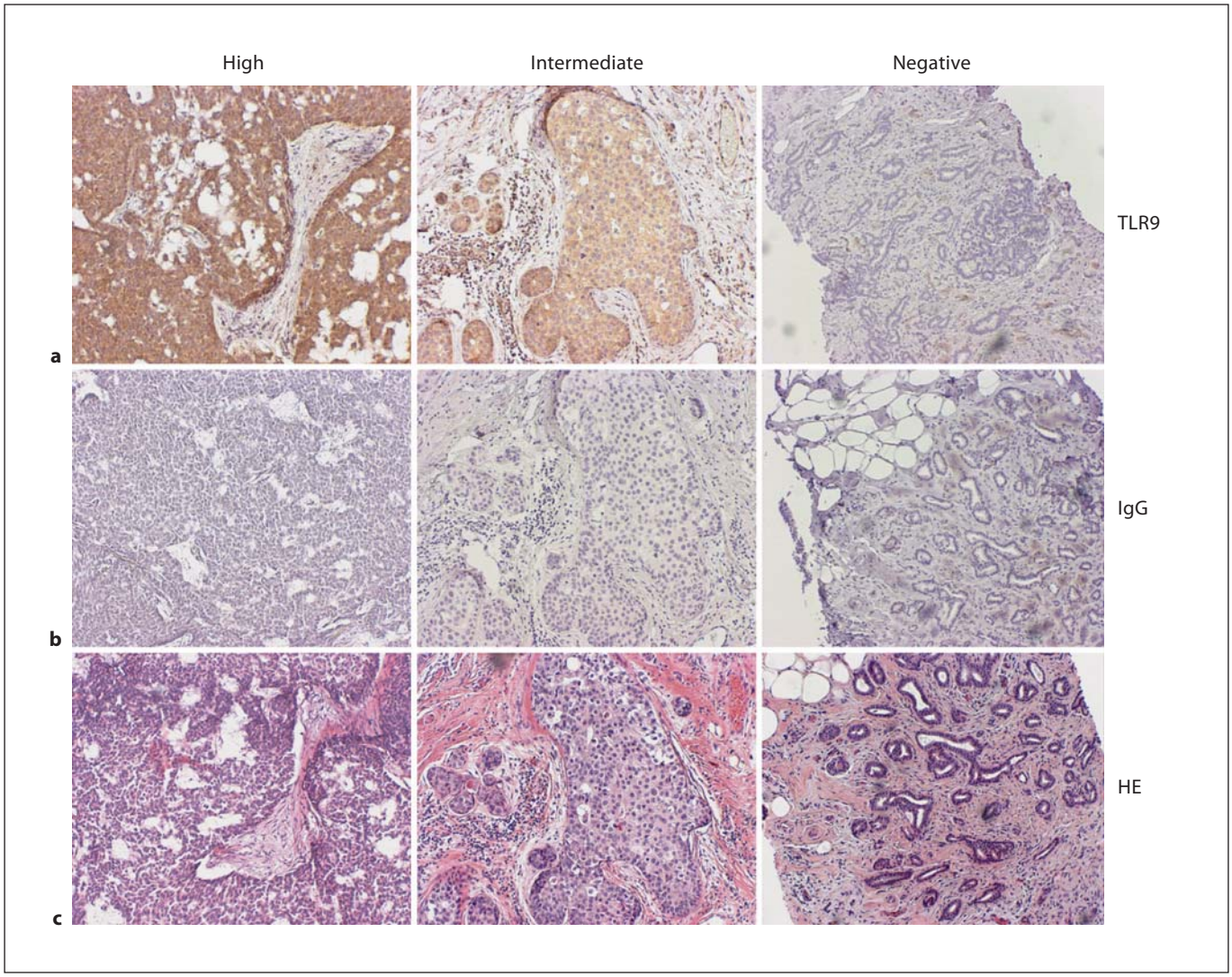


Fig. 2. TLR9 is expressed in breast cancer epithelium. Immunohistochemical detection of TLR9 was seen mostly in epithelial tumor cells and not in the stroma cells in the breast cancer specimen (**a**). Omission of the primary anti-TLR9 antibody and the use of IgG instead resulted in no staining (**b**). HE staining of the corresponding tumors (**c**). Shown are staining results representing high (16), intermediate (7) and negative (0) staining scores of three different specimens.

in the mean TLR9 expression levels when the tumors were stratified according to their invasive characteristics, the mean TLR9 expression levels were higher with the presence of metastatic axillary nodes. The mean TLR9 staining intensity level was the highest in tumors that were 10–19 mm in diameter, and the lowest in tumors that were <10 mm in diameter. The TLR9 staining intensity level was intermediate in the largest tumors (≥20 mm). These differences were not, however, statistically significant (table 2).

TLR9 Expression as a Prognostic Marker for Distant Metastasis-Free Survival

We next analyzed the role of TLR9 expression as a univariate prognostic factor and compared the distant metastasis-free survival in breast cancer patients with TLR9 expression levels in the upper (>8.0) and lower (0.0–8.0) halves of the cohort. The mean follow-up time was 62.7 months (SD 27 months; median 70.0 months). The mean time to distant metastasis was 59.3 months (SD 28.8; median 69.0 months). The probability of distant metastasis-

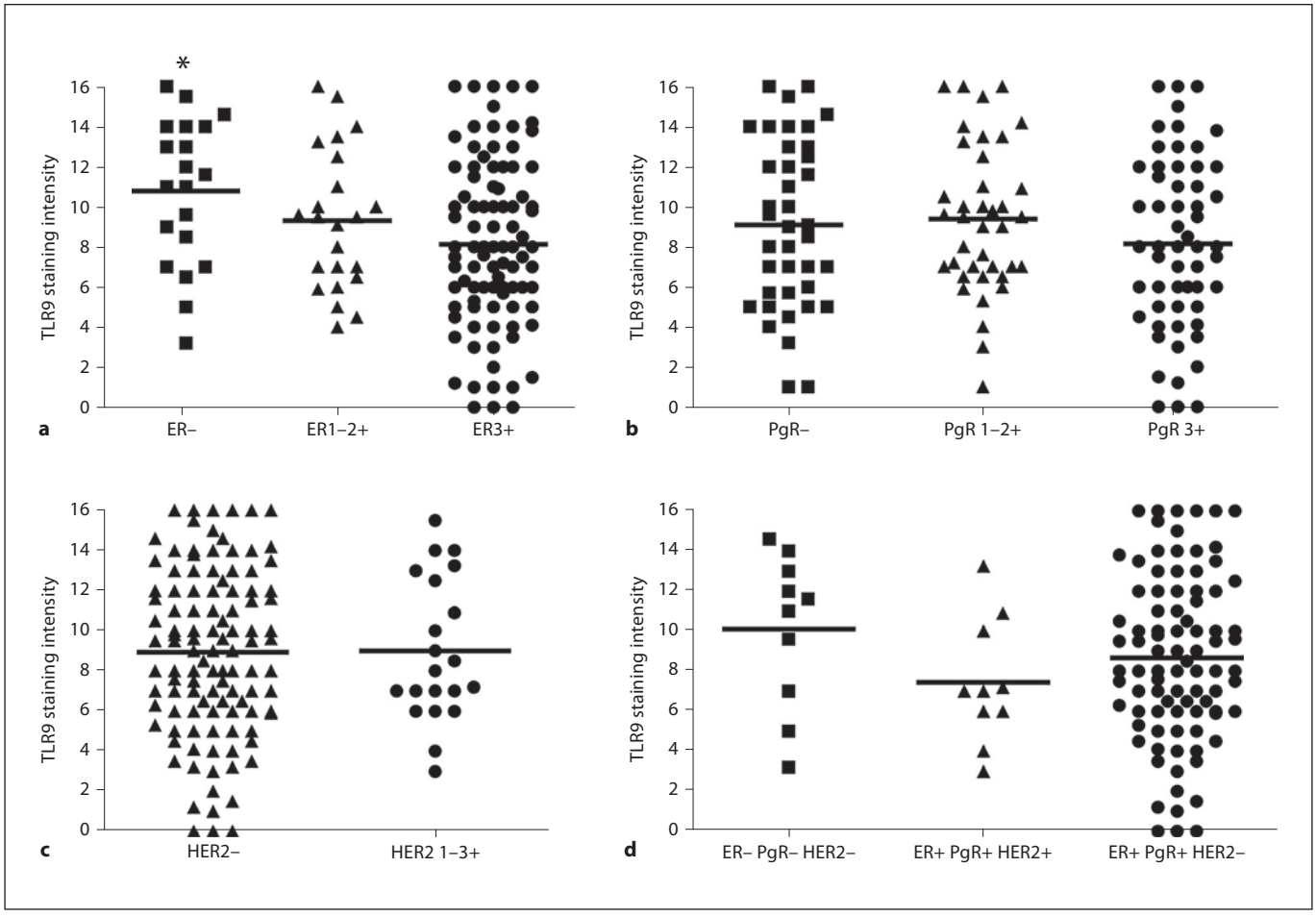


Fig. 3. Expression of TLR9 is significantly higher in ER– cancers than in highly ER+ breast cancers. TLR9 staining intensities within the individual samples were microscopically scored and stratified according to ER (**a**), PgR (**b**) and Her2 expression status (**c**) or according to triple negativity (ER–, PgR–, Her2–; **d**) and a combination of positive ER, PgR and/or Her2 status of the tumor. The mean TLR9 staining intensity was significantly higher (* $p < 0.05$) in the ER– tumors than in highly ER+ (ER3+) tumors.

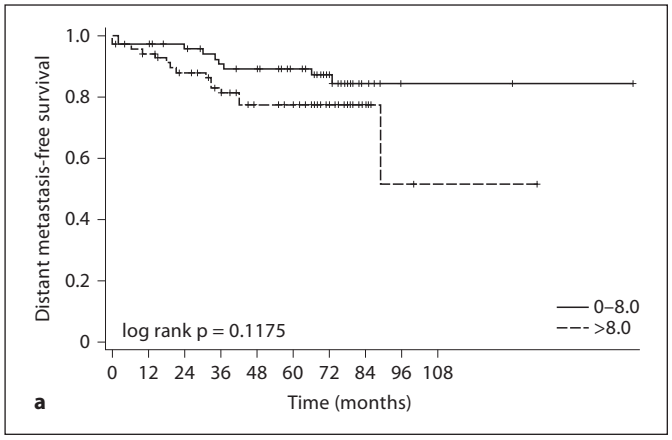
free survival was higher in the group with low TLR9 expression than in the group with higher TLR9 expression. This difference did not, however, reach statistical significance ($p = 0.1175$; fig. 4a).

In a Cox model controlling for ER status, tumor size, nodal status and Her2, the risk of distant metastasis was 2.71 (95% confidence interval 0.92–8.01) times higher for patients with TLR9 levels >8.0 versus patients with TLR9 levels <8.0.

The model showed the expected positive and significant relationship between tumor size (hazard ratio = 2.50, 95% confidence interval 1.07–5.85) and the risk of distant metastasis. The number of patients was too few to test for an interaction between ER status and TLR9. The probability to develop cancer in the contralateral breast ($n = 9$) was independent of the TLR9 expression status (fig. 4b).

Discussion

TLR9 is a protein of innate immunity, which recognizes both microbial and vertebrate (self) DNA [1, 29–32]. It is typically expressed in cells of the immune system and stimulation of TLR9 with its agonistic ligands results in a robust inflammatory reaction, involving the production of various pro-inflammatory cytokines [1]. TLR9 is also expressed in various cancer cell lines, whose in vitro



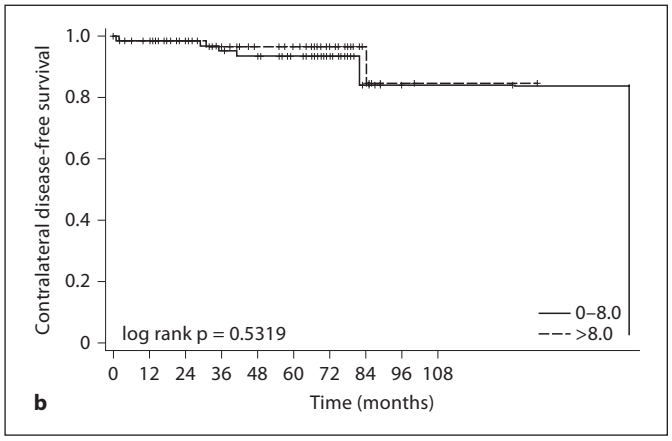


Fig. 4. Kaplan-Meier survival curve for the probability of distant metastasis-free survival (**a**) and contralateral breast tumor development (**b**).

invasion is stimulated by synthetic TLR9 ligands [6, 8, 15, 16, 18]. Whether or not TLR9-mediated invasion contributes to the pathophysiology of any of these cancers is not currently known. Immunohistochemically, TLR9 expression has been detected in clinical prostate and lung cancer specimens and with Western blotting in protein lysates of primary breast tumors [8, 16]. We have also shown that TLR9 expression is increased in breast cancer cells compared with normal breast epithelium [Ilvesaro et al., submitted].

Using an existing cohort of 141 paraffin-embedded breast cancer specimens, we now confirm these previous results and show here that TLR9 is very frequently expressed in breast cancer. Excluding the occasional detection of TLR9 expression within tumor-infiltrating lymphocytes, TLR9 staining was localized only to the epithelial cells and not detected in the stroma cells in cancer.

Table 2. TLR9 levels stratified by characteristics of disease in breast cancer cases

Characteristics	TLR			n	p value
	mean	SD	median		
Age					
<50 years	9.60	3.88	9.60	39	0.1220
≥50 years	8.39	4.17	8.00	92	
Tumor size					
<10 mm	7.40	4.03	7.00	27	0.0899
10–19 mm	9.38	4.06	9.55	64	
≥20 mm	8.14	4.40	8.75	32	
ER					
Negative	11.00	3.62	11.60	19	0.0386*
+	9.07	2.81	9.50	13	
++	9.36	4.27	9.10	11	
+++	8.12	4.17	8.00	92	
PR					
Negative	8.95	4.03	9.05	34	0.4733
+	9.52	3.44	9.55	22	
++	9.31	4.36	9.00	19	
+++	8.14	4.27	8.00	59	
Her2					
Negative	8.59	4.24	8.50	115	0.9064
+	9.14	3.57	8.00	15	
++	7.94	3.99	7.25	4	
+++	10.0	4.24	10.0	2	
Ki67					
Negative	7.69	3.86	6.75	26	0.0850
+	9.01	4.57	9.00	56	
++	8.26	3.78	8.00	31	
+++	11.50	2.88	12.00	10	
Blood vessel invasion					
Yes	1.0	–	1.00	1	0.0704
No	8.66	4.18	8.50	123	
Lymphatic invasion					
Yes	9.75	4.13	8.75	8	0.4132
No	8.49	4.21	8.15	118	
Number of metastatic nodes					
0	8.31	4.24	8.00	110	0.1052
1 or more	9.67	3.63	9.60	31	
Menopausal status					
Premenopausal	9.36	3.64	9.60	46	0.1353
Postmenopausal	8.23	4.27	8.00	81	
Histology					
Ductal	8.72	366	8.0	77	0.0340**
Lobular	8.36	4.05	9.25	40	
Tubular	6.08	4.27	6.75	12	
Mucinous	11.07	6.15	13.80	11	
Grade					
1	9.02	4.72	8.30	39	0.0272***
2	7.73	3.95	7.00	60	
3	10.24	3.80	11.00	29	

p values from one-way ANOVA. * $p < 0.05$ for ER– vs. ER+++; ** $p < 0.05$ for tubular vs. mucinous; *** $p < 0.05$ for grade 2 vs. grade 3.

Applying a well-established method to immunohistochemically assess protein expression levels in tissues [22, 23], we further show that TLR9 expression inversely correlates with a high ER expression status in the tumors. Typically, ER-negative tumors had higher and strongly ER-positive tumors exhibited lower TLR9 expression levels. This finding is in line with our previous results demonstrating high TLR9 expression in the human ER– MDA-MB-231 cells and no TLR9 expression in the human ER+ MCF-7 breast cancer cells [16]. In our cohort, TLR9 expression was similar in tumors from pre- and postmenopausal women. Nothing, however, can be concluded from this particular finding at this point because we have no information about the previous use of hormone replacement therapy, for example, in the postmenopausal group. The mechanisms of how ERα regulates TLR9 expression are unclear and warrant further studies. Female hormone effects on TLR9 function have, however, also been detected in other systems. Especially progesterone, but also estradiol to some extent, inhibits TLR9-mediated inflammation in both human and mouse plasmocytoid dendritic cells [33]. Sex steroids and gender have also been shown to affect the function of other TLRs. For example, TLR7 ligands induce higher inflammatory responses in cells derived from females compared with those detected in males [34]. The effects of lipopolysaccharides have also been shown to be modulated by estradiol in macrophages [35]. Very little is known about the possible mechanisms how sex steroids could regulate the function and expression of these proteins. They may, however, affect downstream signaling proteins in the TLR pathway [33]. In addition to ERα and estradiol possibly regulating TLR9 expression and function, another possible explanation for the inverse correlation between ER negativity and high TLR9 expression is that they both are merely independent indicators of a less differentiated, more aggressive cellular phenotype. Upon epithelial-to-mesenchymal transition, epithelial cells acquire mesenchymal characteristics which allow them to invade the extracellular matrix. This phenomenon is necessary for embryogenesis, but it is also utilized by cancer cells [21]. The fact that TLR9 expression might be a part of epithelial-to-mesenchymal transition is supported by earlier findings which have established a role for Toll, the fruitfly analogue of TLRs, in dorsoventral patterning during Drosophila embryogenesis [19, 20]. Furthermore, human mesenchymal stem cells have recently been shown to express TLR9. Identical with the effects seen in TLR9-expressing cancer cells, the mesenchymal stem cells became more invasive upon stimulation with synthetic TLR9 ligands, too [36].

TLR9 expression was not statistically different when the tumors were stratified according to their invasive characteristics. However, the number of patients with blood or lymphatic vessel invasion was very small and this result cannot be considered conclusive. Tumors that had metastasized to axillar lymph nodes at diagnosis had slightly higher TLR9 expression compared with tumors with no axillary lymph node metastasis. This observation warrants further studies in a larger patient population representing a more advanced stage at diagnosis. We also discovered a trend to shorter distant metastasis-free survival with high TLR9 expression upon diagnosis compared with tumors that exhibited lower TLR9 expression upon diagnosis. This difference persisted when the well-known prognostic factors tumor size, Her2, and nodal and ER status were taken into account. None of these differences were, however, statistically significant and they also need to be confirmed in a larger population. Because the tumors were collected from a time period of 14 years, treatment regimens, which likely varied, were not taken into account in this preliminary study. Nevertheless, combined with the fact that the likelihood to develop cancer in the contralateral breast was similar in both high- and low-TLR9-expressing halves of the study group, these results further support the hypothesis that rather than participating in oncogenesis TLR9 is being utilized during metastasis.

No differences were detected when TLR9 expression was stratified according to PgR or Her2 expression status, tumor size or by proliferative characteristics of the tumors. The poor prognostic triple-negative tumors had slightly higher mean TLR9 expression level compared with tumors that express ER or PgR with or without Her2. However, this difference was not statistically significant and also needs to be reevaluated in a larger population. Of the histologic subtypes, the typically good-prognosis, mucinous types expressed the highest mean levels of TLR9, whereas the tubular types had the lowest levels [37]. The low number of samples of these rarer histological subtypes included in this analysis precludes any conclusion to be drawn.

Finally, the circumstances in which cancer cells could utilize the TLR9-mediated invasive pathway remain to be established. The role of the physiological ligand for TLR9-mediated invasion is also not clear in this context. We showed previously that *Escherichia coli* DNA stimulates prostate cancer cell invasion in vitro [8]. Unlike prostate cancers, which due to their anatomic location are susceptible to microbial infections, breast cancers are very rarely infected [38]. One theoretical source for TLR9 ligands

in breast cancer could, however, be mycoplasmal infections, which have been detected in various cancers, including breast cancer [39, 40]. Interestingly, mycoplasma and TLR9 reside within the same subcellular organelle, at least in theory facilitating the interaction between microbial DNA and TLR9 [41, 42]. Another possible source for TLR9 ligands is the breast cancer tissue itself. Recently, it has been shown that TLR9-mediated recognition of self-DNA can contribute to the pathogenesis of various human diseases. For example, self-DNA-IgG complexes induce TLR9-mediated inflammation in lupus erythematosus and DNA from dead keratinocytes induces TLR9-mediated inflammation in viable dendritic cells, resulting in psoriatic skin lesions [3, 32]. Similarly, we hypothesize that cytotoxic cancer treatment or tissue necrosis could induce the release of DNA from dead breast cancer cells and result in TLR9-mediated invasion in viable breast cancer cells. Obviously, these issues require further characterization in experimental models. Our findings may, however, have several clinical implications. First, if cancer cells truly utilize the TLR9-mediated pathway for invasion, then the anti-invasive efficacy of TLR9 inhibitors, e.g. chloroquine, should be tested. Second, synthetic TLR9 agonists are being tested for cancer immunotherapy [43]. Since these agents may cause invasion in TLR9-expressing cancer cells, caution should be practiced with their use.

In conclusion, TLR9 is frequently expressed in breast cancer and TLR9 expression levels in the cancers inversely correlate with high expression levels of ER. The role of TLR9-mediated invasion in breast cancer needs to be further studied in preclinical models.

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