Biopathological Significance of TLR9 Expression in Cancer Cells and Tumor Microenvironment Across Invasive Breast Carcinomas Subtypes

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Abstract Toll-like receptors (TLRs) are pattern recognition receptors mainly expressed by cells of the immune system but also by epithelial tumor cells. Little is known about expression patterns of TLR genes in breast tumors, and their clinical significance is unclear. The aim of our study was to investigate expression of TLRs pathway components in pre-invasive breast lesions and invasive breast carcinomas (IBCs). We used RT-PCR assays to quantify mRNA levels of the 10 TLR genes and genes involved in TLR pathways in 350 breast tumors from patients with known clinical/pathological status and long-term outcome. Sets of 158 breast samples were also analyzed by immunochemistry including; 40 early noninvasive breast lesions, 38 IBCs and 80 triple negative carcinomas subtype (TNCs). We identified TLR9 as the major TLR gene family member upregulated in breast tumors and more particularly in TNCs. Immunohistochemical studies demonstrated

that TLR9 protein was expressed in tumor epithelial and stromal cells of the TLR9 mRNA-overexpressing tumors. TLR9 overexpression appears very early during breast carcinogenesis. High TLR9 levels were associated with favorable outcome in the TNC sub-group. TLR9 overexpression was associated with alterations of down-stream components of the TLR9 signaling pathway, epithelio-mesenchymal transition (EMT) induction and EGFR pathway deregulation. TNCs with TLR9 overexpression were significantly correlated with development of a fibrous and inflammatory microenvironment with variable status of nuclear phosphoSTAT3. Our results suggest that TLR9 could play a role in TNC carcinogenesis and could be useful as predictive biomarker and therapeutic target.

Keywords Toll-like receptor 9 · Breast cancer subtypes · Tumor microenvironment signaling pathways · EMT

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Abbreviations

RFS	Relapse-free survival
ER	Estrogen receptor
PR	Progesterone receptor
HR	Hormone receptors
RT-PCR	Reverse transcriptase-polymerase chain reaction
DAMPs	Damage-associated molecular patterns
IDC	Invasive breast carcinomas
PAMPs	Pathogen-associated molecular patterns
SBR	Scarff, Bloom and Richardson histopathological grade
MICs	Mononuclear inflammatory cells
CAFs	Cancer-associated fibroblasts
EMT	Epithelio-mesenchymal transition

Introduction

Invasive breast carcinomas (IBCs) are highly heterogeneous tumors, and triple negative subtype (TNC) remains a major cause of death in women. Thus, identification of new prognostic factors is crucial to improve the classical histoprognostic classification of breast tumors. Among breast carcinomas, TNCs represent 10 to 15 % of IBCs and are characterized by an aggressive clinical course and a poor prognosis, owing partly to the lack of targeted therapies [1–3].

Cancer microenvironment is a complex network driving cancer cells fate from invasion to intravasation and metastasis. Reciprocal interactions between neoplastic cells and their microenvironment are pivotal events in carcinogenesis and tumor progression [4]. IBCs and particularly TNCs are usually characterized by establishment of a predominant stromal program with central necrotic/fibrotic areas, and a huge peripheral inflammatory infiltrate [5]. Inflammation has recently emerged as a non-mutational driver of carcinogenesis and is now considered as a new hallmark of cancer. Recent data have underlined the crucial role of the inflammatory microenvironment in IBCs carcinogenesis and particularly in the TNC subtype [6, 7]. Inflammation is usually induced by activation of innate immune receptors such as Toll-like receptors (TLRs) and cytokines receptors.

TLRs belong to the evolutionarily conserved family of Pattern Recognition Receptors (PRR). Ten functional TLRs have been identified in humans and 13 in mice. TLRs function as receptors of the innate immunity system for detection and response to pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), which include a large spectrum of microbial components and endogenous molecules released by injured tissue [8, 9]. Cell surface TLRs (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) recognize microbial membrane lipids, whereas endosomal TLRs (TLR3, TLR7, TLR8, TLR9) recognize pathogen and host-derived nucleotides, including unmethylated CpG motifs of DNA and double or single-stranded RNA [10]. TLRs play a crucial role of molecular sensors, essentially by bridging innate and adaptive immunity. Activation of TLRs results in production of type I interferon (IFNs), cytokines and chemokines that finally initiate inflammation, recruit immune cells and activate adaptive immunity [11, 12]. TLRs are preferentially expressed by immune cells but also by normal and cancer epithelial cells of various origins [13, 14]. In cancer, tumor cells death results in release of numerous proteic or nucleic DAMPs that activate TLRs of both immune/stromal cells and epithelial cancer cells. Increasing evidence has indicated that TLRs expression by cancer cells and immune cells seems to be twofold by either promoting or inhibiting tumor progression [6, 15, 16].

The aim of this study is to investigate TLRs expression in IBCs particularly in TNC subtype and their relationship with clinico-biological data and prognosis.

Materials and Methods

Patients and Samples

Samples of 350 unilateral invasive primary breast tumors excised from women managed at Curie Institute, Saint-Cloud, France from 1978 to 2008 were analyzed. The samples were immediately stored in liquid nitrogen until RNA extraction. A tumor sample was considered suitable for our study if the proportion of tumor cells exceeded 70 %. All patients (mean age 61.7 years, range 31–91 years) met the following criteria: primary unilateral non metastatic breast carcinoma for which complete clinical, histological and biological data were available; no radiotherapy or chemotherapy before surgery; and full follow-up at René Huguenin Hospital.

Treatment consisted in modified radical mastectomy in 221 cases (63.1 %) and breast-conserving surgery plus locoregional radiotherapy in 129 cases (36.9 %). The patients had a physical examination and routine chest radiotherapy every 3 months for 2 years, then annually. Mammograms were performed annually. Adjuvant therapy was administered to 292 patients, consisting in chemotherapy alone ($n = 76$), hormone therapy alone ($n = 150$) and both treatments ($n = 66$). The histological subtype and the number of positive axillary lymph nodes were evaluated at the time of surgery. IBCs were assessed according to Scarff Bloom Richardson's (SBR) histoprognosis grading system. Hormone receptor (HR) (i.e. estrogen receptor (ER) and progesterone receptor (PR) and human epidermal growth factor receptor 2 (ERBB2) statuses were determined at the protein level by using biochemical methods (dextran-coated charcoal method, enzyme immunoassay or immunohistochemistry) and confirmed by real-time quantitative RT-PCR assays [17, 18]. The population was divided into four groups according to HR (ER and PR) and ERBB2 status, as follows: two luminal subtypes [HR+/ERBB2+ ($n = 45$)] and [HR+/ERBB2- ($n = 195$)]; an ERBB2+ subtype [HR-/ERBB2+ ($n = 46$)] and a triple-negative subtype [HR-/ERBB2- ($n = 64$)]. With a median follow-up of 8.9 years (range 6 months to 29 years), 143 patients presented metastases. 10 specimens of adjacent normal breast tissue from breast cancer patients and normal breast tissue from women undergoing cosmetic breast surgery were used as sources of normal RNA. Another series of 80 TNCs was used for prognostic analysis. An additional series of 50 breast tissues; 10 normal breast tissues and 40 early noninvasive breast lesions, including atypical columnar metaplasia (ACM; $n = 10$), atypical ductal hyperplasia (ADH; $n = 10$) and ductal carcinoma in situ (DCIS; $n = 20$), were selected for protein level analysis through immunohistochemistry. Finally, we analyzed 39 breast tumors xenografts. We previously showed that these xenografts analyzed in the present study reproduced the marked heterogeneity of human tumors and generally very closely resemble the patient's tumor in terms of histopathologic and molecular features [19–21]. The experimental protocol and animal housing were in

accordance with institutional guidelines of the French Ethical Committee (Agreement B75-05–18, France).

RNA Extraction

Total RNA was extracted from breast tissue samples by using acid-phenol guanidium as previously described. RNA quality was determined by electrophoresis through agarose gels, staining with ethidium bromide and visualization of the 18S and 28S RNA bands under ultraviolet light.

Real-Time RT-PCR

Quantitative values were obtained from the cycle number (Ct value) at which the increase in the fluorescence signal associated with exponential growth of PCR products started to be detected by the laser detector of the ABI Prism 7900 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, CA), using PE biosystems analysis software according to the manufacturer's manuals. The precise amount of total RNA added to each reaction mix (based on optical density) and its quality (i.e., lack of extensive degradation) were both difficult to assess. Therefore, transcripts of the *TBP* gene (Genbank accession NM_003194) encoding the TATA box-binding protein (a component of the DNA-binding protein complex TFIID) were also quantified as an endogenous RNA control. Each sample was normalized on the basis of its *TBP* content. *TBP* was selected as an endogenous control due to the moderate prevalence of its transcripts and the absence of known *TBP* retropseudogenes (retropseudogenes lead to coamplification of contaminating genomic DNA and thus interfere with RT-PCR, despite the use of primers in separate exons) [17]. Results, expressed as N-fold differences in target gene expression relative to the *TBP* gene and termed “*Ntarget*”, were determined as $N_{target} = 2^{\Delta C_{t\text{sample}}}$, where the ΔC_t value of the sample was determined by subtracting the average Ct value of target gene from the average Ct value of *TBP* gene. The target gene values of the breast tumor samples were subsequently normalized such that the median of the target gene values for the 10 normal breast tissues was 1. The primers for *TBP*, the 10 TLRs and others genes were chosen with the assistance of the Oligo 6.0 program (National Biosciences, Plymouth, MN). dbEST and nr databases were scanned to confirm the total gene specificity of the nucleotide sequences chosen for the primers and the absence of single nucleotide polymorphisms. The primer pairs for each TLR isoform genes were selected to be unique when compared with the sequences of the others TLR genes. To avoid amplification of contaminating genomic DNA, one of the two primers was placed at the junction between two exons or on two different exons. Agarose gel electrophoresis was used to verify the specificity of PCR amplicons. The conditions of cDNA synthesis and PCR were as previously described [22]. To quantify by real-time quantitative RT-PCR the specific human *TLR9* mRNA levels in the total RNAs extracted

from the 39 human breast xenografts, we used a human-specific *TLR9* RT-PCR assay. The human *Hs-TLR9* primer pair was selected to be unique when compared to the sequence of the murine *TLR9* mRNA, whereas the *Total-TBP* primer pair was selected to amplify both the mouse and the human *TBP* genes that used as endogenous RNA control. The *NHs-TLR9* values of the breast tumor xenograft samples were subsequently normalized such that the value for the “basal mRNA level” (smallest amount of *Hs-TLR9* mRNA quantifiable, $C_t = 35$) was 1. *Hs-TLR9* mRNA levels that were totally absence or very low ($C_t > 35$; detectable but not reliable quantifiable) in breast tumor, xenograft samples were scored “0” (no expression).

Immunohistochemistry

Paraffin-embedded tissue blocks, obtained at the time of the initial diagnosis were retrieved from the archives of the Department of Biopathology, René Huguenin Hospital. Sections of 3 μm in thickness were cut with a microtome from the paraffin-embedded tissue blocks of normal breast tissue, pre-invasive lesions and IBCs. Tissue sections were dewaxed and rehydrated through a series of xylene and ethanol washes. Briefly, primary antibodies (abs) against pan-cytokeratin AE1/AE3, vimentin, smooth muscle actin, E-cadherin, ZEB2 and EGFR were used. Immunostaining was processed by using a Dako automated device except for TLR9 assay that was performed with a primary monoclonal antibody (Imgenex, Cambridge, UK) at a dilution of 1/50 associated with an antibody diluent (Invitrogen 00–3218) and incubated overnight at 4 °C. The specificity of the TLR9 abs was confirmed via the same protocol on paraffin-embedded human tissue sections containing lymphocytes. In addition, we realized positive and negative TLR9 test controls on normal breast tissue - terminal duct lobular unit (TDLU) (Supplemental Figure 1). For each case of TLR9 immunostaining, two sections of paraffin-embedded breast tissue of IBCs and TNCs were performed; one section in the center of the tumor and second section including tumor microenvironment and invasion margins. Then, TLR9 immunostaining slides were anonymized and blinded for the interpretation of immunostaining score. A semi quantitative intensity score (H score) was used for TLR9 (score 0: negative staining, score 1: weak cytoplasmic staining, score 2: moderate cytoplasmic staining, score 3: strong cytoplasmic staining in most of tumor cells, score 4: strong cytoplasmic and sub membranous staining in almost all tumor cells). Slides were scored by three pathologists in two working groups. The pathologists did not know neither hormone receptors nor HER2 status before slides interpretation. TLR9 normal breast tissue expression levels were observed with H score between > 1 and < 2 . TLR9 underexpression was defined with H score between 0 and 1 and TLR9 overexpression with a H score between 2 and 4.

For anti-STAT3 antibody (phosphoS727, abcam, ab32143, pH6, 1/200) and anti-STAT3 antibody (phosphor Tyr705, Cell

Signaling, D3A7, pH9, 1/50), only tumors that showed more than 10 % nuclear staining in cancer cells and stromal/immune cells were considered as positive.

We performed immunohistochemical study of the tumor microenvironment by using TLR9 immunostaining in stromal cancer associated fibroblasts (CAFs) and mononuclear inflammatory cells (MICs). Then, we used several abs to distinguish different subpopulations of MICs (CD3, CD20, CD4, FoxP3, CD8/CD45RO and CD68), with a semi quantitative score (score 0: negative, score 1: <5 %, score 2: 5 to 20 %, score 3: > 20 %). We also evaluated the density of microvessels by using a CD31 abs with the same semi quantitative score. All immunostaining was processed using a Dako abs (Dako, Glostrup, Denmark), except of CD8 and CD4 (Novocastra, Newcastle, UK).

Statistical Analysis

The distributions of target mRNA or protein levels were characterized by their median values and ranges. Relationships between mRNA levels of the different target genes, and between mRNA levels and clinical parameters, were identified using nonparametric tests, namely the chi-square test (relation between two qualitative parameters), the Mann–Whitney *U* test (relation between one qualitative parameter and one quantitative parameter) and the Spearman rank correlation test (relation between two quantitative parameters). Differences were considered significant at confidence levels greater than 95 % ($P < 0.05$). In order to evaluate the efficacy of a molecular marker (TLR9 level) in two populations (metastatic versus non metastatic patients) in the absence of an arbitrary cut-off value, data were summarized in an ROC (receiver operating characteristic) curve [23]. The AUC (area under curve) was calculated as a single measure for discriminate efficacy. Metastasis-free survival (MFS) was determined as the interval between initial diagnosis and detection of the first metastasis. Survival distributions were estimated by the Kaplan–Meier method, and the significance of differences between survival rates were ascertained with the log-rank test.

Results

Endosomal TLR8 and TLR9 are Overexpressed in IBCs

We measured mRNA levels of the 10 TLRs isoforms in a first series of 84 patients (21 HR+/ERBB2+, 21 HR+/ERBB2-, 21 HR-/ERBB2+ and 21 HR-/ERBB2-) with unilateral invasive breast tumors, and in 10 normal breast tissues by using quantitative RT-PCR assays (Supplemental table 1). For the 10 TLR genes, the mRNA values of the breast cancer samples were normalized such that the median of the 10 normal breast tissue mRNA values was 1. To determine the cut-off point for altered TLR isoforms expression in breast cancer tissues, the *NTLR* value, calculated as described in Materials and

Methods, was determined for 10 normal breast RNA samples. As these values were consistently between 0.02 (TLR9) and 2.76 (TLR6), values of 3 or more were considered to represent overexpression of these 10 genes in tumor RNA samples. We previously used the same approach to determine cut-off points for tumor gene overexpression [24, 25]. Only *TLR9* gene (and to a lesser extent *TLR8*) exhibited marked overexpression in breast tumors, mainly in the HR-/ERBB-/TNC subgroup (*TLR8*: med 3.15, min 0.38, max 11.33; *TLR9*: med 6.07, min 0.54, max: 52.05). In the present study, the overexpressions of *TLR8* ($P = 0.047$) and *TLR9* ($P = 0.00098$) in TNC subgroup were statistically significant (Table 1). Consequently, we further studied TLR9 (and stopped studying the 9 others TLR genes) in the subsequent analyzes.

TLR9 Protein is Overexpressed in Breast Cancer Cells

TLR9 mRNA Expression is Correlated With Amount of the Protein in IBCs

IHC assay performed from a subgroup of 38 breast tumors belonging to the series of 84 tumors tested by quantitative RT-PCR, confirmed TLR9 protein localization in breast epithelial cancer cells. Carcinoma cells exhibited a diffuse cytoplasmic TLR9 staining with various intensity and sub membranous (endolysosomal localization) accentuation in some cases (Fig. 1). Stromal cells, including immune cells, endothelial cells, fibroblasts and adipocytes were used as internal positive controls. TLR9 overexpression (Supplemental Table 2) was observed at protein level in our first series of 38 IBCs ($n = 8/38$; 21 %), including 13 TNCs ($n = 5/13$; 38.5 %). TLR9 protein overexpression in the TNC subgroup was confirmed in a larger series of 80 TNCs ($n = 32/80$; 40 %). A statistically significant correlation was identified ($P = 0.0086$; Mann–Whitney *U* test) between *TLR9* mRNA and protein expression levels, suggesting that TLR9 deregulation is mainly transcriptional in breast tumors.

TLR-9 Protein is Expressed in pre-Invasive Breast Lesions

To determine whether TLR9 protein showing marked up-regulation in invasive breast tumors is also expressed at an early step of breast carcinogenesis, we analyzed TLR9 expression by immunohistochemistry in a series of 10 normal breast tissues and 40 pre-invasive neoplastic lesions. A discrete physiological sub-apical and cytoplasmic immunostaining was inconstantly observed in some luminal cells of normal breast tissue (Supplemental Figure 2A), and a diffuse TLR9 cytoplasmic immunoreactivity was observed in pre-invasive breast lesions from the initial stage of atypical columnar metaplasia (IHC score 1; Supplemental Figure 2B) with a stronger staining in atypical ductal hyperplasia (IHC score 2;

Table 1 *TLR1 to TLR10 mRNA levels in 84 IBCs compared with 10 normal breast tissues*

TLR genes	Normal tissues (<i>n</i> = 10)		IBC (<i>n</i> = 84)		HR+/ERBB2- (<i>n</i> = 21)		HR+/ERBB2+ (<i>n</i> = 21)		HR-/ERBB2+ (<i>n</i> = 21)		HR-/ERBB2- (<i>n</i> = 21)		Normal versus IBC		Normal versus HR-/ERBB2-	
	Median (range)	<i>p</i> -value	Median (range)	<i>p</i> -value	Median (range)	<i>p</i> -value	Median (range)	<i>p</i> -value	Median (range)	<i>p</i> -value	Median (range)	<i>p</i> -value	ROC-AUC	<i>p</i> -value	ROC-AUC	<i>p</i> -value
<i>TLR1</i>	1 (0.38–2.02) ^a		0.62 (0.01–3.08) ^a		0.56 (0.18–1.37)		0.55 (0.29–0.96)		0.65 (0.24–2.33)		1.14 (0.01–3.08)		0.11 (NS)		0.343	0.97 (NS)
<i>TLR2</i>	1 (0.31–1.95)		0.71 (0.09–3.74)		0.55 (0.09–0.97)		0.48 (0.27–1.00)		0.92 (0.30–1.61)		1.30 (0.12–3.74)		0.16 (NS)		0.364	0.31 (NS)
<i>TLR3</i>	1 (0.39–2.57)		0.48 (0.05–2.29)		0.49 (0.16–2.29)		0.39 (0.17–1.61)		0.50 (0.19–0.86)		0.52 (0.05–0.93)		0.000083		0.118	0.00028
<i>TLR4</i>	1 (0.07–2.58)		0.51 (0.11–1.83)		0.36 (0.11–0.69)		0.39 (0.24–1.83)		0.60 (0.29–1.06)		0.88 (0.14–1.68)		0.014		0.261	0.18 (NS)
<i>TLR5</i>	1 (0.54–1.42)		0.50 (0.03–2.61)		0.51 (0.09–1.34)		0.50 (0.14–2.21)		0.48 (0.03–1.19)		0.50 (0.16–2.61)		0.00040		0.157	0.0060
<i>TLR6</i>	1 (0.73–2.76)		0.88 (0.18–4.33)		0.80 (0.18–4.19)		0.65 (0.28–1.49)		0.94 (0.34–2.04)		1.43 (0.23–4.33)		0.11 (NS)		0.343	0.75 (NS)
<i>TLR7</i>	1 (0.19–2.03)		0.64 (0.12–3.12)		0.57 (0.12–1.25)		0.55 (0.23–1.76)		0.64 (0.28–3.12)		0.73 (0.12–2.13)		0.081 (NS)		0.330	0.55 (NS)
<i>TLR8</i>	1 (0.36–2.18)		1.16 (0.14–11.33)		0.70 (0.14–2.30)		0.68 (0.31–3.89)		1.58 (0.40–6.66)		3.15 (0.38–11.33)		1.00 (NS)		0.500	0.047
<i>TLR9</i>	1 (0.02–2.33)		1.62 (0.00–52.05)		0.75 (0.00–6.35)		1.05 (0.15–11.60)		1.71 (0.35–19.51)		6.07 (0.54–52.05)		0.11 (NS)		0.654	0.00098
<i>TLR10</i>	1 (0.31–1.36)		0.62 (0.04–12.29)		0.49 (0.07–2.15)		0.65 (0.04–1.81)		0.49 (0.06–7.24)		0.86 (0.12–12.29)		0.12 (NS)		0.349	0.87 (NS)

^a Median (range) of gene mRNA levels. The values are normalized such that the median of the TLR gene values for the 10 normal breast tissues was 1

Supplemental Figure 2C) and ductal carcinoma in situ (IHC score 3; Supplemental Figure 2D).

TLR9 mRNA Levels of Expression are Observed in Human Breast Tumor Xenografts

To confirm *TLR9* expression in the cancer epithelial cells, we specifically quantified the human *TLR9* transcript in a series of 39 human breast tumors xenografted in mice. We used this species-specific PCR array to distinguish human/epithelial *TLR9* expression from murine/stromal *TLR9* expression. We observed marked human *TLR9* transcripts (Ct < 35) in 92.3 % (*n* = 36/39) of the human breast tumor xenografts. The *NHs-TLR9* values ranged from 1 to 60.4 (median = 7.2).

TLR9 mRNA Expression is Correlated With Classical Clinicopathological Prognosis Factors and Metastasis-Free Survival

In order to determine the prognosis significance of expression pattern of *TLR9* in human breast tumors, we analyzed *TLR9* mRNA levels in a large series of 350 primary breast tumors from patients with known clinical/pathological status and long-term outcome (Table 2). Among the 350 breast tumor RNA samples tested, 50 IBCs (14.3 %) showed *TLR9* mRNA overexpression (*NTLR9* from 3.02 to 49.9). We sought links between *TLR9* mRNA level status and standard clinicopathological and biological factors in breast cancer. Significant positive associations were observed between the tumor group showing *TLR9* overexpression and histopathological grade III (*P* = 0.000083), estrogen receptor negative (*P* = 0.00000067), progesterone receptor negative (*P* = 0.00056) and molecular subtypes (*P* = 0.000012). Among the 64 TNC (RH-/ERBB2-) samples tested, 19 tumors (29.7 %) showed *TLR9* mRNA overexpression, compared to the total breast tumor population (14.3 %).

Recent literature data suggested that *TLR9* is implicated in induction of EMT mainly through NF-κB activation [6]. EMT plays an important role in breast cancer metastasis, especially in the TNC subtype. We hypothesized that *TLR9* overexpression could promote EMT in TNCs. We analyzed 9 major candidate transcripts involved in EMT (Supplemental Table 3) in the series of 71 TNCs (Supplemental Table 4). We observed a significant positive association between *TLR9* expression and *ZEB2*, and a trend towards significance with *SNAIL*, *TWIST1* and *VIM* (only significantly using the Spearman rank correlation test). Significant positive correlations in the total population of 350 breast tumor RNAs were confirmed between *TLR9* and *TWIST1* (*r* = +0.167; *P* = 0.002) and *VIM* (*r* = +0.203; *P* = 0.0002). It is noteworthy that no correlation was observed between *TLR9* and *CDH1/E-cadherin* (*r* = −0.010;

Fig. 1 IHC expression of TLR9 by epithelial cancer cells in IBCs of the TNC subgroup. TLR-9 IHC score 0 **a**, TLR9 IHC score 1 **b**, TLR9 IHC score 2 **c**, TLR9 IHC score 3 **d**

NS) in the series of 350 breast tumors, as in the series of 71 TNCs (Supplemental Table 5).

Interactions between TLR9 and EGFR pathways were recently identified in colorectal cancer [26]. We previously observed by RT-PCR and IHC only a low percentage (11 %) of *EGFR* overexpression in the TNC subgroup and mainly *EGFR* under-expressions in the 3 other subgroups [27]. In the present study, we observed a positive statistical correlation between *TLR9* and *EGFR* mRNA levels ($r = 0.437$; $P < 10^{-7}$) in the series of 350 breast tumor RNAs.

To further investigate whether *TLR9* mRNA expression could be of prognosis relevance, the log-rank test was used to identify link between MFS (metastasis-free survival) and *TLR9* mRNA expression. Firstly, the relationship between RFS and *TLR9* overexpression status was analyzed using the Log-rank test at the threshold of 3. Results showed that MFS was not significantly influenced by *TLR9* overexpression status ($P = 0.30$) (data not shown). Area under the curve analyzes were performed to identify the cut-point allowing to divide the cohort into relevant *TLR9* expression subgroups. We therefore compared tumors with low *TLR9* expression ($NTLR9 \leq 0.59$; $n = 138$; 39.4 %), with those with moderate and high *TLR9* expression ($NTLR9 > 0.59$; $n = 212$; 60.6 %). High *TLR9* mRNA levels showed a trend towards significance association with better MFS ($P = 0.05$) (Fig. 2a). At the protein level, we could identify in our series of 80 TNCs a statistically significant correlation between high TLR9 protein levels with better MFS ($P = 0.000011$) (Fig. 2b). As we observed *TLR9* overexpression more frequently associated with histopathological grade III and estrogen receptor negative, but associated with favorable outcome in the total population (Table 3), we tested relationship between *TLR9* expression and MFS in these three tumor subgroups of poor prognostic. High *TLR9* mRNA levels of expression showed significance association with

favorable MFS in histopathological grade III ($P = 0.043$) and estrogen receptor negative ($P = 0.007$) (data not shown).

TLR9 Signaling Pathways are Activated in TLR9-Overexpressed TNCs and Induce Overexpression of NF- κ B, Cytokines and Chemokines

To explore the possible activation of the TLR9 signaling pathways in the TLR9-overexpressed breast tumors, we tested relationship between expression of 25 genes involved in the *MyD88* and *TICAM1/TRIF* pathways in 36 low *TLR9*-expressing TNCs and 35 high *TLR9*-expressing TNCs. We selected a homogenous population of tumors (only TNCs), as it represents the breast cancer subtype with the highest proportion of *TLR9*-overexpressed tumors. We selected 25 major candidate genes involved in the *TLR9* downstream/interconnected signaling pathways from literature's data: (i) endosomal TLR7, 8 and 9 ($n = 3$), (ii) molecular adaptors *MYD88* and *TICAM1/TRIF* ($n = 2$), (iii) downstream *TICAM1/TRIF* and *MyD88* pathways ($n = 4$), (iv) transcriptional factors *IRF* ($n = 2$) and *NF κ B* ($n = 8$) and (v) inflammatory cytokines and chemokines ($n = 6$). By using both the Mann–Whitney *U* test (relation between one qualitative parameter and one quantitative parameter) and the Spearman rank correlation test (relation between two quantitative parameters), we found a significant positive association between (i) *TLR9* expression and (ii) *TLR7*, *TLR8* (Toll-like receptors), *MYD88* (molecular adaptors), *IKBKE*, *IRAK1*, *IRAK3* (downstream pathways), *IRF7*, *IRF3* (IRF transcription factor), *RELB*, *NFKB2*, *NFKB1*, *NFKIB*, *NFKBIA*, *REL* (*NF κ B* complex) and *TNF*, *IFN γ* , *IL1 β* and *IL6* (inflammatory cytokines and chemokines) (Supplemental Table 5). A part of these results was confirmed by RT-PCR in our large series. Significant positive correlations in the total population of 350 breast

Table 2 Characteristics of the 350 patients and relation to metastasis-free survival

Total	Number of patients (%) 350 (100)	Number of relapse (%) 143 (100)	P-value ^a
Age			
≤ 50	80 (22.9)	35 (43.7)	0.18 (NS)
> 50	270 (77.1)	108 (40.0)	
SBR histological grade^{b,c}			
I	41 (12.0)	8 (19.5)	0.018
II	165 (48.2)	72 (43.6)	
III	136 (39.8)	60 (44.1)	
Lymph node status^d			
0	83 (23.8)	26 (31.3)	0.0000018
1–3	179 (51.3)	62 (34.6)	
> 3	87 (24.9)	55 (63.2)	
Macroscopic tumor size^e			
≤ 25 mm	154 (44.6)	50 (32.5)	0.0027
> 25 mm	191 (55.4)	92 (48.2)	
ER-status			
Negative	111 (31.7)	49 (44.1)	0.050
Positive	239 (68.3)	94 (39.3)	
PR status			
Negative	166 (47.4)	74 (44.6)	0.06 (NS)
Positive	184 (52.6)	69 (37.5)	
ERBB2 status			
Negative	259 (74.0)	104 (40.2)	0.42 (NS)
Positive	91 (26.0)	39 (42.9)	
Molecular subtypes			
RH- ERBB2-	64 (18.3)	25 (39.1)	0.14 (NS)
RH- ERBB2+	46 (13.1)	23 (50.0)	
RH+ ERBB2-	195 (55.7)	79 (40.5)	
RH+ ERBB2+	45 (12.9)	16 (35.6)	

^a Log-rank test. NS: not significant^b Scarff Bloom Richardson classification^c Information available for 342 patients^d Information available for 349 patients^e Information available for 345 patients

tumor RNAs were identified between *TLR9* and *IKBKE* ($r = +0.401$; $P < 10^{-7}$), *NFKB1* ($r = +0.296$; $P = 0.0000002$) and *TNF* ($r = +0.582$; $P < 10^{-7}$).

TLR9 is Also Overexpressed by Immune/Inflammatory and Stromal Cells of the Tumor Microenvironment

Because TLR9 signaling pathways induce overexpression of cytokines and chemokines, we analyzed different subtypes of stromal cells in our series of 80 TNCs (Supplemental Table 6). We performed TLR9, CD4, CD8, FoxP3, CD45RO and CD31 immunostaining of cancer-associated fibroblasts (CAFs), mononuclear inflammatory cells (MICs), cytotoxic and memory T lymphocytes and endothelial cells. We could observe in TNCs significant positive correlations between cancer cells overexpressing TLR9 and fibrous ($P = 1.2 \times 10^{-6}$) and inflammatory stromal profiles ($P < 10^{-7}$) with accumulation of CAFs ($P = 7 \times 10^{-3}$) and MICs ($P = 4.2 \times 10^{-4}$) overexpressing TLR9, CD4 lymphocytes ($P = 3.8 \times 10^{-5}$), Treg lymphocytes ($P = 6.7 \times 10^{-5}$), CD8 CD45RO memory cells ($P = 0.016$) and high microvessel density ($P < 10^{-7}$).

Immunohistochemical analysis of TLR9 expression in epithelial cancer cells and stromal cells suggests the existence of phenotypic profiles with prognosis significance, depending on TLR9 expression pattern by cancer cells and immune/stromal cells, presence of an inflammatory/immune infiltrate and microvessel density. Three subtypes could be identified (Supplemental Figure 3): (i) a first subtype ($n = 32/80$; 40 %) associating high TLR9 expression by cancer cells, a desmoplastic and hypercellular stroma, numerous CAFs and MICs overexpressing TLR9 (score 3), and a high microvessel density (score 3), (ii) a second subtype ($n = 27/80$; 33 %) associating low TLR9 expression by cancer cells, a predominantly edematous stroma, rare CAFs and MICs overexpressing TLR9 (score 1) and a low microvessel density (score 1), and (iii) a third subtype ($n = 21/80$; 27 %) associating low TLR9 expression by cancer cells, a predominantly fibrotic and hypocellular stroma, moderate number of CAFs and MICs overexpressing TLR9 (score 2) and a moderate microvessel density (score 2).

We found that TNCs with overexpressed TLR9 protein were associated with a better MFS ($P = 0.0000011$) and that

Fig. 2 Breast tumors expressing TLR9 and MFS. Breast tumors with high TLR9 are associated with a better prognosis compared to breast tumors with TLR9 low expression (Fig. 2a: Global population-ARN/RT-PCR, Fig. 2b: TNC population-Protein/IHC)

TNCs with an inflammatory microenvironment with numerous CAFs, MICs and CD8 lymphocytes were associated with a better prognosis (respectively $P = 0.000012$, $P = 0.000013$, $P = 0.0000042$ and $P = 0.00000076$).

During cancer progression, development and maintain of cancer-associated inflammation is provided by a chemokine inducer initiated by simultaneous activation of NF- κ B and signal transducer and activator of transcription 3 (STAT3). In order to explore these 2 co-activators located both in stromal/immune cells of the tumor microenvironment and in epithelial cancer cells, we finally performed phosphoSTAT3 immunostaining (Tyr705 and Ser727) in our series of 80 TNCs. We could observe that in TNCs with TLR9 overexpression ($n = 32/80$; 40 %), nuclear phosphoSTAT3 (Tyr705) was observed in 81.2 % ($n = 26/32$) of epithelial cancer cells (Supplemental Figure 4) as compared to nuclear phosphoSTAT3 (Tyr705) immunostaining of cancer cells belonging to the 48 normal or low TLR9 expressed TNCs ($n = 15/48$; 31.2 %; $P = 0.000012$). Conversely, by using

phosphoSTAT3 Ab (Ser727), we showed that in TNCs with TLR9 overexpression, nuclear positivity was observed in 21.9 % ($n = 7/32$) of epithelial cancer cells as compared to nuclear immunostaining of cancer cells belonging to the normal or low TLR9 expressed TNCs ($n = 32/48$; 66.7 %) ($P = 0.000086$). PhosphoSTAT3 (Tyr705) and phosphoSTAT3 (Ser727) were also variably expressed by immune and non-immune stromal cells.

Discussion

TLRs are actually considered as major actors implicated in chronic inflammatory-driven carcinogenesis [28, 29]. In the present study, we hypothesized that a functional TLR pathway may exist in IBCs, particularly in the TNC subtype and be activated by both epithelial cancer cells and stromal cells. We analyzed expression levels of TLRs and downstream and interconnected signaling pathways molecules at the mRNA level by real time quantitative RT-PCR and at the protein level by immunohistochemistry. Among the 10 TLRs analyzed, TLR9 and to a lesser extent TLR8 endosomal TLRs were overexpressed in our series of IBCs. TLR9 mRNA overexpression was observed in 14.3 % ($n = 50/350$) of breast tumors but interestingly in 29.7 % ($n = 19/64$) of the TNC subtype. By using IHC, TLR9 was not only expressed by the neoplastic epithelial population but also by several subtypes of stromal and immune/inflammatory cells belonging to the tumor microenvironment. Epithelial cancer cells expressed TLR9 with varying frequency (0 to 100 %), intensity levels (score 0 to 4) and topography, including cytoplasmic/endosomal and rarely nuclear or sub membranous localization. Significant statistical positive correlation was observed between TLR9 mRNA and protein expression levels ($P = 0.0086$). We also confirmed TLR9 expression in cancer epithelial cells by quantifying specifically the human TLR9 transcript in a large series of 39 human malignant breast tumours xenografted in mice. Taken together, our results showed that TLR9 transcripts are translated into TLR9 protein in tumor epithelial cells, with a strong correlation between TLR9 mRNA overexpression and TLR9 protein abundance, suggesting regulation at transcriptional level. Moreover, TLR9 overexpression in pre-invasive breast lesions is indicative that TLRs may play a pivotal role at early stages of breast carcinogenesis.

EMT was recently identified as a crucial morphomolecular process in cancer progression. NF κ B family of transcription factors plays pivotal roles in both promoting and maintaining an invasive phenotype mainly by controlling master regulators of EMT and by repressing epithelial phenotype. In our series, significant positive correlations were observed between TLR9 and ZEB2, TWIST1 and VIM. Ultimately, as an interconnection was recently identified between TLR9 and EGFR pathways, we also explored EGFR at mRNA level and identified a

Table 3 Relationship between *TLR9* mRNA status and standard clinicopathological and biological factors

	Total population (%)	Number of patients (%)		<i>p</i> ^a
		TLR9 mRNA expression ≤3	TLR9 mRNA expression >3	
<i>Total</i>	350 (100)	300 (85.7)	50 (14.3)	
<i>Age</i>				
≤ 50	80 (22.9)	68 (22.7)	12 (24)	0.84 (NS)
> 50	270 (77.1)	232 (77.3)	38 (76)	
<i>SBR histological grade</i> ^{b,c}				
I	41 (12.0)	38 (12.8)	3 (6.5)	0.000083
II	165 (48.2)	154 (52.0)	11 (23.9)	
III	136 (39.8)	104 (35.1)	32 (69.6)	
		4	4	
<i>Lymph node status</i> ^d				
0	83 (23.8)	67 (22.4)	16 (32)	0.25 (NS)
1–3	179 (51.3)	154 (51.5)	25 (50)	
> 3	87 (24.9)	78 (26.1)	9 (18)	
		1	0	
<i>Macroscopic tumor size</i> ^e				
≤ 25 mm	154 (44.6)	132 (44.6)	22 (44.9)	0.97 (NS)
> 25 mm	191 (55.4)	164 (55.4)	27 (55.1)	
		4	1	
<i>ER-status</i>				
Negative	111 (31.7)	80 (26.7)	31 (62)	0.00000067
Positive	239 (68.3)	220 (73.3)	19 (38)	
<i>PR status</i>				
Negative	166 (47.4)	131 (43.7)	35 (70)	0.00056
Positive	184 (52.6)	169 (56.3)	15 (30)	
<i>ERBB2 status</i>				
Negative	259 (74)	227 (75.7)	32 (64)	0.082 (NS)
Positive	91 (26)	73 (24.3)	18 (36)	
<i>Molecular subtypes</i>				
RH- ERBB2-	64 (18.3)	45 (15.0)	19 (38)	0.000012
RH- ERBB2+	46 (13.1)	34 (11.3)	12 (24)	
RH+ ERBB2-	195 (55.7)	182 (60.7)	13 (26)	
RH+ ERBB2+	45 (12.9)	39 (13.0)	6 (12)	

^aχ² Test^b Scarff Bloom Richardson classification^c Information available for 342 patients^d Information available for 349 patients^e Information available for 345 patients^f Mann Whitney

statistically significant correlation between *TLR9* and *EGFR* expression levels.

We further investigated whether *TLR9* overexpression by cancer epithelial cells was associated with TLR signaling pathway overexpression/activation [30]. By using real time quantitative RT-PCR in the TNC subgroup of IBCs, we confirmed that TNCs overexpressing *TLR9* were characterized by activation of numerous genes implicated in *TLR9* downstream signal transduction pathways: adaptors (*MYD88*), intracellular multimolecular mediators (*IKBKE*, *IRAK1*, *IRAK3*) and transcription factors (*IRF7*, *IRF3*, *NFκB* complex). In accordance with the literature, we did not observe overexpression of *TICAM/TRIF* and *TRAF6* usually activated after dimerization of endosomal *TLR3* and cell membrane *TLR1* to *TLR6* and *TLR10* [31].

Functional and activated TLRs play a pivotal role in promoting reciprocal interactions between cancer epithelial cells and their microenvironment during tumor progression. Cancer epithelial and stromal/immune cells overexpressing TLRs release numerous cytokines, chemokines, metalloproteases and integrins [32–34]. This resulting profile is associated with immune tolerance, tumor progression and promotion of angiogenesis. By using RT-PCR in our series of TNCs, we could observe overexpression of pro-inflammatory cytokines, including *TNF*, *IFNγ*, *IL1β* and *IL6*, in accordance with activation and nuclear translocation of transcription factors NF-κB, IRF7 and IRF3. These data were consistent with morphological characteristics of the tumor microenvironment observed in TNCs [28].

TLR9 levels of expression may have statistical prognostic value. *TLR9* overexpression by cancer epithelial

cells previously showed to be related to high grade or late stage of evolution in several carcinomas, including breast cancer [35–41]. Results from our study are in agreement with those reported in the literature [42]. Indeed, we found an association between *TLR9* gene overexpression by tumor epithelial cells and SBR histopathological grade III and hormone receptor negativity. We observed a trend for IBCs with high *TLR9* expression to have better MFS after surgery than IBCs with low *TLR9* mRNA expression in the total population ($P=0.05$), and in various subgroups of poor prognosis including histopathological grade III ($P=0.043$) and estrogen receptor negative ($P=0.007$). We also observed at the protein level that TNCs with *TLR9* overexpression were associated with a better prognosis. Interestingly, TNCs with *TLR9* overexpression were significantly correlated with development of a fibrous ($P=1.2\times 10^{-6}$) and inflammatory microenvironment ($P<10^{-7}$) with accumulation of CAFs and MICs overexpressing *TLR9* and numerous CD4 lymphocytes, Treg lymphocytes and CD8 CD45RO memory cells ($P=0.016$). Moreover, high contents of stromal inflammation, CAFs and MICs overexpressing *TLR9* and numerous cytotoxic CD8 lymphocytes were also associated with a better prognosis [43].

Cancer-associated chronic inflammation initiated by epithelial cancer cells and stromal/immune cells overexpressing *TLR9*, is amplified and sustained by a chemokine inducer composed of activated NF- κ B and STAT3 [44, 45] which may promote cancer progression and therapeutic resistance [46]. Furthermore, several studies identified TLRs, such as *TLR9*, as crucial activators of the JAK–STAT3 pathway. Recent data showed that *TLR9* is highly expressed in glioblastoma stem-like cells (GSCs) [46] and that activated STAT3 binds to the *TLR9* promoter and contributes to *TLR9* expression in GSCs [47], providing a potential mechanism by which infection or inflammation enhances carcinogenesis. In our series of 80 TNCs, we observed that in TNCs with *TLR9* overexpression, nuclear phosphoSTAT3 (Tyr705) was identified in 81 % of cancer cells as compared to 31 % of cancer cells in TNCs with normal or low *TLR9* level of expression. Conversely, by using phosphoSTAT3 (Ser727), a highly positive nuclear score was observed in 22 % of TNCs with *TLR9* overexpression and 67 % of TNCs with normal or low *TLR9* expression. PhosphoSTAT3 (Tyr705) and phosphoSTAT3 (Ser727) were also variably expressed by immune and non-immune stromal cells belonging to the tumor microenvironment. We identified a population of CD15+, phosphoSTAT3+ myeloid-derived suppressor cells (MDSCs) with granulocytic morphology that accumulate in peri-vascular and/or within vascular lumen of breast cancer tissues (data not shown). MDSCs are important inhibitors of T-cell responses in

solid tumors. It seems that preexisting STAT3 activity in MDSCs, can skew *TLR9* signaling toward supporting tumor angiogenesis while blocking antitumor immunity [48–50].

Recently, *TLR9* stimulation by CpG-ODN has been shown to activate NF- κ B pathway, leading to Th1 immunostimulation and antitumor immune responses [51, 52]. A promising targeted combined therapy should associate T-cell therapy and STAT3 inhibitors linked to CpG/*TLR9* that could silence STAT3 in cancer cells and stromal /immune cells and favor down-regulation of cancer stem cells and antitumor immune responses [53, 54].

In addition, IBCs microenvironment contains tertiary lymphoid structures (TLSs), different subtypes of cancer-associated fibroblasts (CAFs) and dendritic cells (DCs) which are derived from myeloid (MDCs) or lymphoid precursors (plasmacytoid DCs, pDCs). It has been shown that pDC-infiltrating primary breast tumors represent an independent prognostic factor associated with poor outcome [55]. Investigating these subtypes of microenvironment cells will provide important clues in breast carcinogenesis and tumor progression.

In summary, our study contributed to a better biopathological characterization of IBCs subtypes by identifying an activated endosomal *TLR9* signaling pathway in 15 % of IBCs, mainly belonging to the TNC subgroup (30–40 %). Moreover, our results suggest that *TLR9* expression is a prognosis biomarker in TNCs, and highlight the interest of initiating clinical trials combining *TLR9* agonists and STAT3 inhibitors in TNCs. These results also warrant studies of other altered genes identified in the present study as putative predictive biomarkers for selecting patients most likely to benefit from these drugs [56].

Compliance With Ethical Standards

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Conflict of Interest The authors declare no conflict of interest.

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