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Original Article

TLR3 correlated with cervical lymph node metastasis in patients with papillary thyroid cancer

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Abstract: Papillary thyroid cancer (PTC) is the most rapidly increasing endocrine malignancy worldwide. Although less aggressive than the majority malignancies, PTC exhibits extensive cervical lymph node metastasis in early stage of PTC. However, the underlying molecular mechanism of this early-metastasis remains unknown. Toll like receptors (TLRs) constitute a crucial component of the innate immune response to bacterial and viral pathogens. Emerging evidence suggests that TLRs play important roles in cancer progression, invasion and immune evasion, whereas whether TLRs have any role in PTC remains to be clarified. In this study, we found that TLR3 was present in both PTC specimen and various thyroid cancer cell lines. Further IHC analysis of 63 PTC patients revealed that TLR3 expression was associated with cervical metastasis, but not correlated with patients' TNM staging, extrathyroidal invasion. In addition, TLR3 promoted migration of K1 cells in vitro. Activation of TLR3 increased cancer stem cell marker and migration promoting CD44 expression in vitro, indicating that TLR3 might promote metastasis of PTC via modulating CD44 expression. Taken together, our data revealed that TLR3 is correlated with cervical metastasis of PTC and might be an essential prognostic indicator and target for PTC metastasis.

Keywords: Papillary thyroid cancer (PTC), metastasis, Toll-like receptor (TLR), CD44

Introduction

Papillary thyroid cancer (PTC) is the most common endocrine malignancy worldwide [1]. The incidence of which has increased dramatically over the past decades, being the "most rapidly growing" cancer among women [1, 2]. Although indolent and less aggressive compared with other endocrine malignancies such as pancreatic carcinoma, extensive cervical lymph node metastasis occurs in early stage of this disease (stage I or stage II). Over 45% of PTC patients were found to have concurrent cervical metastasis when first diagnosed [3], which may contribute to the principle of routine central neck dissection. Moreover, the clinically undetectable "micro-metastasis" to the central or even lateral compartment of the neck still hinders the curability of this disease [4]. Pre-operational ultrasound screening is limited in differentiating inflammatory lymph nodes from "micro-metastatic" lymph nodes. Unfortunately however, molecular markers that are suitable for predicting "early metastasis" or "micro-metastasis" are still under exploration.

TLRs are enzymatically-inactive single membrane-spanning proteins that were first found on immune cells for recognizing pathogen-associated molecular patterns (PAMPs) and fighting against pathogen infection [5, 6]. Recent reports revealed them "double-edge sword" as they might expressed on tumor cells and play important roles in promoting cancer proliferation, invasiveness and immune evasion [7]. Our previous study showed that TLR4 attenuates tumor inflammation by targeting pro-inflammatory cytokine IL-6 through MP contained miR-let-7b pathways, thus regulates the inflammatory microenvironment so as to better service the proliferation of hepatic cancer cells [8]. Moreover, TLR stimulated cancer cells up-regulate B7H1 and B7H2 and secrete substances to inhibit T cell proliferation and NK cell activity

[9]. A growing body of evidence showed that TLR might also be involved in metastasis in breast, ovarian and colon cancer [10]. However, whether TLRs have any roles in carcinogenesis, development or metastasis of PTC is still unclear.

In this study, expression of TLR3 was first determined in PTC patients and cell lines. Then, its relationship with patients' clinical data such as TNM staging, cervical lymph node metastasis and extrathyroidal invasion was analyzed, revealing its close relationship with cervical metastasis in PTC. Finally, in vitro assays using TLR3 specific agonist showed that upon stimulation, TLR3 promoted migration of PTC cell line, upon which metastasis associated molecule expression was modulated, partially revealed some underlying mechanism.

Materials and methods

Patients and control subjects

A total of 63 patients who underwent total thyroidectomy for PTC were included in this study from Dec, 2013 to Jun, 2014. Patient's diagnosis was confirmed by pathological examination after surgical operation. The thyroid function of patients included were summarized in **Table 1**. The study was approved by the ethics committee of Tianjin Medical University, and was conducted according to the ethical guidelines of our institution. Informed consents were obtained from all participants.

Cell lines

Human PTC cancer cell lines (K1, BCPAP), anaplastic thyroid cancer cell line (8505c, 8305c) and normal thyroid epithelium cell line (Nthy-ori 3-1) were obtained from the Cell Bank of the Chinese Academy of Medical Sciences (Beijing, China) and cultured according to their guidelines.

RT-PCR and real-time PCR

Total RNA was isolated(TRIzol, Invitrogen), reverse transcribed (TOYOBO) and subjected to polymerase chain reaction (PCR) for 30 cycles at 94°C for 0.5 min, 55°C for 0.5 min, and 72°C for 1 min. Primers were as follows: for human TLR3, forward: 5'-GCCAACTTCACAAGGTATAG-3', reverse: 5'-GACAAGCCATTATGAGACAG-3'; human CD44, forward: 5'-CTACTTCAGACAACCACAAG-3', reverse: 5'-TCCATATCCATCCTTCTTCC-3'; human GAPDH, forward: 5'-ACCACAGTCCATGCCATCAC-3', reverse: 5'-TCCACCACCCTGTTGCTGTA-3'; human CCR4, forward: 5'-GAAATTCTGTGGTGGTTCTG-3', reverse: 5'-TGCCAGGTATCTATCAATGC-3'; human ICAM-1, forward: 5'-GCCAGCTTATACACAAGAAC-3', reverse: 5'-CCACAGTGATGATGACAATC-3'; human SGK1, forward: 5'-ATGGTGGAGAGTTGTTCTAC-3', reverse: 5'-GAGGCTTGTTCAGAATGTTG-3'. Real-time PCR was performed with a FastStart Universal SYBR Green Master Kit (Roche, USA) on an ABI 7900 system. mRNA levels were normalized to GAPDH (glyceraldehyde 3-phosphate dehydrogenase). Protocols of real-time PCR were described previously [8].

Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.4) for 48 h, followed by embedding into paraffin sections (4 mm). The endogenous peroxidase activity was inactivated in a solution containing 3% hydrogen peroxide (H_2O_2) in methanol. Then, the sections were blocked for 2 h at room temperature with 1.5% blocking serum. Sections were incubated with anti-TLR3 (1:100, Abcam) antibody overnight at 4°C. Labelled horseradish peroxidase was applied for 30 min at room temperature, followed by application of diaminobenzidine solution until color developed. Slides were counterstained with haematoxylin. Negative control slides were performed without primary antibody. The staining intensity was graded as follows: 0 (no staining), 1 (weak staining), 2 (moderate staining), 3 (intense staining).

Cell migration assay

Migration assays were performed in Transwell (Corning Inc., 8.0 μm pore size). For migration assay, 3×10^4 K1 cells in serum-free medium (with 10 μg/mL poly I:C, or 1 μg/mL LPS or PBS) were added to the upper wells. Media containing 10% FBS (with 10 μg/mL poly I:C, or 1 μg/mL LPS or control PBS) were added to the lower wells. Cells that migrated through the filter after 6 or 12 h were stained with 0.2% crystal violet and counted by phase microscopy.

Western blot

Cell lysates and pre-stained molecular weight markers were separated by SDS-PAGE followed

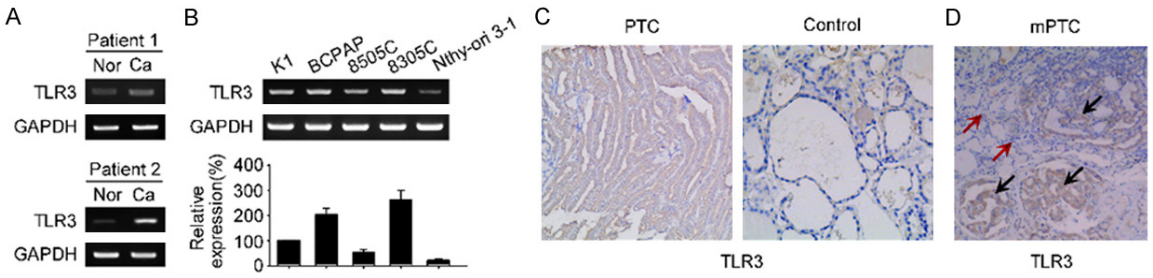

Figure 1. TLR3 is present in patients with PTC and PTC cell lines. (A and B) mRNA expression of TLR3 in thyroid cancer tissue (A) and cell lines (B). Fresh specimens were obtained within 3 hours of surgical operation, homogenized to isolate RNAs and subjected to reverse-transcriptase PCR (A and upper panel of B) or quantitative real-time PCR (lower panel of B) analysis for TLR3 determination. (C) Representative IHC analysis of TLR3 in paraffin embedded PTC and control thyroid tissue. (D) IHC analysis of heterogeneous TLR3 staining in a representative mPTC. The black arrows show staining of TLR3 in the cancerous nests, yet red arrows indicate no staining of TLR3 in the normal thyroid epithelium in the same individual. Nor, normal thyroid tissue; Ca, thyroid cancer; PTC, papillary thyroid cancer; mPTC, multifocal PTC.

Table 1. Clinical characteristics of patient's thyroid function (N=63)

Thyroid function	TLR3 expression		*P* value
	−~++	+++~++++	
TSH (mIU/L)	2.66±2.01	2.85±1.09	0.632
fT3	4.22±0.72	4.33±0.68	0.579
fT4	18.2±3.32	17.5±3.64	0.328
Tg (mIU/L)	23.6±34.3	27.9±37.1	0.662
A-Tg (IU/mL)	47.8±88.3	54.8±101.6	0.776
A-TPO (IU/mL)	55.7±71.3	39.8±62.3	0.350

Data were expressed as mean ± standard deviation. fT3, free triiodothyronine; fT4, free tetraiodothyronine.

by transfer onto nitrocellulose membranes. The membranes were blocked in TBST (Tris-buffered saline with 0.5% of Triton X-100) containing 5% nonfat milk, and probed with antibodies. After incubation with the secondary antibody conjugated with horseradish peroxidase, membranes were extensively washed, and the immunoreactivity was visualized by enhanced chemiluminescence according to the manufacturer's protocol (ECL kit, Santa Cruz, Santa Cruz, CA). Antibody β-actin (SC-47778) was purchased from Santa Cruz, CD44 (ab157107) from Abcam.

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM) and interpreted by Mann-Whitney Rank Sum Test or repeated measure ANOVA. Significant levels were defined as p values less than 0.05.

Results

TLR3 is present in patients with PTC and PTC cell lines

To ascertain whether TLR3 is expressed in PTC, fresh PTC specimens and normal thyroid tissues were obtained and subjected to reverse-transcriptase PCR for the detection of TLR3. Representative expression patterns were shown in **Figure 1A**. TLR3 was differentially but specifically expressed in PTC specimens, whereas little or no TLR3 expression was found in normal thyroid tissues. Then, TLR3 expression status was further determined in vitro in PTC cell line K1 and BCPAP, anaplastic thyroid cancer cell line 8505 c, 8305 c and normal thyroid epithelial cell line Nthy-ori 3-1 (**Figure 1B**). In accordance with the fresh tissues, RT-PCR and quantitative real-time PCR showed that TLR3 is expressed in all cancerous cell lines but normal thyroid epithelial cell line Nthy-ori 3-1, suggesting its specificity for malignancies.

IHC analysis of TLR3 further confirmed its presence. Although varied in staining degree, TLR3 expressed only in PTC samples but not normal thyroid controls (**Figure 1C**). Of note, TLR3 staining was homogenous within the cancerous lesion in single loci PTC (one cancerous lesion in two thyroid lobes) specimen; whereas an obvious heterogeneous expression pattern of TLR3 was observed across the multifocal PTC (mPTC, ≥ two cancerous lesions in two thyroid lobes) (**Figure 1D**). TLR3 staining was most prominent and specifically within the scattered

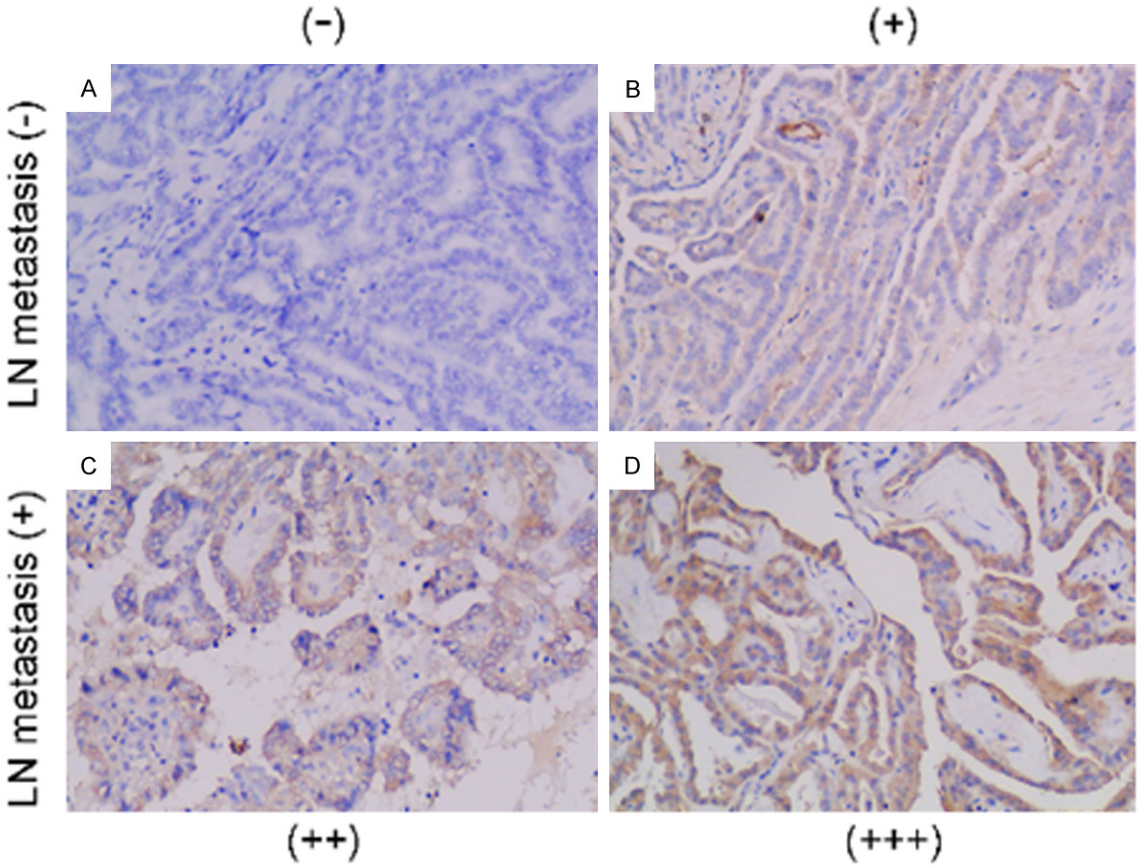

Figure 2. TLR3 expression is correlated with cervical micro-metastasis in papillary thyroid cancer patients. (A) Examples of different degrees of staining in tissue sections: − (A), + (B), ++ (C), and +++ (D). Magnification: (A-D) 100× magnification. (B) TLR3 expression correlates strongly with cervical metastasis in PTC patients.

Table 2. Clinical characteristics of patient's clinicopathological factors (N=63)

Clinicopathological factors	No. of patients	TLR3 expression		*P* value
		−~++	+++~++++	
Gender				
Female	47	21	26	0.6161
Male	16	6	10	
Age				
<45 years	32	16	16	0.2444
≥45 years	31	11	20	
TNM staging				
I-II	41	20	21	0.1946
III-IV	22	7	15	
Extrathyroidal invasion				
Yes	19	6	13	0.2346
No	44	21	23	
Lymph nodes metastasis				
Yes	30	8	22	0.0133
No	33	19	14	

cancerous loci, whereas interestingly, the surrounding normal parenchymal thyroid tissue is free of TLR3 staining, thus providing convincing evidence showing its specificity in differentiating benign and malignant origins (**Figure 1D**).

TLR3 is correlated with cervical metastasis in patients with PTC

Since TLR3 is present specifically in PTC, we thus further asked whether this expression pattern correlates with patients' clinical data. A total of 63 patients were included in this study. The clinical characteristics of these patients were summarized in **Table 1**.

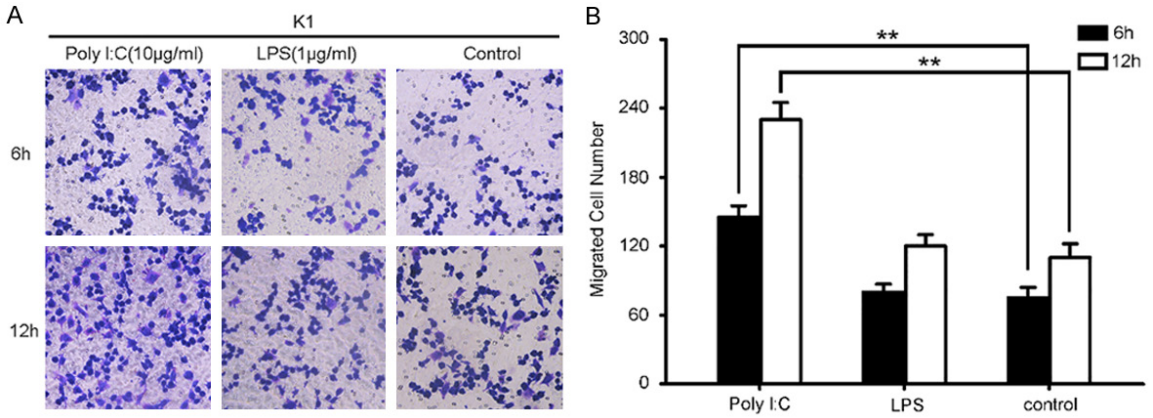

Figure 3. Activation of TLR3 promotes migration of PTC in vitro. A. Representative Transwell migration assay shows significant improvements in cell mobility upon activation of TLR3. 3×10^4 K1 cells were implanted into the upper chamber of the well and treated with 10 μg/mL poly I:C, 1 μg/mL LPS or PBS as controls. B. Quantization of Transwell migration assay was counted in a blinded fashion by light microscopy at 20× magnification. Data is representative of three independent experiments. **, P<0.01.

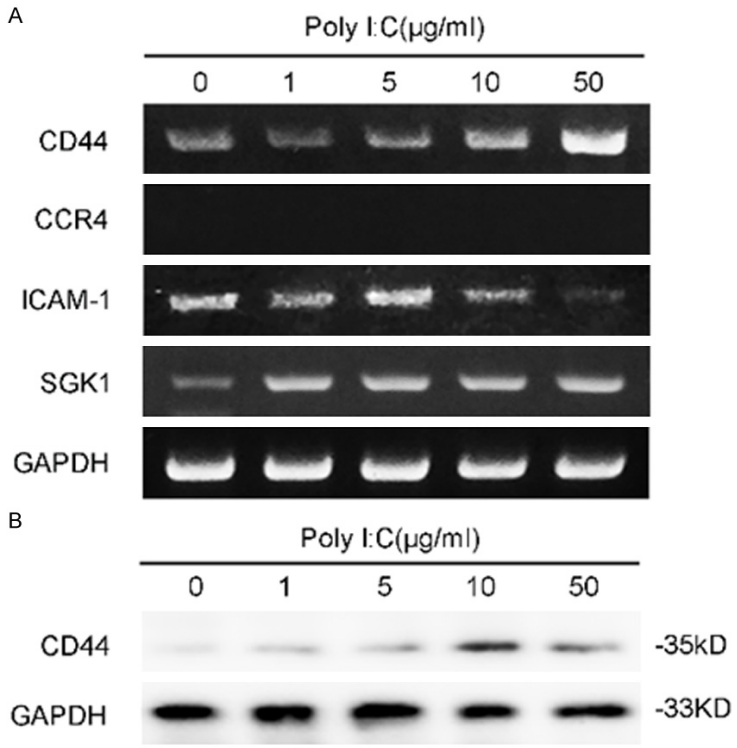

Figure 4. Expression of selected migration associated molecules upon stimulation of TLR3. A. Reverse-transcriptase PCR shows the expression of CD44, ICAM-1, SGK1 and CCR4. K1 cells were treated with various concentration of poly I:C (as indicated) or PBS for 24 h and then subjected to total RNA isolation and PCR. B. Western blot shows the expression of CD44 at 36 h when treated with poly I:C.

TLR3 staining varied in degree in all these PTC patients. Representative pictures showing various degree of TLR3 were shown in **Figure 2**. We found that TLR3 expression was closely correlated with cervical metastasis in PTC patients (**Table 2**). The TLR3 staining was strong in 73.3% (22/30) of PTC patients with cervical lymph node metastasis, whereas only 42.4% (14/33) of patients without cervical lymph node metastasis showed strong staining of TLR3. Thus, higher expression of CD44 was more frequently detected in patients with cervical lymph node metastasis in contrast to those who without. However, TLR3 expression was not correlated with extrathyroidal invasion, TNM staging, gender or age, demonstrating that TLR3 activation might contribute to only metastasis but not aggressiveness in patients with PTC.

Activation of TLR3 promotes migration of PTC in vitro

Next, to further confirm whether TLR3 plays any direct role in promoting cancer metastasis in PTC, in vitro migration assay was thus performed using TLR3 specific agonist poly (I:C) at indicated time period. As shown in **Figure 3A** and **3B**, TLR3 activation significantly increased the K1 cell's mobility. The mean migrated cell increased from 75±9 to 145±11 cells per sight

at 6 h, and 110±10 to 230±30 cells at 12 h, respectively ($P<0.01$), while control or TLR4 activation had no such effect.

TLR3 activation resulted in up-regulation of CD44 in PTC cells

Since TLR3 activation promotes migration of PTC cells, it is reasonable to hypothesize that migration and adhesion associated molecular events might play a role in this process. In this setting, the expression of CD44, ICAM-1, SGK1 and CCR4 upon the stimulation of TLR3 was tested via RT-PCR. As shown in **Figure 4A**, ICAM-1 and CCR4 showed no significant change. However, CD44 RNA expression were increased with respect to the poly I:C stimulation, which reached a maximal effect on the 50 μg/mL. On the other hand, SGK1 expression was also up-regulated but not correlated with the degree of TLR3 stimulation (**Figure 4A**). Western blot analysis of CD44 further confirmed the above result (**Figure 4B**), indicating that up-regulation of CD44 might be responsible for this migration promoting effect.

Discussion

TLRs were first found on immune cells responsible for initiating innate and acquired immune responses [11]. Then, tumor TLRs were identified and was being indicated as a major culprit of cancer progression and immune evasion [12]. Our study provides evidence that the TLR3 specifically expressed in PTC tissue, even a heterogeneous expression pattern was observed within multifocal PTC specimen that showing both strong staining cancerous tissue and non-staining normal thyroid tissue, suggesting a potential helpful diagnostic tool in making difficult pathological diagnosis.

Early lymph node metastasis is one of the characteristics of PTC biological behavior and accounts for the majority of the cancer related death throughout the course [13]. Even the "microcarcinomas of thyroid" (less than 1cm in diameter) have a tendency to metastasis in 45% of all papillary thyroid microcarcinomas (PTMCs) [14]. Although remarkable progress has been made in understanding molecular pathogenesis of thyroid cancer, yet only a few studies have shed light on thyroid cancer early metastasis. González-Reyes et al reported that tumor with high TLR3 expression was significantly associated with higher probability of metastasis in breast cancer [15]. Moreover, LPS may potentiate the ability of colon cancer cells to metastasis via Nox enzyme mediated redox signaling in colon cancer [16]. Recently, Fabbri et al reported that tumor-secreted miR-21 and miR-29a also can function by binding as ligands to receptors of the murine TLR7 and human TLR8 in immune cells, triggering a TLR-mediated pro-metastatic inflammatory response that may ultimately lead to tumor growth and metastasis [17]. Our data provides direct evidence that TLR3 expression is correlated with PTC cervical lymph node metastasis. It is of more clinical significance to predict whether there is lymph node metastasis in the lateral compartment. In this setting, clinical strategy taken TLR3 expression into consideration might be of great importance in surgery management decision.

In our study, we demonstrated in vitro that TLR3 promoted metastasis of PTC cells, although the underlying mechanisms and molecular events require further demonstration. Interestingly, upon stimulation of TLR3, SGK1 as well as CD44 expression was up-regulated. CD44 and its variants are glycosylated transmembrane glycoproteins that are implicated in tumor growth and metastasis [18, 19]. Bourguignon et al reported that CD44 phosphorylates Rho GTPase, thus further phosphorylates myosin light chain II (MLC II) to promote tumor progression [20]. In our previous study, we found that TLR4 signaling directly activated MyD88/myosin light chain II (MLC II) pathway and transformed cytoskeleton in H22 cancer cells [8]. It is thus reasonable to hypothesize that TLR3 promotes metastasis via up-regulation of CD44 expression. Moreover, since human CD44 has various isoforms, which subtype of CD44 variants plays a predominant role still needs to be unfolded.

In sum, we found TLR3 expression is closely related with cervical lymph node metastasis. And, in vitro activation promoted migration of PTC cells, which suggests a potential prognostic and intervention target for the management of PTC metastasis.

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Disclosure of conflict of interest

None.

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