Increased expression of Toll-like receptor 5 during progression of cervical neoplasia


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The purpose of this study was to determine whether Toll-like receptor 5 (TLR5) expression was associated with disease progression in cervical neoplasia. TLR5 expression was evaluated by immunohistochemistry (IHC) in 55 formalin-fixed paraffin-embedded cervical tissues; 10 normal cervical specimens, 9 low-grade cervical intraepithelial neoplasias (CINs), 12 high-grade CINs, and 24 invasive squamous cell carcinomas (ISCCs). TLR5 expression was also evaluated at the RNA level, in fresh, frozen cervical carcinoma tissues by real-time quantitative RT-PCR. TLR5 expression, which was mainly observed as cytoplasmic staining, gradually increased in accordance with the histopathologic grade in the following order: low-grade CIN less than high-grade CIN less than ISCC (P < 0.001). Immunohistochemical staining showed that TLR5 expression was undetectable (80%) or weak (20%) in normal cervical squamous epithelial tissues. However, moderate expression was detected in 33.3% of low-grade CIN (3/9), 41.7% of high-grade CIN (5/12), and 45.8% of ISCC (11/24). Strong expression was detected in as much as 33.3% of high-grade CIN (4/12) and 50% of ISCC (12/24). Contrary to IHC results, real-time quantitative RT-PCR revealed that TLR5 expression in tumors was not statistically different compared to normal cervical tissues (P = 0.1452). The IHC result suggests that TLR5 may play a significant role in tumor progression of cervical neoplasia and may represent a useful marker for malignant transformation of cervical squamous cells.

KEYWORDS: cervical neoplasia, squamous epithelium, Toll-like receptor 5.

Cervical cancer progresses slowly in a multistep process, which involves the transformation of normal cervical epithelium to preinvasive cervical intraepithelial neoplasia (CIN) to ultimately invasive cervical cancer(1,2). Although it is commonly accepted that high-risk human papillomaviruses (HPVs) are associated with cervical cancer(1,2), HPV infection alone is thought to be insufficient for malignant transformation of HPV-infected cells. Hence, other genetic alterations are likely to be required in addition to an HPV infection for the development of cervical cancer. Moreover, the identification of such gene alterations, if they exist, could be of considerable importance for cervical cancer screening and treatment.

Toll-like receptors (TLRs) are members of the interleukin-1 receptor superfamily that share significant homology in their cytoplasmic region, for example, the Toll/IL-1R (TIR) domain(3,4). The structures of TLRs are leucine-rich repeat domains in their extracellular regions and a TIR domain in the intracellular regions. Originally identified in the early Drosophila larvae(5), 12 members of the TLR family have been identified in mammals to date(6). They are essential for the discrimination between self and nonself. Their critical role in the innate immune system is through recognizing the unique, highly conserved molecular structures of pathogens that are not expressed by host, which are referred to as pathogen-associated molecular patterns(7). Pathogen-associated molecular patterns are characterized by bacterial lipoproteins(8), viral

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double stranded RNA/poly (I:C), lipopolysaccharides, flagellin, and bacterial DNA, which engage TLR2, TLR3, TLR4, TLR5, and TLR9, respectively. The TLR signaling pathway activates several different signaling elements, including nuclear factor κB (NF-κB) and extracellular signal-regulated kinase/c-Jun-NH2-kinase/p38, which regulate many immunologically related proteins. Many of these elements are also involved in tumorigenesis and tumor growth, suggesting that TLRs may affect tumor growth.

It was previously reported that some human reproductive tract epithelial cell lines (endocervical, ectocervical, vaginal cell lines) express several TLR genes. The first report of in vivo protein expression or distribution of TLR in the female reproductive tract in humans was made by Fazeli et al. He showed that TLR 1, 2, 3, 5, and 6 were present in the epithelia of different regions of female reproductive tract. Although there are a limited number of studies on the association between TLR expression and human malignancy, several recent reports on the expression of TLRs and cancers have been published, including prostate cancer, stomach cancer, lung cancer, and breast cancer. There is also a report that flagellin signaling through TLR5 activates NF-κB and mitogen-activated protein kinase in colon cancer cell lines. The relationship between TLR expression on a malignant epithelial tumor and its precursor lesion was first reported by Schmausser et al. He found that there was a diffuse TLR4 and TLR5 expression in the tumor cells of gastric carcinoma as well as its precursor lesion, but not in noninflamed gastric mucosa. Based on the above research that the TLR5 is present in female reproductive tract and altered expression of TLR5 is associated with malignancy in other organs, we tried to find out if there were any reports between TLR5 and cervical carcinogenesis. But to our knowledge, no report on TLR5 in cervical cancer has been made.

So, the goal of this study was to evaluate the expression of TLR5 in normal cervical epithelium and cervical neoplasia, in order to determine whether altered expression of this protein is associated with neoplastic progression in the uterine cervix.

Materials and methods

Tissues samples

A total of 55 paraffin-embedded, formalin-fixed tissue specimens were used in this study. These included 9 low-grade CINs (CIN 1), 12 high-grade CINs (CIN 2, 3), and 24 invasive squamous cell carcinomas (ISCCs) (Table 2) in addition to 10 normal cervical specimens. All specimens were obtained from the Department of Pathology, Samsung Medical Center, Sungkyunkwan University School of Medicine. All tissue specimens were obtained from either conization procedures or radical hysterectomies. Sections (5 μm) were cut from formalin-fixed, paraffin-embedded blocks and were used to prepare routine histopathology and immunohistochemistry (IHC) slides. Representative tumor sections and sections of adjacent normal mucosa were studied by IHC. One dedicated gynecological pathologist reviewed all cases with regard to histologic type, grading, and lymph node status.

For real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR), fresh, frozen tumor biopsy specimens (n = 13) from ISCC patients were obtained at the time of surgery after Institutional Review Board approval was obtained for the study (these 13 specimens were different from the tissues used for IHC). Also normal cervical tissues (n = 10) were obtained as control specimens from the women who underwent hysterectomy for benign disease that was confirmed by normal cytology reports. To obtain the normal cervical tissue, fresh biopsies (5–8 mm3) were obtained before undertaking any surgical procedures. And we used Dispase II (2.4 U/mL, Roche, Mannheim, Germany) to obtain the normal epithelial tissues only from the entire cervical tissues including stroma. These biopsies were washed in sterile phosphate-buffered saline (PBS) for a few minutes and incubated in Dispase II at 37°C, stromal side down, for 1 h. The epithelial sheets were then gently removed from the stromal layers and washed two times in sterile PBS before extraction of total RNA.

Immunohistochemical studies

Immunohistochemical staining was performed using the standard avidin–biotin complex peroxidase method (DakoCytomation, Glostrup, Denmark) on formalin-fixed, paraffin-embedded tissue sections. Sections (5 μm) were mounted on poly-L-lysine coated glass slides and dried at 37°C overnight, deparaffinized in xylene, washed in graded ethanol, and finally in PBS (pH 7.4). To increase specificity and sensitivity, samples were pretreated with Target Retrieval Solution (pH 6, S 2367; DakoCytomation) in a pressure cooker (15 Pa) at 121°C for 3 min. After cooling and rinsing in distilled water, endogenous peroxidase activity was blocked with 3% H2O2 for 15 min and samples were then preincubated with a protein blocking solution for 10 min. Primary antibody (goat polyclonal anti-human TLR5, ab1654, Abcam plc,
Cambridge, UK) was applied in a dilution of 1/400 in PBS at 4°C overnight in a humid chamber. Slides were washed three times in PBS and then incubated with secondary biotinylated antibody for 30 min at room temperature. Antibody–antibody complexes were detected using the streptavidin–peroxidase method using diaminobenzidine as a chromogen substrate (Vectastain ABC-Kit, Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Tissue sections were lightly counterstained with hematoxylin and then examined by light microscopy. Mouse serum was used in place of the primary antibody as a negative control.

One dedicated gynecological pathologist (S.Y.S) and one gynecological oncologist (W.Y.K) blindly reviewed slides and evaluated the immunohistochemical data. The distribution and intensity of cell staining were assessed. The percentage of cells expressing TLR5 was estimated by dividing the number of positively stained tumor cells by the total number of tumor cells per high-power field. The staining was scored on a scale from 0 to 3+ as follows: 0, no staining; 1+, less than 50% with weak intensity; 2+, more than 50% with weak or moderate intensity; 3+, more than 50% with strong intensity.

**RNA isolation and reverse transcription**

Total RNA was isolated from tissues using an easy-spin (genomic DNA free) Total RNA Extraction Kit (iNtrON Biotechnology, Seoul, Korea), according to the manufacturer’s instructions. Total RNA concentrations were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE). We synthesized first-strand complementary DNA (cDNA) from 4 μg of total RNA using 2.5 μM of random hexamer primers (Applied Biosystems, Foster City, CA) and 200 U of Superscript III Reverse Transcriptase (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer’s instructions. Samples were incubated at 25°C for 5 min, 50°C for 60 min, and inactivated at 70°C for 15 min. To measure TLR5 messenger RNA (mRNA) expression levels in sample tissues, we used cDNA mixtures of six cervical cancer cell lines (CaSki, ME-180, C-33A, HT-3, MS751; American Type Culture Collection [ATCC]) as controls.

**Real-time quantitative RT-PCR of TLR5 mRNA expression**

The TLR5 mRNA expression was measured using the TaqMan technology on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer’s instructions. TaqMan probes and primer pairs for TLR5 and glyceraldehyde phosphate dehydrogenase (GAPDH) were Assay-on-Demand gene expression products (Applied Biosystems). To avoid amplification of genomic DNA, segments were chosen to hybridize to sequences at the junction between two exons, as follows: TLR5 (Hs00152825_m1, probe 5’-CAGTCACAAACCAGGGATGTCA-T-3’), TaqMan Gene Expression Assays (6-carboxyfluorescein); GAPDH (4310884E), and a human GAPDH endogenous control (VIC/carboxytetramethylrhodamine probe). PCRs were prepared in a final volume of 20 μL, with a final concentration of 1× TaqMan universal PCR master mix (Applied Biosystems) and cDNA equivalent to 10 ng input RNA. Reaction mixtures were assembled at 4°C, followed by PCR consisting of 50°C for 2 min, AmpliTaq Gold activation at 95°C for 10 min, followed by 95°C for 15 sec and 60°C for 1 min, for 40 cycles. Each PCR run included three points of the calibration curve for TLR5 and GAPDH (3-fold diluted human cervical cancer cell line RNA mixtures), a no-template control, the calibrator cDNA mixtures, and patient samples, all in triplicate. The relative expression of TLR5 mRNA was normalized to the amount of GAPDH in the same cDNA using the standard curve method described by the manufacturer.

**Statistical analysis**

Statistical calculations were carried out with SPSS for Windows version 11.0 (SPSS, Inc, Chicago, IL). The Jonckheere-Terpstra test was used to correlate the cumulative TLR5 expression with cancer progression, and a linear by linear association was used to compare the groups. The Wilcoxon two-sample test was used to compare the mean TLR5 expression in fresh, frozen ISCC tissues with normal cervical samples, as determined by real-time quantitative RT-PCR. P values of <0.05 were considered significant.

**Results**

**Immunohistochemical staining**

TLR5 expression was undetectable (80%) or weak (20%) in normal squamous epithelial tissue (Fig. 1A). In contrast to no moderate or strong immunoreactivity (2+–3+) detected in normal squamous epithelium, moderate expression was detected in 33.3% of low-grade CIN (3/9), 41.7% of high-grade CIN (5/12), and 45.8% of ISCC (11/24) (Fig. 1B–D). And strong expression was detected in as much as 33.3% of high-grade CIN (4/12) and 50% of ISCC (12/24) (Table 1).
Statistical analysis showed that TLR5 expression was significantly higher in high-grade CINs and ISCCs compared with low-grade CINs or normal controls (Table 1). When the four specimen grades (ie, normal, low-grade CIN, high-grade CIN, and ISCC) were compared with regard to the frequency of positive staining, TLR5 expression was found to gradually increase with increasing histopathologic grade in the following order: low-grade CIN less than high-grade CIN less than ISCC \((P, 0.001, \text{Table 1})\). However, the TLR5 immunostaining scores were not found to correlate with patient age, FIGO stage, tumor size, local recurrence, or lymph node metastasis (data not shown). When invasive cervical carcinoma was compared with noninvasive cervical pathology regarding moderate to strong \((2+–3+)\) TLR5 expression, there was a significant statistical difference \((P < 0.001)\) between two groups.

**TLR5 mRNA expression using real-time quantitative RT-PCR**

To obtain highly sensitive measures of TLR5 expression in cervical carcinoma and normal cervical tissues, we used real-time quantitative RT-PCR. As shown in Figure 2, TLR5 gene expression was quantified in 13 ISCCs and in 10 normal squamous cervical tissues. Contrary to IHC results, we found that TLR5 mRNA expression was not statistically different between ISCC samples and normal cervical controls after comparison with the normal standard GAPDH \((P = 0.1452)\). In addition, no correlations were found between TLR5 expression levels and the above-mentioned clinical parameters.

### Discussion

Immunohistochemical staining shows that TLR5 expression increases in accordance with histopathologic grade of preinvasive condition and also shows that ISCC of cervix has more moderate to strong expression \((2+–3+)\) of TLR5 than noninvasive pathology of cervix \((P = 0.001)\). The expression of TLR5 in normal squamous epithelial tissue was either undetectable or weak. The level of TLR5 expression increased gradually with increasing grade of CIN, reaching a peak in invasive squamous cell carcinoma \((P < 0.001)\). But the
results of IHC and mRNA expression did not show any concordance. TLR5 mRNA expression was not statistically different between ISCC samples and normal cervical controls after comparison with the normal standard GAPDH ($P = 0.1452$). Abnormalities in the control of translation or accumulation of the TLR5 protein on the tumor cell membrane may account for this phenomenon. Decreased degradation of the protein may also be involved. This discordant result may also suggest that functional changes occur at the mRNA level during cervical neoplasia progression. But a definitive answer to this question is beyond the scope of tools used in this study.

The relationship between TLR expression on a malignant epithelial tumor and its precursor lesion was first reported by Schmausser et al. He found that there was a diffuse TLR4 and TLR5 expression in the tumor cells of gastric carcinoma as well as its precursor lesion, but not in noninflamed gastric mucosa. TLR expression on gastric carcinoma gives Helicobacter pylori the possibility to interact directly with tumor cells. In vitro, Helicobacter pylori induces via TLR5 IL-8 expression by gastric epithelial cell lines. This may have ultimate significance for tumor growth because IL-8 is thought to promote progression of gastric carcinoma by its angiogenic potential as well as by a direct effect on tumor invasion and metastasis via corresponding chemokine receptors CXCR1 and CXCR2.

Likewise, unregulated or inappropriate TLR activation resulting in excessive production of proinflammatory factors is involved in several inflammatory diseases. Tumor cells produce proinflammatory factors including nitric oxide, interleukin-6, and inflammatory cells. Considering this, the role of inflammation, which is thought to be mediated exclusively by immune cells, might need to be reevaluated with regard to cancer development. Therefore, TLR can be important because their primary role is in pathogen recognition.

It is already known that the primary risk factor for CIN and invasive cervical cancer is infection with oncogenic HPV. The oncogenic HPV E6 and E7 prevent cell cycle arrest and intrinsic, p53-dependent apoptosis (programmed cell death), thus allowing uncontrolled cell-cycle progression, resulting in an imbalance between proliferation and apoptosis that has been observed during cervical carcinogenesis.

Although it is commonly accepted that high-risk HPVs are associated with cervical cancer, HPV infection alone is thought to be insufficient for malignant transformation of HPV-infected cells. Hence, other genetic alterations are likely to be required in addition to an HPV infection for cervical cancer development. The biologic significance of the activation of TLR5 expression during the dysplastic cervical process CIN is not known, but it may be related to the implication of TLR5 in intracellular signaling pathways that

![Figure 2. Quantitative comparison of the TLR5 mRNA expression in an ISCC and normal cervical tissues (normal) using real-time quantitative RT-PCR with GAPDH as the control housekeeping gene. The ratios of TLR5/the GAPDH gene expression were calculated from triplicate samples and are presented. A statistically significant difference between TLR5 mRNA expression was not found between ISCCs and normal cervical tissues samples ($P = 0.1452$).](image)

**Table 2.** FIGO stage, lymph node status, HPV, and degree of immunoreactivity of the patients with ISCC

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Degree of immunoreactivity

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NA, not available; L/N, lymph node.
References


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