RESEARCH ARTICLE

Expression of Toll-like Receptor 9 Increases with Progression of Cervical Neoplasia in Tunisian Women - A Comparative Analysis of Condyloma, Cervical Intraepithelial Neoplasia and Invasive Carcinoma

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Abstract

Toll-like receptors (TLRs) are expressed in immune and tumor cells and recognize pathogen-associated molecular patterns. Cervical cancer (CC) is directly linked to a persistent infection with high risk human papillomaviruses (HR-HPVs) and could be associated with alteration of TLRs expression. TLR9 plays a key role in the recognition of DNA viruses and better understanding of this signaling pathway in CC could lead to the development of novel immunotherapeutic approaches. The present study was undertaken to determine the level of TLR9 expression in cervical neoplasias from Tunisian women with 53 formalin-fixed and paraffin-embedded specimens, including 22 samples of invasive cervical carcinoma (ICC), 18 of cervical intraepithelial neoplasia (CIN), 7 of condyloma and 6 normal cervical tissues as control cases. Quantification of TLR9 expression was based on scoring four degrees of extent and intensity of immunostaining in squamous epithelial cells. TLR9 expression gradually increased from CIN1 (80% weak intensity) to CIN2 (83.3% moderate), CIN3 (57.1% strong) and ICC (100% very strong). It was absent in normal cervical tissue and weak in 71.4% of condyloma. The mean scores of TLR9 expression were compared using the Kruskall-Wallis test and there was a statistical significance between normal tissue and condyloma as well as between condyloma, CINs and ICC. These results suggest that TLR9 may play a role in progression of cervical neoplasia in Tunisian patients and could represent a useful biomarker for malignant transformation of cervical squamous cells.

Keywords: Toll - like receptors - TLR9 - cervical cancer - immunohistochemistry

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Introduction

Cervical cancer (CC) is the second most common malignancy in Tunisia and a public health inquiry in women worldwide. In some African and Asian countries like China, its morbidity still ranks the first in gynaecological malignant tumors (Li et al., 2011; Wang et al., 2013; Wang et al., 2013; Mvundura and Tsu, 2014), leading to a revising scheme of preventive strategy (Al Moustafa et al., 2014) including vaccination (Othman et al., 2014; Hanley et al., 2014). Human Papillomaviruses (HPVs) have been recognized as its etiologic factor (Zur Hausen, 2009) and are classified as low risk and high-risk HPVs (HR-HPVs), the latter being associated with cervical intraepithelial neoplasia (CINs), mainly HPV16 and 18 that possess a high potential for progression into invasive squamous cells cervical carcinoma (ISCC). Development of CIN and ISCC from normal cervical tissue is a gradual process and is directly linked to persistent HR-HPV infection (Wang et al., 2013) but determinant factors for viral persistence and tumorogenic progression are ill-understood. Tumor associated inflammation have a key role in CC development (Boccardo et al., 2010) and an inflammatory related biomarker that can be predictive for CC progression and development could give good opportunities for novel therapeutic approaches. Toll-like receptors (TLRs) are important families of pattern recognition receptors, which recognize conserved components of microorganisms and trigger the immune response against invading pathogens (Zhou et al., 2013). The essential role of TLRs in the activation of the immune system was a revolutionary finding awarded in 2011 by the Nobel Prize for medicine (Wagner, 2012). Among them, TLR9 has been demonstrated as an important receptor of recognition against DNA invading pathogens and especially several DNA virus, inducing inflammatory

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and immune responses (Lund et al., 2003; Krug et al., 2004). Most of the previous reports have focused on their expression and function in cells of the immune system like B-lymphocytes and plasmacytoid dendritic cells. Current works are focusing on their association with cancers (Domingos-Pereira et al., 2013; Bodelon et al., 2014) and the use of vaccine with TLR agonist or antagonist in cervical cancer is now undergoing extensive investigations (Geller et al., 2010; Mai et al., 2013; Sajadian et al., 2014). Several previous works have shown that TLR9 signaling promotes tumor growth, survival and immune evasion in CC as in many other cancer types, such as gastric cancer (Schmausser et al., 2005), prostatic cancer (Gonzalez-Reyes et al., 2011) breast cancer (Bhattacharya and Yusuf, 2012), lung cancer (Ren et al., 2007), glioma (Wang et al., 2010), and oesophageal squamous cell carcinoma (Sheyhidin et al., 2011). But it has also been suggested to have anti-tumor activities (Chang et al, 2014) and whether TLR9 promotes or suppress cancer development remains under discussion, leading to investigations about TLR polymorphism and genetic variations of TLR pathway (Oliveira et al., 2013; Bodelon et al.,2014). The present study aims to evaluate the expression levels of TLR9 on Tunisian cervical samples in gradual stages of CINs and ISCC using normal cervical tissues and condyloma as control cases.

Materials and Methods

Tissues samples

53 archival (2008-2012) formalin fixed and paraffin-embedded tissues derived from cervical biopsies, conization or hysterectomy specimens collected from the files of the Department of Human and Experimental Pathology of Pasteur Institute of Tunis and five departments of pathology from the Northern, Central and Southern Tunisian hospitals were used in this study. All tissue sections were rechecked for pathological assessment of diagnosis in the Laboratory of Human and Experimental Pathology of Pasteur Institute of Tunis before the immunohistochemical assay. Eighteen subjects were diagnosed with different stages of CIN, including 5 CIN1, 6 CIN2, 7 CIN3 and were compared to 22 cases of ISCC, 7 condyloma and 6 samples of normal cervical squamous epithelium to serve as control cases.

Immunohistochemistry

Tissue sections 4μm thick were cut from formalin-fixed, paraffin-embedded blocks and were used to prepare routine histopathology and immunohistochemistry (IHC) slides. Sections were mounted on glass slide, deparaffinised in toluene (2×15 min) and rehydrated with ethanol. To increase specificity and sensitivity, slides were processed for antigen retrieval by boiling in 10 mmol/L citrate buffer (pH 6.0), 30 min in 96°C bain-marie and then left to cool for 25 min, rinsed in running water for 1min and dipped in distilled water for 2-3 dips. Endogenous peroxidase activity was blocked by a15 min incubation in peroxydase block (Leica kit NovolinkTM Max polymer detection system). Sections were washed in PBS (7.75 g Tris-HCl pH 7.5, 8.78 g NaCl ad 1 liter H_2O) and incubated for 15 min in protein block buffer provided in the kit. They were then incubated with an anti-TLR9 monoclonal antibody (Imgenex Cat.No IMG-305A, clone 26C593) at a 1/250 dilution for 45 min at room temperature, washed in PBS and incubated in post- primary Block buffer (Novocastra Leica Microsystems) for 30 minutes. Sections were rinsed in 1×Tris and incubated with Novocastra polymer for 30 min at room temperature. Bound antibodies were stained with AEC solution (Sigma) and counterstained with Hemalun (Merck). The slides were rinsed 3 times in bidistilled water and mounted on glass slides using Eukitt (Merck).

Two histopathologists blindly reviewed the slides and evaluated the immunohistochemical data under light microscopy with photography. Red or purple-red color in the cytoplasm of cells was defined as TLR9 positive staining. Based on the staining intensity and the distribution and the proportion of TLR9 positive cells in squamous epithelium, TLR9 immunoreactivity was scored on a scale from 0 to 4+ as follow: 0: no positive staining cells; 1+: less than 50% of cells with weak intensity; 2+: more than 50% of cells with moderate intensity; 3+: more than 50% of cells with strong intensity; 4+ : more than 80% of cells with very strong intensity.

Statistical analysis

SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis of the data. The mean scores of TLR9 expression in normal cervical epithelia, condyloma, CIN1, CIN2, CIN3 and ISCC were compared with the Kruskal-Wallis test ($p<0.05$).

Results

Immunohistochemistry

TLR9 immunoreactivity was intracellular (Figure 1). None of the 6 normal cervical tissue samples was stained (Figure 1B). TLR9 expression was detected in all slides of condyloma, CINs and ISCC. The intensity of immunostaining was constantly increasing from condyloma to ISCC. Staining intensity was weak in 71.4% of condyloma, and involved koïlocytic cells and some of the medium layer epithelial cells (Figure 1D). Weak intensity of staining was observed in 80% of CIN1 and was mostly involving the lower third epithelial layer (Figure 1F). 83.3% of CIN2 expressed TLR9 protein with a moderate intensity in the two lower thirds epithelial layers. Immunostaining was strong in 71.4% of CIN3, involving all epithelial layers (Figure 1J). All samples of ISCC displayed a very strong and diffuse staining (Figure 1L). There was also a strong expression of TLR9 in many of the stromal inflammatory cells. Distribution of TLR9 scoring according to the degree of immunoreactivity is detailed in Table 1.

Statistical results

The mean scores of TLR9 expression were clearly increasing from condyloma to CIN1, 2, 3 and ISCC (Figure 2). Statistical analysis of the immunostaining scores of TLR9 in the different cervical samples using the non parametric kruskall-Wallis test (Table1) showed

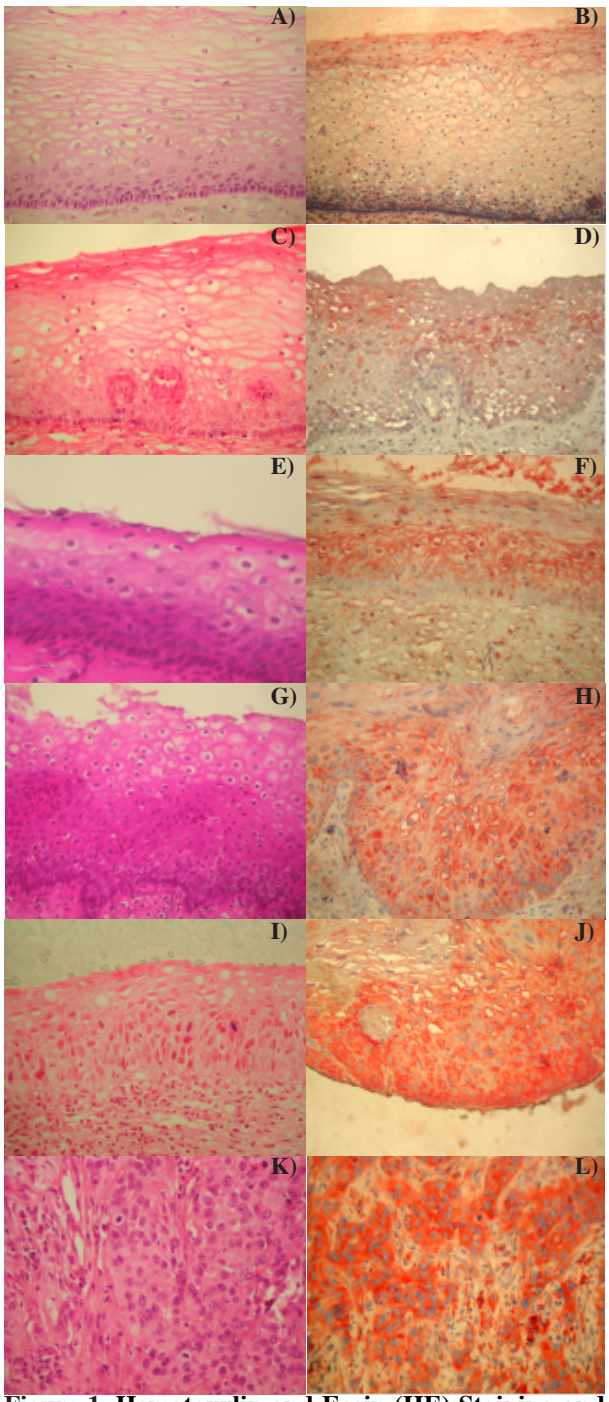

Figure 1. Hematoxylin and Eosin (HE) Staining and Expression of TLR9 Detected by Immunohistochemistry in the Tested Samples (High Power field x400). A) Normal cervical squamous epithelium serving as control cases; **B)** No TLR9 staining was detected in the control; **C)** Koilocytic changes in cervical epithelium of condyloma; **D)** Some of the koilocytic cells were weakly stained in condyloma; **E)** Atypical cells are restricted within lower third of squamous epithelium in CIN1; **F)** Moderate immunoreactivity of TLR9 within lower third of squamous epithelium in CIN1; **G)** Atypical cells are seen in lower two thirds of squamous epithelium in CIN2; **H)** immunoreactivity of TLR9 within two lower third of squamous epithelium in CIN2; **I)** Atypical cells exceeded lower two thirds of squamous epithelium in CIN3; **J)** Strong immunoreactivity of TLR9 diffusely distributed within the atypical layers of the cervical epithelium in CIN3. **K)** Cancer nests invaded into cervical wall in ISCC; **L)** Very strong and diffuse TLR9 expression in tumor epithelial cells of invasive carcinoma. Stromal inflammatory cells were stained too

Table 1. Expression of TLR9 in Normal, Condyloma, CIN1, 2, 3 and ISCC

Tissues	Degree of immunoreactivity				
	0	1+	2+	3+	4+
Normal	6/6(100)	0/6(0)	0/6(0)	0/6(0)	0/6(0)
Condyloma	1/7(14.2)	5/7(71.4)	1/7(14.2)	0/7(0)	0/7(0)
CIN1	0/5(0)	4/5(80)	1/5(20)	0/5(0)	0/5(0)
CIN2	0/6(0)	1/6(16.6)	5/6(83.3)	0/6(0)	0/6(0)
CIN3	0/7(0)	1/7(14.2)	1/7(14.2)	4/7(51.7)	1/7(14.2)
ISCC	0/22(0)	0/22(0)	0/22(0)	0/22(0)	22/22(100)

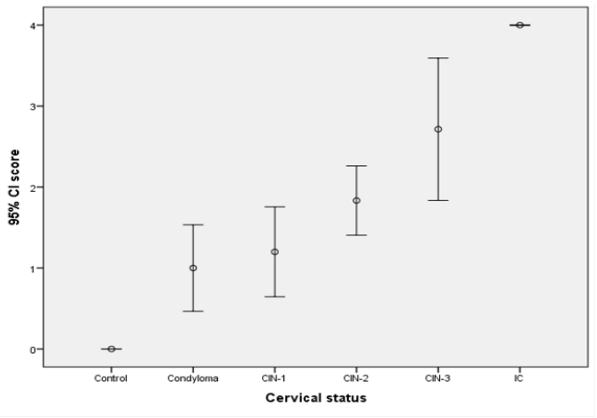

Figure 2. Mean Scores of TLR9 Expression According to the Grade of Neoplasia of the Cervix. The expression of TLR9 was increasing from normal cervical tissue to condyloma and CINs to invasive carcinoma (IC), Error bars: 95,00 CI

Table 2. Results of Multiple Comparaison of P value

Lesion	p value
normalxcondylome	<0.05
nomalxCIN1	<0.05
normalxCIN2	<0.05
normalxCIN3	<0.05
normalxISCC	<0.05
condylomexCIN1	>0.05
condylomexCIN2	>0.05
condylomexCIN3	<0.05
condylomexISCC	<0.05
CIN1xCIN2	>0.05
CIN1xCIN3	>0.05
CIN1xISCC	<0.05
CIN2xCIN3	>0.05
CIN2xISCC	<0.05
CIN3xISCC	>0.05

a significant increasing from normal cervical tissue to condyloma, CINs and ISCC, from condyloma to CIN3 and ISCC and from CIN1 and CIN2 to ISCC ($p<0.05$). The intensity of TLR9 staining in CIN3 and ISCC was similar. The mean scores of TLR9 imunoreactivity that were increasing from CIN1 to CIN2 and CIN3 was not statistically significant (Table 2).

Discussion

Cervical cancer is a major health inquiry in developing countries where Pap smear screening has not been successful to control the disease and HPV vaccine implementation is lacking cost-effectiveness studies. Immunotherapeutic approaches could give alternative

means for CC control and Toll-like receptors pathway appears as a good field of research. It has been demonstrated that under some conditions, TLRs expressed by tumors cells do play a pivotal role in regulating tumor growth, activating innate immunity against invading pathogens and cytokine production (Yu et al., 2013). TLR9 is an important receptor of recognition against DNA viruses (Zolini et al., 2014) and although a first study was consistent with the lack of relation between HPV and TLR9 expression and function (Andersen et al., 2006), it has been later demonstrated to be expressed in HPV-positive cervical neoplasia on human foreskin, vaginal and cervical keratinocytes cell lines maintaining episomal copies of HPV16 and 18, in several CC derived cell lines and in formalin-fixed paraffin-embedded or frozen cervical tissue sections (Karim et al., 2011; Werner et al., 2012). In this series, immunohistochemical scoring of TLR9 staining using a monoclonal antibody has shown that TLR9 expression is detected in all condyloma, CINs and ISCC with a significant gradual increasing level of expression from CIN1 to ISCC. Staining was weak in 80% of the CIN1 and moderate in 83.3% of CIN2, becoming strong in 71.4% of CIN3 to very strong in all ISCC. There was not any TLR9 expression among 6 normal cervical tissues and staining was weak in most of condyloma. Our findings were consistent with other reports (Lee et al., 2007; Hasimu et al., 2011; DeCarlo et al., 2012) which also used formalin-fixed and paraffin-embedded tissue specimens providing from Korean, Chinese and Canadian women. They have shown an increasing expression of TLR9 in neoplastic cervical epithelia in the course of progression of the disease, suggesting that TLR9 upregulation could be beneficial to tumor progression and invasiveness of cervical tissue. An increasing expression of TLR5 and more recently of TLR4 in CC is also reported (Kim et al., 2008; Chen et al 2012; Mai et al 2013). The possible way of action could be an increase of tumor pro-growth conditions through a selective pressure of the pro-inflammatory local microenvironment. TLR9 recognizes the ODN with CpG motif and after binding with the ligand, it may facilitate an immune evasion through inhibitory cytokines, inflammatory factors, proteinases, and other small molecules such as nitric oxide, IL-6 and IL-12 mimicking some characteristics of inflammatory cells. Their action can lead to resistance of tumor cells to CTL and natural killer attacks and evasion from immune surveillance (Zhou et al., 2013). The existence of opposite data in different ethnic population specimens makes a major problem in understanding the role of TLR9 signaling pathway in CC (Yu et al 2010; DeCarlo et al., 2012). It has been reported a decrease of TLR9 mRNA levels with cervical disease severity (Yu et al., 2010) or a gradual decrease in TLR9 expression along with the grade of CIN suggesting TLR9 expression as an early event in the development of CC in Mexican women (Moreno-Eutimio et al., 2013). Other studies have demonstrated an increase of TLR9 expression with HPV clearance (Daud et al., 2011). TLR9 expression and function can be abolished by HPV16 when using a quantification of TLR9 on cancer derived cell lines infected by HPV16 E6 and E7 proteins (Hasan et al., 2007; Hasan et al., 2013) and speculate that HPV may use TLR9 dysregulation as a strategy to avoid viral recognition. The interaction between HPV and components of the tumor microenvironment could impair TLR9 signaling and downregulate TLR9 gene expression leading to the reduction of the innate immune responses during chronic viral infections. New therapeutic approaches based on TLR agonists have then been proposed (Hirsch et al., 2010; Ohlschlager et al., 2011; Domingos-Pereira et al., 2013). The same inequity has been shown in other cancer types. Some authors has suggested an abnormal activation of TLRs on tumor cells that are associated with immune tolerance, tumor progression and propagation in oesophageal squamous cell carcinoma (Sheyhidin et al., 2011), pancreatic carcinogenesis (Ochi et al., 2012), colorectal cancer (Doan et al., 2011), breast cancer (Yang et al., 2010), lung cancer (Ren et al., 2007) and glioma (Wang et al., 2010). At the opposite, a protective action of TLR9 and TLR3 has been described in experimental models of glioma (El andaloussi et al., 2006), in a mouse model of colitis-associated colon cancer (Liu et al., 2010) and in chronic lymphatic leukemia (Spaner and Masellis, 2007). TLRs pathway is supposed here to provide signals that are necessary for the resolution of inflammation and TLR agonists have also been proposed as a good opportunity for cancer treatment as effective immune adjuvants or combined immunotherapy or as a modulator of cervical cancer cells chemosensitivity (Schmidt, 2006; Weng et al., 2011; Mason and Hunter, 2012). TLRs pathway could then induce different signals in CC as in other cancer types that could promote or inhibit tumour progression. The complicated interaction between tumor cells, immune cells pathogen-associated molecular patterns in the tumor microenvironment could promote either tumor progression or apoptosis and cancer regression. The reasons for those discrepancies are not clear but genetic variations of TLR9 pathway through a polymorphism in some TLR9 regions is now under question (Pandey et al., 2011; Roszak et al., 2012; Chen et al., 2012; Oliveira et al., 2013; Lai et al., 2013; Bodelon et al., 2014) and it seems now likely to talk about cancer susceptibility and TLR9 polymorphism (Chen et al., 2012; Dai et al., 2014). Our results suggest that TLR9 may play a role in progression of cervical neoplasia in Tunisian patient and could represent a useful biomarker for malignant transformation of cervical cells.

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