Exploring *TLR2* Gene Polymorphisms in Cervical Cancer Development

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Abstract

Human papillomavirus is a pathogen that directly infects cervical keratinocytes and may cause persistent infection that leads to cervical cancer. Toll like receptors (TLRs) play an essential role in initiating antiviral immune responses. Therefore, polymorphisms in *TLR* gene may contribute to cancer susceptibility. This study aimed to explore the *TLR2* gene distribution and susceptibility to cervical cancer. In this case-control study, cervical cancer patients and their controls were recruited from the Department of Obstetrics and Gynecology, Dr. Hasan Sadikin General Hospital. Genomic DNA was extracted from blood of patients with histopathologically confirmed cervical cancer (n=100) and from unrelated, healthy female controls (n=100) during 2011. Three single nucleotide polymorphisms (SNPs) of the *TLR2* gene were genotyped on the BeadXpress Reader system (Illumina)®. Chi square test was used to calculate the role of *TLR2* and susceptibility to cervical cancer. Only subjects with complete clinical and genetic data were analyzed. Analysis of TLR2 rs3804099, rs4696480 and rs5743708 of cervical cancer patients and controls showed no significant association with the cervical cancer risk (p 0.424, p 0.275, p 0.209, respectively). Further classification in the FIGO (*Fédération Internationale de Gynécologie et d'Obstétrique*) criteria for lower stage (FIGO I/II) and higher stage (FIGO III/IV) showed a lack of association between *TLR2* and cancer development, suggesting the possibility that *TLR2* polymorphism does not play a role in the susceptibility to cervical cancer in this study. Other toll like receptors may be involved in the cancer susceptibility. The significance of TLR polymorphism should be further studied. [**MKB.** 2013;45(4):257–62]

Key words: Cervical cancer, toll like receptor, TLR2

Polimorfisme Gen TLR2 dan Perkembangan Kanker Serviks

Abstrak

Kanker serviks disebabkan oleh human papillomavirus (HPV), patogen yang dapat langsung menginfeksi keratinosit serviks secara persisten dan dapat berkembang menjadi kanker. Toll like receptors (TLR) berperan dalam merangsang respons imun, sehingga polimorfisme gen TLR dapat berkontribusi dalam kerentanan terhadap kanker. Tujuan penelitian ini adalah untuk mengetahui distribusi gen TLR2 dan peranannya terhadap kerentanan kanker serviks. Pada studi kasus kontrol, DNA genomik diekstraksi dari darah penderita kanker serviks yang terdiagnosis secara histopatologi (n=100) dan kontrol dengan *Pap smear* normal (n=100) tahun 2011 di Departemen Obstetri dan Ginekologi RSUP Dr. Hasan Sadikin Bandung. Pemeriksaan tiga *single nucleotide polymorphisms* (SNP) gen TLR2 dilakukan menggunakan *BeadXpress Reader system* (Illumina). Hanya subyek dengan data klinik dan genetik lengkap yang dianalisis dengan menggunakan uji chi square. Analisis dari TLR2 rs3804099, rs4696480 dan rs5743708 antara pasien dan kontrol tidak menunjukkan perbedaan yang bermakna (p 0.424, p 0.275 dan p 0.209). Di antara pasien dengan klasifikasi FIGO (*Fédération Internationale de Gynécologie et d'Obstétrique*) tingkat rendah (FIGO I/II) dan tingkat tinggi (FIGO III/IV) juga tidak tampak perbedaan yang bermakna. Dari penelitian ini terbukti polimorfisme TLR2 tidak berperan dalam proses kerentanan maupun perkembangan terjadinya kanker serviks. Kemungkinan TLR yang lain seperti TLR 1, 3, 9 lebih berperan dalam perkembangan terjadinya kanker serviks. [**MKB.** 2013;45(4):257–62]

Kata kunci: Kanker serviks, toll like receptor, TLR2

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Introduction

Cervical cancer is the second most common cancer in women worldwide with approximately 500,000 new cases and 250,000 deaths each year.[1] In Indonesia, this cancer is considered the most frequent primary malignancy among women.[2] There is evidence that the immune system is of importance in the etiology of cervical cancer since human papillomaviruses (HPVs) are the main cause of the cancer.[3] HPV directly infects cervical keratinocytes and interferes in the toll-like receptor (TLR) signaling. Therefore, TLRs have been established to play an essential role in sensing and initiating the antiviral immune responses.[4] The TLR signaling pathways have also been known to play roles not only in cancer, but also in several infections, inflammation, and autoimmune diseases.[5] HPVs modulate TLR expression and interfere in the TLR signaling pathways which lead to persistent viral infection and carcinogenesis.[6]

There are 11 types of TLR with each type recognizes specific pathogens.[7] A recent study in Northern India demonstrated that common nucleotide variations in TLR pathways may be associated with the risk of cervical cancer.[8] This study showed that TLR expression in cervical cancer increases with HPV infection incidence. Nevertheless, there are no consistent associations between *TLR* gene polymorphism and infection risk.[9] To shed light on the effect of HPV infection and the role of TLR2, we conducted this study in Bandung, West Java, Indonesia to compare *TLR2* gene polymorphim in cervical cancer patients and controls.

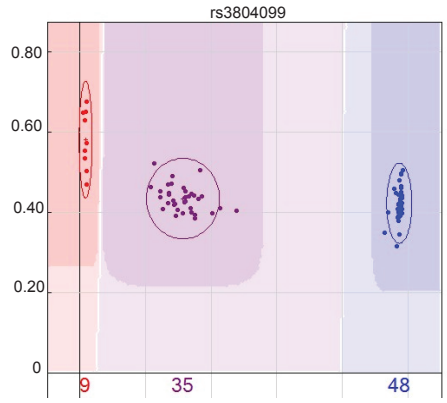

Figure 1 Distribution of TLR2 Gene rs3804099 as shown in the GENOME STUDIO program. Every dot is designated as one individual to point the genotype TT, TC or CC.

Methods

In this case-control study, peripheral blood samples were collected in Ethylenediaminetetraacetic acid (EDTA) tubes after informed consent was gained from histopathologically confirmed squamous cervical carcinoma patients (n=100). As controls, unrelated, age-matched healthy female of similar ethnicity (n=100) who participated in Pap smear screening with no abnormalities were recruited from the outpatient clinic of the Department of Obstetrics and Gynecology, Dr. Hasan Sadikin General Hospital, Bandung Indonesia during the period of 2011.

This study was a part of the Oncology Working Group research, and clearance of the protocol was granted by the Ethics Committee of the Faculty of Medicine Universitas Padjadjaran.

Genomic DNA was extracted according to the manufacturer's protocol (Qiagen, blood minikit, Germany) and was subjected to genotyping tests using BeadsXpressReader (Illumina®). Data on this system can be downloaded from http://support.illumina.com/array/array_instruments/beadxpress.ilmn). Using this genotyping system, 48 Single Nucleotide Polymorphisms (SNPs) were detected in total, with three *TLR2* SNPs were examined. These SNPs (rs3804099, rs4696480 and rs5743708) were taken from the literature reviews that are associated with cervical cancer development.

The chi-square test with a p-value of $p<0.05$ was performed. To assess the risk for cervical cancer of each SNP, odds ratio (OR) and 95% confidence intervals (CI) were calculated.

Results

Of all participants recruited only patients (n=71) and controls (n=38) with complete clinical records data were included in the study. The patients were staged according to the FIGO staging into stage I with 12 (16.9%) patients, stage II with 24 (33.5%) patients, stage III with 34 (47.9%) patients, and stage IV with 1 (1.4%) patient (Table 1).

The mean age of the patients was 48.4 (± 9.6) years old and the control group mean age was 45.6 (± 14.0) years old. Most of them were multiparous (2-4 pregnancies) and no significant difference was seen between patients and controls (66.2% and 68.4%, respectively). The clinical characteristics for age, parity and abortion of both groups showed no statistical significance in this study (Table 1).

The distribution of the *TLR2* polymorphism in cervical cancer patients for rs3804099 was shown

Table 1 Clinical Characteristic of Cervical Cancer Patients and Controls from Dr. Hasan Sadikin General Hospital year 2011

	Patients (n=71) n%	Control (n=38) n%	p value
Age			
Mean±SD (years)	48.4±9.06	45.6±14.0	0.198
Parity			
Nulliparous	1 (1)	4 (11)	0.084
Primiparous (1 parity)	5 (7)	3 (88)	
Multiparous (2-4 parity)	46 (65)	26 (68)	
Grand Multiparous (≥5)	19 (27)	5 (13)	
Abortion			
No abortion	38 (54)	25(66)	0.469
Once	28 (39)	11(29)	
Twice	3 (42)	2(5)	
Thrice	2 (3)	0 (0)	
FIGO stage *			
I (%)	12 (17)	-	
II (%)	24 (34)	-	
III (%)	34 (48)	-	
IV (%)	1 (1)	-	

Note: * FIGO staging I, II, III and IV only in the cervical cancer patients

in Figure 1. The result for genotype TT, TC and CC in patients were 43 (60.6%), 25 (35.2%) and 2 (4.2%), respectively, and 20 (52.6%), 16 (42.1%) and 2 (5.3%) in control group, respectively. The *TLR2* rs 3804099 genotype data in this study did not present any significant correlation (p=0.424). Further distribution of TLR2 polymorphisms is depicted in Table 2.

The *TLR2* gene polymorphism distribution in cervical cancer patients was further classified according to the FIGO staging (Table 3). Stages were merged because of the low number of variables in each stage. Stage I-II were designated as *lower stage* in which tumor may extend through the upper 2/3 vaginal wall and parametria but does not reach the pelvic wall. Stage III-IV were designated as *higher stage* in which carcinoma has extended onto the pelvic wall and may extend beyond the true pelvis. The comparison between distribution of *TLR2* genotypes in the lower stage and the higher stage is depicted in Table 3, that shows no significant difference between the two groups.

Discussion

The host immune system mechanism to prevent and control human papillomaviruses (HPVs) infection, which is the causative agent for the development of cervical cancer, remains poorly understood.[10] The polymorphisms of the toll-like receptor gene is probably helpful to dissect the immunobiological mechanism that is associated with cervical cancer susceptibility because TLRs are specific pattern recognition molecules that bind to the virus components and trigger innate immunity and direct adaptive immunity.[6]

The cancer patients in our study were mostly multiparous with a mean age of mid forty. Results from a previous study has shown associations between HPV infection with high parity in developing cervical cancer.[11] Multiparity may increase the risk for maintaining transformation zone in the ectocervical region of the cervix. There was also evidence on the increased number of squamous metaplasia during pregnancy.[12] This could result in carcinogenic cellular growth in the transformation zone. Interestingly, HPV16 infection is very common among young sexually active women. However, the majority of these women mount an effective immune response and may be able to clear the infection.[13]

Our study showed that the *TLR2* gene was not associated with cervical cancer development. One may suggest that this different result might

Table 2 The Distribution of TLR2 Gene Polymorphism in Cervical Cancer Patients and Control from Dr. Hasan Sadikin General Hospital year 2011

TLR2	Genotype	Patients (n=71) n (%)	Control (n=38) n (%)	p value	OR	CI 95%	
						Lower	Upper
rs3804099	TT	43 (61)	20 (53)	Reff			
	TC	25 (35)	16 (42)	0.424	1.38	0.62	0.31
	CC	3 (4)	2 (5)				
rs4696480	TT	8 (11)	6 (19)	Reff			
	TA	56 (79)	20 (65)	0.272	0.53	0.17	1.68
	AA	7 (10)	5 (16)				
rs5743708	AA	18 (25)	14 (37)	Reff			
	AG	19 (27)	11 (29)	0.209	0.58	0.26	1.36
	GG	34 (48)	13 (34)				

Note: * pvalue < 0.05 set as significant (x^2 test). ** the wild type was designated as reference

be caused by diversities in the different types of TLR distribution in epithelia.[6] For example, TLR2 is expressed on the cervical epithelium surface and also presents in active pouchitis and keratinocytes.[14] TRL4, which is another type of TLR, was expressed on the pulmonary epithelium while TLR5 expressions are more prominent in the ileal mucosa.[14]

In cervical cancer development, infections caused by human papillomavirus is an essential factor in cervical carcinogenesis and cervical intraepithelial neoplasia (CIN) is a key stage in cervical cancer development that confirms chronic infection and inflammation as the most important factors contributing to cancer development and growth.[10] Meanwhile, various TLR expressions in different stages of cervical cancer tissue had also been reported, showing that TLR2 expressions are more frequently found on cervical tissue epithelium.[13] As the cervical cancer progresses, it

Table 3 The Distribution of TLR2 Gene Polymorphism in Cervical Cancer Patients from Dr. Hasan Sadikin General Hospital year 2011, Stratified by FIGO Classification

TLR2	Genotype	FIGO I/II (n=36) n (%)	FIGO III/IV (n=35) n (%)	p value	OR	CI 95%	
						Lower	Upper
rs3804099	TT	21 (58)	22 (63)	Reff			
	TC	13 (36)	12 (34)	0.697	0.83	0.32	2.15
	CC	2 (6)	1 (3)				
rs4696480	TT	4 (11)	5 (14)	Reff			
	TA	26 (72)	26 (74)	0.688	0.75	0.18	3.06
	AA	6 (17)	4 (11)				
rs5743708	AA	9 (25)	9 (26)	Reff			
	AG	11 (31)	8 (23)	0.945	0.95	0.33	2.81
	GG	16 (44)	18 (51)				

Note: * pvalue < 0.05 set as significant (x^2 test). ** the wild type was designated as reference

penetrates to the deeper layer of the cervical lining. In higher FIGO stage, the tumor progression has breached the cervical epithelial layer. TLR 2 may not be capable to recognize the tumor anymore as it has already passed the epithelial layer. A similar study has also found that *TLR* polymorphism is associated with advanced stage of cancer which, in this case, is the higher FIGO stage.[15]

Evidence from a population in North India suggested that *TLR2* genes played a significant role in cervical cancer development. However, *TLR2* alone did not contribute to the susceptibility to cervical cancer.[8] Study on *TLR3* polymorphism showing association with the risk for developing cervical cancer was previously reported.[15] There were various studies showing involvement of *TLR*4,[16] *TLR*1, *TLR*3, *TLR*7, *TLR*8 and *TLR*9[17] in early and late cervical carcinogenesis suggesting that stromal up-regulation of TLRs plays a role in cervical disease progression.[18] Interestingly, a recent study on *TLR* 9 gene polymorphism that is involved in the risk for developing cervical cancer[15] was contradictive to the results of other groups showing that the promoter region of *TLR*9 gene did not seem to have a mediating role in the natural history of the HPV infection.[9]

Data on *TLR* polymorphisms offer further insights regarding the association between HPV infection and TLR signaling during cervical cancer carcinogenesis. A recent study also showed that the TLR up-regulation expression had been detected in many other tumor cell lines or tumors, especially in the epithelial derived cancer.[17] In addition to TLRs, other genetic polymorphisms in the cytokines pathways might contribute to cancer development,[19] including cytokine IFN, which is a key cytokine involved in the immunity.[20] IFNG polymorphisms had previously shown significant correlation through an increase in higher stage (FIGO III/IV) cervical cancer.[21] However, our previous study showed that IFNG is not a risk factor for cancer susceptibility.[22]

The limitation of this research was the small number of subjects with complete medical record data. Incomplete medical record data frequently becomes the problem in large clinical studies which might be due to patient's failure in giving complete information or the physician's neglect in filling out data in the medical records. Better and complete medical record data are essential as one of the requirements in cancer registry for cancer control planning since it will be able provide various essential data regarding cancer incidence, mortality, and survival. Furthermore, low number of the research subjects might reduce the statistical power of the research.

To conclude, *TLR2* genotype in our study does not seem to show any significant correlation with cervical cancer susceptibility. As cervical epithelium contains diverse types of TLRs, other TLR genes need to be further explored since TLR is a promising target for the development of anticancer therapy. Future well-designed studies with a large sample size and complete clinical data should shed light on the significance of TLR polymorphisms for cancer prevention.

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