

RESEARCH ARTICLE

Local Cervical Immunity in Women with Low-grade Squamous Intraepithelial Lesions and Immune Responses After Abrasion

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Abstract

Minor trauma to the uterine cervix is supposed to induce local immunity to prevent cervical lesions caused by human papillomavirus (HPV) infection. This study aimed to investigate the local cervical immunity in women with low grade squamous intraepithelial lesion (LSIL) and effects of abrasion after cryosurgery or Pap smear. One hundred women with LSIL and known results of HPV detection were recruited. HPV positive women were randomly divided according to abrasion into cryotherapy and Pap smear observation groups. Cervical tissues and cervico-vaginal lavage (CVL) were collected at 6 and 12 months after allocation. The levels of cytokines at first recruitment were compared with cytokine levels at 6 months after abrasions. The mRNA of IFN- γ , TNF- α and IL-10 in cervical tissues and these cytokines secreted in CVL were determined using real time PCR and ELISA, respectively. Anti-HPV16 IgG and IgA antibodies in CVL were assessed by western blotting. At first recruitment of women with LSIL (100 cases), IL-10 mRNA and cytokine in HPV positive group (60 cases) was significantly higher than negative group (40 cases). IFN- γ and TNF- α mRNA level in both groups were comparable but their secretions in CVL were significantly increased in HPV negative group. After abrasion for 6 months in HPV-positive women, all mRNA and secreted cytokines were changed, but no significant difference was observed between cryotherapy and observation groups. When individuals were compared between first recruitment and after abrasion for 6 months, IFN- γ mRNA and anti-HPV16 L1 IgA antibodies were significantly increased in the cryotherapy group. The results suggest that modulation of local cervical immunities by abrasion might promote different effects in clearance of HPV-related cytological abnormalities.

Keywords: LSIL - IFN- γ - TNF- α - IL-10 - anti-HPV16 antibody - cryotherapy

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Introduction

Most human papillomavirus (HPV) infections are cleared without further consequences for the host, especially in adolescents and young women (Winer et al., 2011). In some subjects, however, HPV infection, especially with high-risk HPV types (HR-HPV) can cause low-grade cervical lesions and persistent infections. A certain proportion of untreated infections with HR-HPV types may give rise to high-grade cervical intraepithelial neoplasia (CIN II–III) and cervical cancer in a few years (Koshiol et al., 2008; Jaisamrarn et al., 2013).

There is evidence that cell mediated immune responses of the host, both systemic and local, are important determinants of the course of infection (Amador-Molina et al., 2013). This immune response is mediated through the release of various cytokines, which can influence the synthesis and actions of other cytokines; the setting known as an immunoregulating cytokine network. Cytokines in immune responses to infection are often classified as immunostimulating cell mediated immunity that is induced by Th1-type cytokines such as interferon

γ (IFN- γ), tumor necrosis factor α (TNF- α), interleukin 2 (IL-2), and IL-12, to generate cytotoxic CD8+T cells that are the effectors for the clearance of viral infected cells. On the other hand, humoral immunity is mediated by Th2-type cytokines, IL-4, IL-5, IL-6, IL-8, and IL-10, and those Th2-cytokines are inhibitory to the cell mediated responses. The role of humoral responses against HPV infection is unclear, but neutralizing antibodies generated by a strong “induced” response may protect infection (Spellberg and Edwards, 2001).

Genital HPV infection frequently induces antibody production, mostly directed against the viral capsid. The HPV capsid is composed of two structural proteins, a largely internal minor capsid protein (L2) and a major capsid protein (L1). L1 is an important target of the immune system. HPV-specific IgA and IgG are suggested to be protective against subsequent HPV infection and the presence of antigen-specific CD4+ T cells is potentially important for the long-term anti-tumor effects (Marais et al., 2008).

Several studies reported that the minor trauma caused by taking a Pap smear may reduce the risk of cervical

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cancer by triggering an inflammatory response to HPV infection (Passmore et al., 2007; Boccardo et al., 2010). HPV infections elicit the production and release of several inflammatory cytokines from keratinocytes, the main target cells of HPV, from skin fibroblasts and from various immunocompetent cells such as macrophages, natural killer cells and lymphocytes. TNF- α , IL-1 and IFN- γ are known to inhibit HPV oncogene transcription and inhibit the growth of cell lines harboring the viral genome (Clerici et al., 1997; Stanley, 2008).

In low-income countries, cryotherapy provided by physicians is a safe and effective method for the outpatient treatment of cervical intraepithelial neoplasia (CIN), with cure rates exceeding 86% for CIN confined to the visible part of the cervix (Poomtavorn et al., 2009; Saxena et al., 2012). Cryotherapy was successful in the treatment of subclinical HPV infection and prevented the development of clinical disease (Mohanty and Lowe, 1990; Jacob et al., 2005). Cryotherapy or loop electrosurgical excision procedure (LEEP) was suggested to be effective for removal of CIN (Moore and Tajima, 2004).

No information is available, however, on the immune modulation after abrasion cryotherapy for histologically diagnosed subclinical HPV infection of the cervix. The modulation of local cervical immunity may partly predict the side effect of cryotherapy or on cervical disease. This study aimed to determine local cervical immunity and immune modulation that occurred after abrasion by cryotherapy or Pap smear in women with histologically diagnosed low-grade squamous intraepithelial lesion (LSIL) with or without HPV infection.

Materials and Methods

Samples

Cervico-vaginal lavages (CVL) and fresh cervical tissue biopsies used in this study were obtained from 100 eligible women with a histological diagnosis of LSIL who participated in the research entitled “Cryotherapy for HPV clearance in women with biopsy-confirmed cervical low-grade squamous intraepithelial lesions” (Chumworathayi et al., 2010). They received verbal and written information and gave written informed consent approved by the Institutional Review Boards of Khon Kaen University, Khon Kaen Hospital, and Roi Et Hospital, Thailand (IRB00001189, FWA00003418). The eligibility criteria of recruited women who were consecutively recruited were: aged older than 30 years, had results of HPV testing, no contraindication of cryotherapy. HPV positive women were randomly allocated by blocks of 2, 4, and 6 using computerized randomization to a cryotherapy group, treatment and abrasion with cryotherapy, and an observation group who were investigated by Pap smear as described in Figure 1.

CVL and fresh biopsies were collected from 100 eligible women at the first recruitment to obtain base line data and at 6 months after allocation and receiving abrasion by Pap smear and cryotherapy. Only CVLs were collected at 12 months after cryotherapy. RNA was extracted from fresh biopsies by All Prep DNA/RNA purification kits (Qiagen, Valencia, USA) for detecting

cytokine gene expression of IFN- γ , TNF- α , IL-10. Anti-HPV16 L1 IgG and IgA and amounts of IFN- γ , TNF- α , and IL-10 were determined in CVL samples.

Detection of IFN- γ , TNF- α and IL-10 gene expression by quantitative real time polymerase chain reaction (qRT-PCR)

The extracted RNA was investigated for mRNA of TNF- α , IL-10, IFN- γ and β -actin by quantitative reversed transcription polymerase chain reaction (qRT-PCR) using specific primers and hydrolysis probes (Invitrogen Life Technologies, Carlsbad, CA, USA) (Table 1). The cDNA was generated from the extracted RNA using the SuperScript™ III First-Strand Synthesis System for the RT-PCR kit (Invitrogen, Milan, Italy) according to the manufacturer’s protocol and qualified by amplifying 550 bp fragments of GAPDH cDNA. The target transcript levels were normalized to the expression levels of β -actin.

Detection of cytokine levels by enzyme-linked immunosorbent assay (ELISA)

The levels of IFN- γ , TNF- α and IL-10 in CVL samples were detected by commercial kits (PeproTechInc, Rocky Hill, USA) according to the manufacturer’s protocol. Then color development was monitored with an ELISA plate reader (Tecan, Mannedorf, Switzerland). The cytokine concentration was estimated from standard curves.

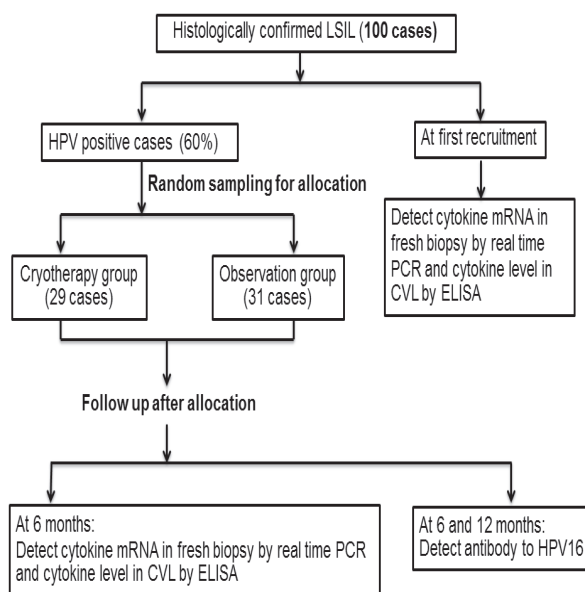


Figure 1. Flow Chart Demonstrating Sample Collection and Study Design

Table 1. Primer and Probe Sequences Used in RT-PCR

Genes	Primer sequences	PCR product size (bp)
IFN- γ	F: TCAGTCTGCATCGTTTTGG	120
	R: GTTCCATTATCCGCTACATCTGAA	
	TP: FAM CCAAGACCCAGACATCAAGGCGCA TAMRA	
TNF- α	F: TCTTCTCGAACCCCGAGT GA	150
	R: CCTCTGATGGCACCACCAG	
	TP: FAM TAGCCCATGTTGTAGCAAACCCTCAAGCT TAMRA	
IL-10	F: GTGATGCCCAAGCTGAGA	138
	R: CACGGCCTTGCTCTTGTITT	
	TP: FAM CCAAGACCCAGACATCAAGGCGCA TAMRA	
β -actin	F: TCACCCACACTGTGCCCATCTACGA	294
	R: CAGCGGAACCGCTCATTGCCAATGG	
	TP: FAM ATGCCCTCCCCAYGCCATC TAMRA	

Detection of anti-HPV16 L1 IgG and IgA antibodies by western blotting

Anti-HPV16 L1 IgG and IgA antibodies in CVL were detected by western blot using HPV16 L1 protein from HPV16 virus-like particles (VLP16) prepared as described previously (Kim et al., 2010). A 1:2 dilution of each sample was incubated with viral proteins on the nitrocellulose membrane. HRP conjugated goat anti-human IgG or IgA (Invitrogen Life Technologies, Carlsbad, CA, USA) and SuperSignal West Pico Substrate (Thermo scientific, Rockford, IL, USA) were used as the detection system.

Results

Prevalence of HR-HPV and patient allocation

Most of 60 HPV positive women with LSIL (100 cases) were infected with HR-HPV (83.3%) and HPV16 was the most common type. HPV positive women were separated into two groups; a cryotherapy group of 29 women who received abrasion by cryotherapy and an observation group of 31 women monitored by Pap smear. After abrasion in both groups, cervical biopsies and CVL were collected at 6 months; CVL samples were collected again at 12 months.

IFN- γ , TNF- α and IL-10 gene expression

A total of 100 biopsy samples from women with LSIL at the recruitment were investigated for the expression of cytokine genes; IFN- γ , TNF- α and IL-10 to obtain the base line data. The results showed that IFN- γ , TNF- α and IL-10 genes were expressed in 93%, 97% and 93% of the samples, and there was no significant difference between women with HPV DNA positives (60 cases) and HPV DNA negatives (40 cases) (Table 2). The median mRNA expression level of IL-10 in HPV positive cases was higher than that in HPV negative cases, whereas, the

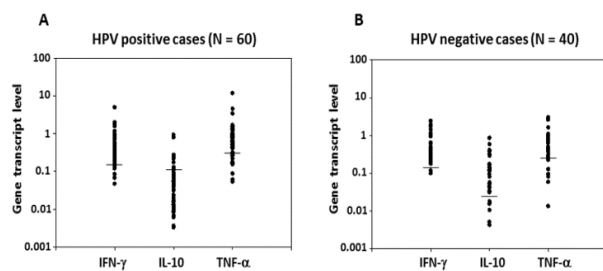


Figure 2. The mRNA Expression Levels of IFN- γ , IL-10 and TNF- α in 100 Women with LSIL at the First Recruitment

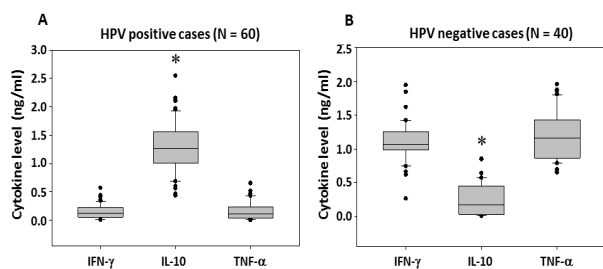


Figure 3. Cytokine Secretion Median Levels in CVL of 100 Women with LSIL at the First Recruitment. *p value <0.05

expression levels of IFN- γ and TNF- α were comparable between the two groups (Figure 2).

Cytokine levels in cervico-vaginal lavage

The secreted cytokine levels of IFN- γ , TNF- α and IL-10 were detected in all 100 CVL samples by ELISA. The IL-10 levels in CVL corresponded to the mRNA expression levels and were significantly higher ($p < 0.001$) in the HPV DNA positive group than in the HPV DNA negative group (Figure 3). Conversely, IFN- γ and TNF- α levels were significantly lower in HPV DNA positive group than in HPV DNA negative group ($p < 0.001$). Six months after abrasion by cryotherapy, cervical biopsies and CVLs were collected again. The cytokine mRNA levels in biopsied specimens and secreted cytokines in CVL were determined and compared with those at first recruitment. The cytokine mRNA expression levels tended to increase in abrasion by both cryotherapy and observation groups but without a statistical difference (Table 3). When the fold changes of the cytokine mRNA expression levels before and 6 months after abrasion of each patient were plotted (Figure 4), the increases of IFN- γ mRNA became apparent ($p = 0.001$) in the cryotherapy group, whereas other cytokine mRNA expression levels were slightly increased but not statistically significantly different.

Figure 5 showed the cytokines levels in CVLs of the cryotherapy group and the observation group before and after abrasion. The IL-10 levels at 6 months significantly decreased ($p < 0.000$) from the base line in both groups. In contrast, IFN- γ and TNF- α significantly increased ($p < 0.001$) in both cryotherapy and observation groups. The changes of these cytokines in both groups, however, were not significant between the groups. These results

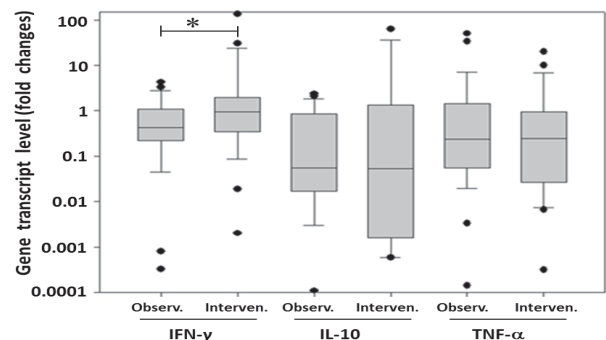


Figure 4. The Fold Change of Cytokine mRNA Expression before and 6 Months after Abrasion in Each Patient of Observation (O) and Cryotherapy (I) Groups. *p value <0.05

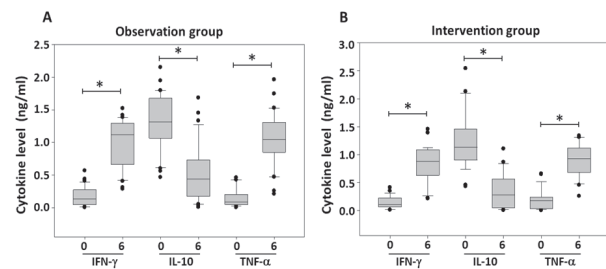


Figure 5. Cytokine Levels in CVL of Each Patient in Observation and Cryotherapy Groups before (0) and 6 Months after Abrasion (6). *p value <0.05

Table 2. Cytokine mRNA Expression in 100 Women with LSIL at the First Recruitment

LSIL cases (N=100)	Cytokine mRNA expression					
	IFN- γ		TNF- α		IL-10	
	ve+ (%)	ve- (%)	ve+ (%)	ve- (%)	ve+ (%)	ve- (%)
HPV ve- (40)	34 (85)	6 (15)	39 (97.5)	1 (2.5)	36 (90)	4 (10)
ve+ (60)	59 (98.3)	1 (1.7)	58 (96.7)	2 (3.3)	57 (95)	3 (5)
Total (100)	93 (93)	7 (7)	97 (97)	3 (3)	93 (93)	7 (7)

Table 3. The Median mRNA Expression Levels of the IFN- γ and TNF- α in Cryotherapy and Observation Groups at Baseline and after 6 Months

Cytokine gene	Cryotherapy group			Observation group		
	At base line	6*	p value	At base line	6*	p value
IFN- γ	0.311	0.638	0.158	0.331	0.563	0.201
IL-10	0.05	0.121	0.451	0.054	0.087	0.061
TNF- α	0.575	0.517	0.154	0.551	0.654	0.606

*Months

suggest that abrasion by both cryotherapy and Pap smears affected local immunity in a similar manner.

Response of anti-HPV16 L1 IgG and IgA antibodies

In a total of 60 CVL samples, 35 samples were HPV16 DNA positive that were investigated for the presence of anti-HPV16 L1 IgG and IgA antibodies using western blotting. The results show that the prevalence of anti-HPV16 L1 IgG and IgA in the HPV16 positive group at first recruitment was 25.8% (9/35) and 14.3% (5/35). In the observation group, IgG was detected at a slightly increased rate after abrasion, whereas IgA was absent. Conversely, the detection rates of anti-HPV16 L1 IgG antibody were not changed after cryotherapy, but those of the IgA antibody were increased after abrasion by cryotherapy.

Discussion

This study demonstrated that IFN- γ , TNF- α and IL-10 genes are expressed in most of women with LSIL. The increase in IL-10 corresponded with HPV DNA detection in contrast to the levels of IFN- γ and TNF- α in CVL, which were increased in cases of the HPV negative group. The expression and secretion levels of IFN- γ , TNF- α and IL-10 were slightly increased at 6 months after abrasion by cryotherapy and Pap smear without a significant difference. Only IFN- γ was significantly increased in the cryotherapy group when compared between individuals. In addition to the specific immune response to HPV16 L1, a change of IgA antibodies was observed and increased in the cryotherapy group. These results defined the effects of abrasion by cryotherapy on the alteration of cervical cytokines and viral immunity.

In this study, most of women with LSIL expressed TNF- α , IFN- γ , and IL-10 genes. HPV positive women with LSIL (60 cases) were found gene expression level of IL-10 higher than HPV negative women, whereas TNF- α , and IFN- γ gene expression were in similar level. The study of cytokine network; TNF- α , IFN- γ , IL-2, IL-4, IL-10, and IL-12 was reported that they are activated by infection of HR-HPV without expression of cervical dysplasia (Bais et al., 2007). The levels of cytokines

IFN- γ , TNF- α , IL-6, and IL-10, were determined in 57 HR-HPV positive cases with mild dysplasia or less. The results showed that only IFN- γ positive results were significantly associated with clearance of HR-HPV after 12 months of follow-up (Song et al., 2008). The micro-dissections of 11 normal cervixes and 25 HPV-16 positive CIN cervixes were studied and the researchers suggested that reduced epithelial and sub-epithelial IFN- γ , as well as increased sub-epithelial IL-10 synthesis, may play a role in the development and progression of HPV16 associated cervical pre-cancer (El-Sherif et al., 2001). Alterations of the local cervical immune environment in cervical cancer were demonstrated in a study that analyzed the presence of various cytokines in CVLs of 22 healthy volunteers, 63 CIN patients and 33 cervical cancer patients. IL-10 and TNF- α level were significantly higher in patients with cervical cancer than in controls and CIN patients whereas the levels of IFN- γ were not different (Tjiong et al., 2001). IL-10 also showed significant direct association with progression of prostate cancer while inverse relation with survival duration and survival rate (Dwivedi et al., 2011).

This present study showed that 93-97% of women with LSIL had expressions of IFN- γ , TNF- α and IL-10 mRNA at the baseline of first recruitment, and there were no differences between the HPV DNA positive and negative groups (Table 2). The median levels of secreted IL-10 in CVL of women that were HPV DNA positive, however, was significantly higher than those in the negative group (Figure 2). This pattern of cytokine levels in CVL may suggest evidence of immune evasion in HPV positive women with LSIL. In addition, IL-10 expression in cervical tissues was determined in Mexican women according to the severity of the malignancy and its association with HPV infection. This study suggested a clear relationship between IL-10, HPV and the stage of cervical cancer (Bermudez-Morales et al., 2008).

In a randomized controlled trial followed Pap smears, the inflammatory responses were observed and markers of cell mediated immunity were significantly elevated (Passmore et al., 2007). A study of gastric cancer suggested that IFN- γ might slow proliferation of some gastric cancer cells by affecting the cell cycle to play a negative role in the development of gastric cancer (Zhao et al., 2013). Corresponding to the present study, at 6 months after abrasion by both Pap smear and cryotherapy showed significant increased (p<0.001) IFN- γ and TNF- α mRNA expression, whereas the secreted IL-10 levels significantly decreased (p<0.001) in comparison with the base line at first recruitment as in Figure 5. A study of cytokines that may predict HR-HPV clearance or persistence in untreated patients with mild or less dysplasia of the uterine cervix suggested that intralesional IFN- γ might be a prognostic marker for clearance of HR-HPV (Song et al., 2008). The effects of Pap smear or cryotherapy also suggested as possible ways to eliminate HPV and prevent cervical lesions (Chumworathayi et al., 2010). The present study showed that increased IFN- γ mRNA up-regulation was seen more frequently in the cryotherapy group than in observation group. This abrasion may effect to the clearance activity triggered by cryotherapy more effective than Pap smear to activate local cytokines such as IFN- γ

and TNF- α (Table 3, Figure 3 and 4).

IgA and IgG antibodies in cervical secretions and sera were examined using a newly developed capsomer-based ELISA. The results showed that the duration time of IgA in cervical secretions and sera was shorter than the duration time of serum IgG ($p=0.007$ and 0.001) (Onda et al., 2003). In this present study, the detection rate of anti-HPV16 IgA antibody was increased in the cryotherapy group but it was reduced in the observation group. An increase of IgA may partly support the clearance rate of HPV infection in women with LSIL; however, more of this information is needed to be investigated in a larger sample size.

In conclusion, local cervical immunity induced in women with LSIL may be modulated by different abrasions and may affect to clear HPV infection and to rapidly eliminate cervical lesions.

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