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Original Article

Diagnostic and clinicopathological significance of Ki67 mRNA expression in cervical cancer tissue

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Abstract: Ki67 is a key biomarker associated with cancer cell proliferation and poor prognosis. We previously evaluated the diagnostic potential of quantitatively measured Ki67 mRNA levels in formalin-fixed paraffin-embedded (FFPE) cervical cancer tissue samples. In the present study, we continued this avenue of research using quantitative reverse transcriptase PCR (RT-qPCR) to measure Ki67 mRNA levels in FFPE cervical tissues and performed an assessment with each clinical prognostic factor of patients. We obtained 190 FFPE cervical tissue samples that comprised of 80 squamous cell carcinoma (SCC), 10 adenocarcinoma (ADC), 30 HSIL, 30 LSIL, and 40 normal cervical tissue samples. And using this assay, we also evaluated the predictive value of Ki67 in cases with a low-grade squamous intraepithelial lesion (LSIL) and those with a high-grade squamous intraepithelial lesion (HSIL). As a result, Ki67 mRNA levels were increased in SCC and ADC cervical cancer tissues (n = 90) compared to those in normal cervical tissues (n = 40) ($P < 0.001$). The diagnostic validity of the Ki67 mRNA assay was evaluated and demonstrated a sensitivity of 93.3% (95% confidence interval (CI) = 86.1 to 97.5) and a specificity of 97.5% (95% CI = 86.8 to 99.9). Ki67 mRNA positivity was 93.3% for cervical cancer, 40.0% for HSIL, 13.3% for LSIL, and 2.5% for normal tissue samples. Furthermore, we found that high levels of Ki67 mRNA expression in cervical cancer were associated with lymph node status ($P = 0.01$). In conclusion, Ki67 mRNA assay can provide an additional accurate approach for molecular diagnosing cervical cancer, and also predict prognosis of cervical cancer depending on LSIL and/or HSIL status.

Keywords: Cervical cancer, Ki67 mRNA, HPV E6/E7, RT-qPCR, molecular diagnosis

Introduction

Cervical cancer is the third most common malignancy in women globally and one of the leading causes of morbidity and mortality in women worldwide. The World Health Organization estimates that approximately 527,600 women are newly diagnosed and there are 265,700 deaths from cervical cancer every year [1]. Human papillomavirus (HPV) is a major cause of cervical cancer and is the most common sexually transmitted pathogen among women and men [2]. Therefore, the detection of HPV is routinely performed in exfoliated infected cervical cells or tissues of patients with cervical cancer or patients with precancerous lesion [3-7].

However, most high-risk HPV infections resolve spontaneously within 1 to 2 years [2]. Several studies have investigated the molecular mechanisms underlying cervical cancer carcinogenesis to identify potential diagnostic or prognostic biomarkers for cervical cancer [8-11]. For example, putative molecular markers such as p16, p53, and Ki67 have been identified in cervical carcinogenesis. Their respective coding genes and proteins have been characterized, and their roles in the process have been studied with the aim of improving diagnosis and treatment of cervical cancer.

Several studies found that among these different markers, immunohistochemical (IHC) staining of Ki67 was an effective method for the

Table 1. Clinical characteristics of patients

	Cancer (n = 90) n (%)	HSIL (n = 30) n (%)	LSIL (n = 30) n (%)	Normal (n = 40) n (%)
Age				
≤50 years	41 (45.6)	23 (76.7)	20 (66.7)	24 (60.0)
>50 years	49 (54.4)	7 (23.3)	10 (33.3)	16 (40.0)
HPV DNA chip				
Negative	7 (7.8)	1 (3.3)	11 (36.7)	
Positive	66 (73.3)	21 (70.0)	10 (33.3)	
Unknown*	17 (18.9)	8 (26.7)	9 (30.0)	
Histology				
SCC	80 (88.9)			
ADC	10 (11.1)			
FIGO stage				
< IIB	34 (37.8)			
≥ IIB	43 (47.8)			
Unknown	13 (14.4)			
Lymph nodes				
Negative	37 (41.1)			
Positive	37 (41.1)			
Unknown	16 (17.8)			
Tumor size				
≤4 cm	43 (47.8)			
>4 cm	33 (36.7)			
Unknown	14 (15.5)			

ADC, adenocarcinoma; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; HPV, human papillomavirus; SCC, squamous cell carcinoma. *Unknown stands for cases referred to other institution after biopsy without any baseline study.

prognosis of different tumor types [12-15]. Ki67 is associated with cell cycle activity and is expressed at varying levels during G1, S, G2, and M phases, but is not expressed in G0 [10, 16]. In our previous study, we demonstrated the potential diagnostic value of Ki67 in cervical cancer by quantitatively measuring Ki67 mRNA levels in formalin-fixed paraffin-embedded (FFPE) cervical cancer tissue samples [17]. A purpose of the current study is to investigate the relationship between Ki67 mRNA level and clinicopathological measures of patients with cervical cancer.

Low-grade squamous intraepithelial lesion (LSIL) and high-grade squamous intraepithelial lesion (HSIL) are important precancerous courses in the development of cervical cancer, but their carcinogenesis is not well-known [16, 18, 19]. Cox et al, performed a meta-analysis and concluded that the likelihood of progression from LSIL to HSIL was approximately 10% within 2 years [20]. However, there is no accurate method or approach to identify which patients with LSIL will progress to HSIL. Therefore, a better understanding of cervical cancer progression is necessary for improved management of patients with either LSIL or HSIL.

In this study, we evaluated the discriminatory power of a Ki67 mRNA assay using quantitative reverse transcriptase PCR (RT-qPCR). We determined threshold cutoff for the diagnosis of cervical cancer using one hundred and ninety FFPE cervical cancer tissues and studied their clinical relevance. Additionally, the predictive prognostic value of Ki67 mRNA was assessed in cases with LSIL or HSIL.

Materials and methods

Patients and samples

We retrospectively obtained 190 FFPE cervical tissues from patients who underwent pathological testing at the Department of Pathology, Yonsei University Wonju Severance Christian Hospital between January 2010 and December 2014. This study was approved by the Institutional Ethics Committee of Yonsei University Wonju College of Medicine (approval no. CR315052), and all subjects were provided written informed consent. Of the 190 FFPE tissue samples collected, 40 (21.1%) were normal, 30 (15.8%) were LSIL, 30 (15.8%) were HSIL, 10 (5.3%) were adenocarcinoma (ADC), and 80 (42.1%) were squamous cell carcinoma (SCC) (**Table 1**). Using a prior grading system for diagnosis, we categorized grade 1 cervical intraepithelial neoplasia (CIN 1) tissue as LSIL and grade 3 cervical intraepithelial neoplasia (CIN 3) tissue as HSIL. FFPE normal tissue samples included 40 chronic cervicitis specimens obtained from patients who underwent a hysterectomy for other benign gynecological diseases such as leiomyoma and adenomyosis.

Table 2. Ki67 mRNA expression in matched non-cancerous and cancerous lesions

	Age	BMI	Cytology	Histology	Ki67 mRNA expression			*P* value
					Non-cancerous lesion	Cancerous lesion	Fold change	
P001	62	33.3	ADC	ADC	0.06	0.22	3.67	*P* = 0.0005*
P002	39	22.1	ASCUS	SCC	0.26	22.32	85.85	
P003	41	25.8	SCC	SCC	0.04	7.01	175.25	
P004	49	20.5	SCC	SCC	0.04	5.9	162.10	
P005	36	22.8	ADC	ADC	2.62	38.85	14.82	
P006	30	21.6	HSIL	SCC	0.64	1.18	1.84	
P007	40	16.4	SCC	SCC	3.12	35.02	11.22	
P008	57	21.4	ADC	ADC	0.19	11.79	62.05	
P009	30	19.6	ADC	ADC	0.01	4.35	435.00	
P010	57	22.7	SCC	SCC	0.19	53.34	280.74	
P011	46	20.7	SCC	SCC	3.43	131.6	38.37	
P012	35	27.6	HSIL	SCC	0.25	24.59	98.36	
P013	42	21.9	SCC	SCC	1.19	39.95	33.57	
P014	57	26.2	ADC	ADC	0.2	34.54	172.70	
P015	67	26.6	HSIL	SCC	0.03	35.02	1167.33	
P016	65	27.9	SCC	SCC	0.28	29.86	106.64	

*Ki67 mRNA levels were higher in canceroustissuelesion than that found in matched Non-cancerous tissuelesion (Wilcoxon matched-pairs test). ADC, adenocarcinoma; ASCUS, Atypical squamous cells of undetermined significance; BMI, body mass index; HSIL, high-grade squamous intraepithelial lesion; SCC, squamous cell carcinoma.

Deparaffinization of FFPE tissue and total RNA extraction

Three 10-μm sections from each paraffin block of cervical tissue were used for total RNA extraction. Extractions were performed using the Qiagen RNeasy FFPE mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA purity and concentration were determined by measuring absorbance at 260 nm and 280 nm using a spectrophotometer (Infinite 200, Tecan, Salzburg, Austria). All RNA preparation and handling was performed in a laminar flow hood under RNase-free conditions. Isolated RNA was stored at -70°C.

cDNA synthesis

Complementary DNA (cDNA) was synthesized using the M-MLV Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) and random hexamers (Invitrogen) according to the manufacturer's recommendations. Briefly, 10 μL of total RNA was added to a master mix containing 10 mM dNTPs at neutral pH, 0.25 μg random hexamers, and 5-μL DEPC-treated water. Reactions were incubated at 65°C for 5 min and chilled on ice. A mixture of 4 μL 5× First-Strand Buffer, 2 μL 0.1 M dithiothreitol, and 1 μL M-MLV reverse transcriptase (RT) was added, and cDNA synthesis was synthesized at 25°C for 10 min, followed by 37°C for 50 min, and 70°C for 15 min.

Ki67 mRNA RT-qPCR assay

Quantitative real-time PCR amplification of the OPTIMYGENE Ki67 mRNA assay (Optipharm, Osong, Republic of Korea) was performed in 10 μL 2× Thunderbird probe qPCR mix (Toyobo, Osaka, Japan), 3 μL primer and TaqMan probe mixture, 2 μL template cDNA, and distilled water (DW) to a final volume of 20 μL per sample. Positive and negative controls were included. No-template controls were included in each run and consisted of sterile DW instead of template DNA. PCR cycling was 95°C for 3 min, followed by 40 cycles of 95°C for 3 seconds, and 55°C for 30 seconds. mRNA levels were quantified by determining the cycle threshold (C_T), which is defined as the number of PCR cycles required for fluorescence to exceed a value significantly higher than that of the background fluorescence. To avoid false negatives because of mRNA degradation, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The amount of Ki67 mRNA was determined using the comparative C_T method ($\Delta\Delta C_T$ method) [21],

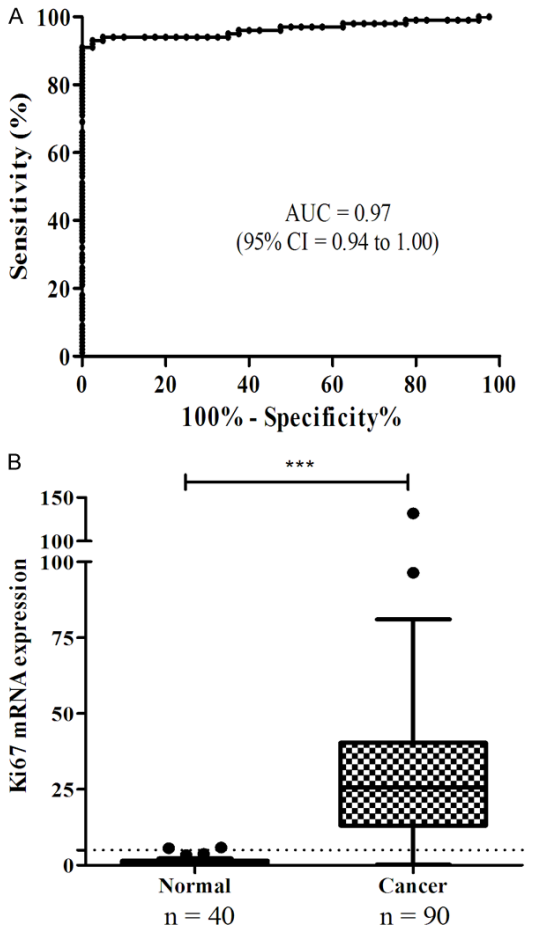

Figure 1. Receiver operating characteristics (ROC) curve analysis. A. The area under the ROC curve (AUC) was 0.97 (95% CI = 0.94 to 1.00, $P < 0.001$). B. Ki67 mRNA levels were significantly higher in FFPE cancer tissues compared to that found in FFPE normal tissues (*t*-test, $P < 0.001$) using a diagnostic threshold of 5 (shown as a horizontal dotted line). ***$P < 0.001$.

Table 3. Sensitivity, specificity, NPV, and PPV of Ki67 mRNA levels in cervical cancer and normal tissues

	Ki67 mRNA RT-qPCR assay (n = 130)	95% CI
Sensitivity	93.3%	86.1-97.5
Specificity	97.5%	86.8-99.9
PPV	98.8%	93.6-100.0
NPV	86.7%	73.2-95.0

PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval.

measuring mRNA relative to a reference gene using CFX Manager Software v1.6 (Bio-Rad, Hercules, CA, USA). The amount of Ki67 mRNA was normalized to the internal housekeeping gene GAPDH using the following equation: $\Delta\Delta C_T$ = (ΔC_T [target sample] - ΔC_T [normal sample]).

Statistical analysis

Statistical analyses were performed using GraphPad Prism v5.02 (GraphPad, La Jolla, CA, USA) and SPSS (Statistical Package for the Social Sciences) v23.0 (SPSS Inc., Chicago, IL, USA). The Wilcoxon matched-pairs test was used to compare nonparametric-matched samples, and the Student's *t*-test and 95% confidence interval (CI) were used to determine statistical significance. Receiver operating characteristic (ROC) curves were used to predict cut-off values of the marker. Sensitivity and specificity were calculated using MedCalc v12.5 (MedCalc software, Ostend, Belgium). The Pearson's chi-square test was used to analyze associations between the positivity of Ki67 mRNA expression and histologically diagnosed samples. For all tests, a P value < 0.05 was considered statistically significant.

Results

Patient characteristics

Patient characteristics are summarized in **Table 1**. One hundred and ninety FFPE tissue samples were used in this study of which 90 (47.3%) were cancer, 30 (15.8%) were HSIL, 30 (15.8%) were LSIL, and 40 (21.1%) were normal samples. Especially, for cervical cancer cases, data on histology, FIGO stage, tumor size, and lymph node positivity were retrospectively reviewed from patient medical records (**Table 1**). Among 90 cases of cervical cancer, 80 (88.9%) cases were diagnosed as SCC, 43 (47.8%) cases were more than FIGO stage IIB, 33 (36.7%) cases had more than 4 cm of tumor size, and 37 (41.1%) cases showed positive lymph nodes.

Ki67 mRNA levels for matched FFPE cancerous and non-cancerous tissues

To evaluate the effectiveness of the Ki67 mRNA assay, matched FFPE cancerous and non-cancerous tissues from 16 cervical cancer patients were tested. Using $\Delta\Delta C_T$ to determine mRNA levels in matched tissue samples, we found that levels of Ki67 mRNA in cancer tissues were higher than those in matched normal tissues (P = 0.0005). The Ki67 mRNA levels in

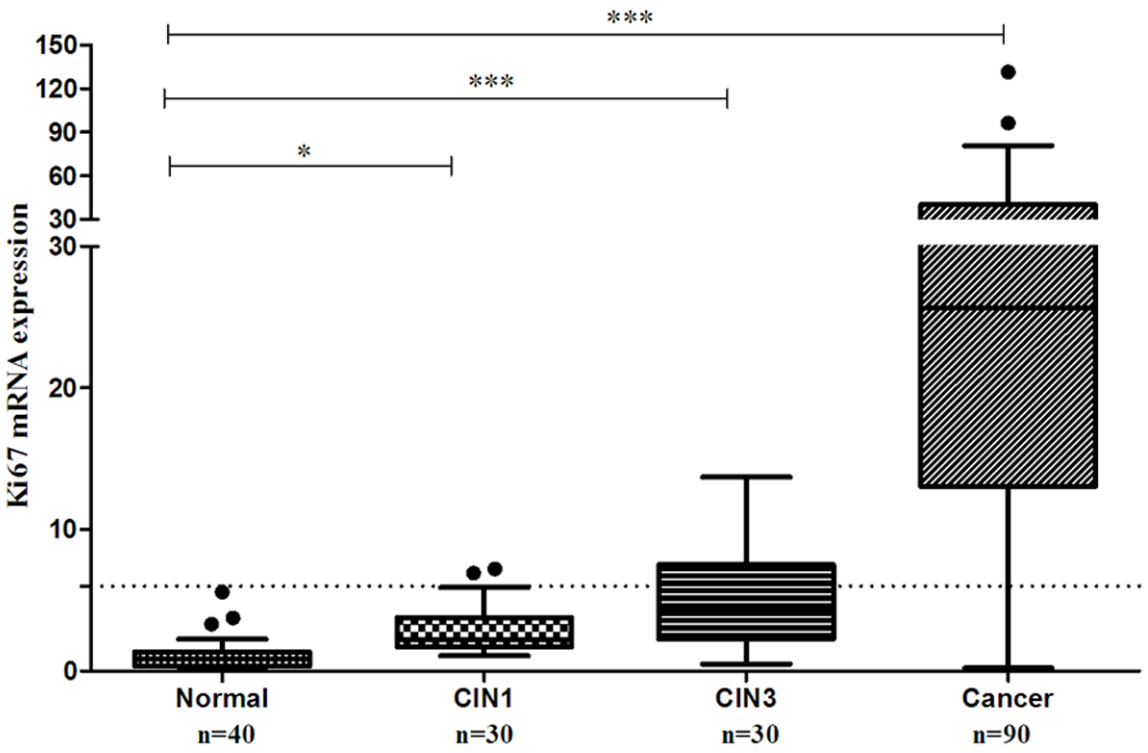

Figure 2. Box and whisker plots of Ki67 mRNA levels in histologically diagnosed FFPE cervical tissues. Ki67 mRNA levels in FFPE normal tissues were significantly lower than that found in LSIL, HSIL, and cancer tissues (*t*-test, $P < 0.0001$). ***$P < 0.001$, *$P < 0.05$.

Table 4. Association between Ki67 mRNA expression levels and histologically diagnosed cervical grades

	Ki67 mRNA RT-qPCR assay		Chi-square (df, *P* value)
	Ki67-Positive Cases n (%)	Ki67 expression levels Median (Min-Max)	
Normal (n = 40)	1 (2.5)	0.8 (0.0-5.6)	Reference
LSIL (n = 30)	4 (13.3)	2.2 (1.1-7.2)	7.4, $P < 0.0001$
HSIL (n = 30)	12 (40.0)	4.5 (0.5-13.7)	17.9, $P < 0.0001$
Cancer (n = 90)	84 (93.3)	26.2 (0.2-131.6)	100.9, $P < 0.0001$

LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; SD: Standard deviation.

normal tissues ranged from 0.01 to 3.43 compared to those in cancer tissues ranged from 0.22 to 131.60. The Ki67 mRNA levels in cervical cancer tissues were 1.84- to 1167.33-fold higher compared to levels found in normal tissues (**Table 2**).

Diagnostic value of Ki67 for cervical cancer

ROC curve analysis was performed to determine the optimal diagnostic cutoff value for the assay to discriminate normal tissues from those with cervical cancer. Ki67 mRNA levels were analyzed in 90 FFPE cancer tissues and 40 FFPE normal tissues, and found that the area under the ROC curve (AUC) was 0.97 (95% CI = 0.94 to 1.00, $P < 0.001$, **Figure 1A**). We also found that the levels of Ki67 mRNA in cervical cancer tissues were significantly increased compared to that found in normal cervical tissues. Based on these findings, we set a diagnostic cutoff (threshold) of 5 ($P < 0.001$, **Figure 1B**). Using a threshold of 5, the assay had a sensitivity of 93.3% (95% CI = 86.1 to 97.5), a specificity of 97.5% (95% CI = 86.8 to 99.9), a positive predictive value of 98.8% (95% CI = 93.6 to 100.0), and a negative predictive value of 86.7% (95% CI = 73.2 to 95.0) (**Table 3**).

Table 5. Ki67 mRNA expression correlated with clinical parameters in 90 cervical cancer patients

	Ki67 mRNA expression			
	Number of cases	Low (Ki67 < 26.2)	High (Ki67 ≥ 26.2)	*P* value
Age (years)				
≤50 years	41	18	23	0.92
>50 years	49	21	28	
HPV DNA chip				
Negative	7	2	5	0.48
Positive	66	28	38	
FIGO stage				
< IIB	34	18	16	0.68
≥ IIB	43	17	26	
Lymph node				
Negative	37	21	16	0.01
Positive	37	12	25	
Tumor size				
< 4 cm	43	23	22	0.30
≥ 4 cm	33	13	20	

Ki67 mRNA levels in histologically diagnosed FFPE cervical tissues

Ki67 mRNA levels were evaluated with histologically diagnosed cervical tissues to determine whether Ki67 mRNA levels discriminated between normal, LSIL, HSIL, and cancer tissues. Ki67 mRNA levels were ranged from 0.0 to 5.6 (median 0.8) in 40 normal tissues, from 1.1 to 7.2 (2.2) in 30 LSIL tissues, from 0.5 to 13.7 (4.5) in 30 HSIL tissues, and from 0.2 to 131.6 (26.2) in 90 cancer tissues. The highest Ki67 mRNA levels were expressed in cancer tissues, with progressively lower but still elevated levels were checked in HSIL and LSIL tissues compared to the levels in normal tissues ($P < 0.0001$) (**Figure 2** and **Table 4**). Using a cutoff of 5, positivity for Ki67 was 2.5% (1/40 cases) for normal, 13.3% (4/30 cases) for LSIL, 40.0% (12/30 cases) for HSIL, and 93.3% (84/90 cases) for cancer. We found that this Ki67 mRNA assay using a diagnostic threshold of 5 discriminated between normal and abnormal cervical lesions ($P < 0.001$) (**Table 4**).

Ki67 mRNA expression in relation to clinical prognostic parameters in cervical cancer tissues

The median value of Ki67 mRNA levels in cervical cancer tissues was 26.2. To determine whether there was an association between Ki67 mRNA levels in cervical cancer tissues and clinical prognostic parameters, specifically, age, HPV status, FIGO stage, lymph node positivity, and tumor size, the Ki67 mRNA levelswere divided into two groups: a low Ki67 (below the median Ki67 mRNA level) group and a high Ki67 (above the median Ki67 mRNA level) group. Among the clinical prognostic parameters of cervical cancer,lymph node metastasis showed statistically significant relation with a high Ki67 group ($P = 0.01$) (**Table 5**).

Discussion

Cervical cancer is a leading cause of cancer mortality in 35-55 year old women worldwide. To avoid unnecessary treatment of transient HPV infections and related benign lesions, the optimal screening strategy for cervical cancer should efficiently and accurately identify precursor lesions that will progress to an invasive cancer [22]. The purpose of the present study was to evaluate Ki67 mRNA expression levels with histological grades to identify and understand the relationship between the Ki67 mRNA assay and clinicopathological parameters of cervical cancer, and assess the performance evaluation of the assay as a diagnostic test for the detection of cervical cancer.

Our study compared Ki67 mRNA levels between matched cancerous and noncancerous tissues from 16 patients diagnosed with cervical SCC or ADC, and found that there were significant differences in mRNA levels between matched FFPE tissue samples ($P = 0.0005$) (**Figure 1**). In fact, comparing to normal tissues, Ki67 mRNA expression levels in cancer tissues enabled their discrimination regardless of whether they were ADC or SCC. Yamamoto et al found that Ki67 mRNA expression levels were informative as a Ki67-labeling index in patients with breast cancer [8]. Taking a cue from this, in cervical cancer, this Ki67 mRNA assay was validated as adiscriminative marker.

Using 90 cervical cancer and 40 normal FFPE tissue samples, we studied this Ki67 mRNA assay accurately discriminated between cervical cancer and normal tissues with a high sensitivity of 93.3% (95% CI 86.1 to 97.5) and a high specificity of 97.5% (95% CI 86.8 to 99.9) (**Table 3**). Several studies have demonstrated that Ki67 expression using immunoquantification can provide greater discrimination not only

between normal and cancer tissues but also between LSIL and HSIL. And the reported average positivity rates found in normal, LSIL, and HSIL were 7.9%, 49%, and 90%, respectively [11, 23]. Similarly, in our study, positivity rates of Ki67 mRNA expression were 2.5%, 13.3%, and 40.0% in normal, LSIL, and HSIL respectively. Moreover, the positivity rates of Ki67 mRNA expression in cancer was 93.3%, and these were statistically significant ($P < 0.001$) (**Table 4**).

We also found that increased Ki67 mRNA expression in cancer samples was significantly higher than that found in normal, LSIL, and HSIL samples (**Figure 2**). Since about 10% of cases with LSIL progress to HSIL, cytological and/or histological follow-up are more frequently needed in LSIL patients with tendency of disease progression. Chen et al and Zhou et al were attempt to predict progression using Ki67 immunocytochemistry and immunohistochemistry tests and showed similar results that distinguishing LSIL and HSIL [24, 25]. In addition to burdening the healthcare system, the challenge of using such a broad screening approach is that it reduces overtreatment for follow-up in patients with LSIL, the majority of who do not progress to HSIL.

Through clinicopathological prognostic parameter analysis separating patients into high Ki67 expression (median ≥ 26.2) and low Ki67 expression (median < 26.2) groups, lymph node positivity was associated with Ki67 mRNA levels (**Table 5**). Shokouh studied that Ki67 IHC was correlated to lymph node status in breast cancer [9]. Yang et al showed lymph node metastasis and immunohistochemical markers Ki67 correlated for the predicting lymph node metastasis in endometrial cancer [26]. Cervical cancer in this study as well as breast and endometrial cancer in other studies also showed the interrelation of high Ki67 could be associated with lymph node positivity.

Previous studies demonstrated that IHC staining of Ki67 may be used to complement HPV testing [27, 28]. Because of HPV screening programs, HPV infection status is widely tested, but there are no predictors to determine the risk of high-risk HPV infection causing progression to cancer. We demonstrated that Ki67 mRNA assay can provide an additional accurate approach for molecular diagnosing cervical cancer, and also predict prognosis of cervical cancerdepending on LSIL and/or HSIL status.

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Disclosure of conflict of interest

None.

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