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# Is human papilloma virus DNA and cytokeratin 19 useful as micrometastatic markers in cervical cancer patients only with intermediate risk factors?

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**Summary**

*Purpose:* This study was designed to evaluate high risk human papilloma virus (HPV) DNA and cytokeratin 19 (CK19) as micrometastatic markers in cervical cancer patients only with intermediate risk factors. *Materials and Methods:* Polymerase chain reaction (PCR) for high-risk HPV DNA and immunohistochemistry for CK19 were performed in 335 lymph nodes from 67 patients. *Results:* HPV DNA and CK19 were detected in 23.5% and 2.7% of lymph nodes, respectively. HPV DNA was detected in 31 patients (46.3%), while CK19 was expressed in five patients (7.4%). The five-year disease-free and overall survival rates were 90.5% in the CK19- or HPV DNA-positive group and 76.8% in the negative group. There was no correlation between disease-free survival and CK19 or HPV DNA positivity. *Conclusion:* For the cervical cancer with intermediate risk factors, the presence of high-risk HPV DNA in lymph nodes is not useful as a micrometastatic marker, while CK19 expression requires further study.

*Key words:* Cervical cancer; Intermediate risk factor; Cytokeratin 19; Human papilloma virus.

## Introduction

The survival of patients with early-stage cervical cancer following radical hysterectomy and pelvic lymphadenectomy is associated with several intermediate and high-risk pathological factors for recurrence. For patients with high-risk factors such as positive or close margins, positive lymph node(s), and microscopic parametrial involvement, adjuvant therapy should be considered [1-3]. However, whether adjuvant therapy is required for patients with intermediate-risk factors such as large tumor size, cervical stromal invasion to more than half of stroma, and lymph-vascular space invasion, remains unresolved.

Many gynecologists are reluctant to treat patients with intermediate-risk factors because such patients have a good prognosis and the survival rate without adjuvant treatment is 85–90% [4]. Current treatment modalities for intermediate-risk patients vary from no further treatment to radiotherapy, chemotherapy, or concurrent chemoradiation. There are no randomized prospective studies investigating the appropriate treatment for patients in the intermediate risk factor group. For this reason, treatment modalities are selected according to institutional guidelines or the attending physician. Some studies have reported that concurrent chemoradiation could improve the survival rate in this group; however, concerns surrounding toxicity and over-treatment persist [5]. To address these issues, more clinically useful classifications are needed to determine appropriate treatment for the intermediate-risk factor group. The status of the lymph node is the most independent predictive factor for survival. Patients with negative nodes have a five-year survival rate of 85–90%, while the survival rate for those with positive nodes is 20–74% [6]. While patients classified as having intermediate risk have no pathologically-certified lymph node metastases, lymphatic recurrence or metastasis are sometimes found postoperatively. Therefore, the possibility of undetected micrometastasis or occult metastasis cannot be ruled out.

Human papilloma virus (HPV) DNA and cytokeratin 19 (CK19) have been previously identified as markers of micrometastasis in cervical cancer patients [7-10]. The aim of this study was to evaluate the usefulness of high risk HPV DNA and CK19 as markers of micrometastasis in cervical cancer patients with intermediate risk factors.

## Materials and Methods

This study included 130 patients with pathologically-certified cervical carcinoma and having intermediate risk factors, who underwent radical hysterectomy for cervical cancer between January 2000 and April 2014 at Kyungpook National University Hospital or Medical Center (Daegu, South Korea). All patients were treated with both radical hysterectomy and pelvic lymphadenectomy. The definition of intermediate risk factor in this study included stromal invasion above the middle section, tumor size of > two cm, and

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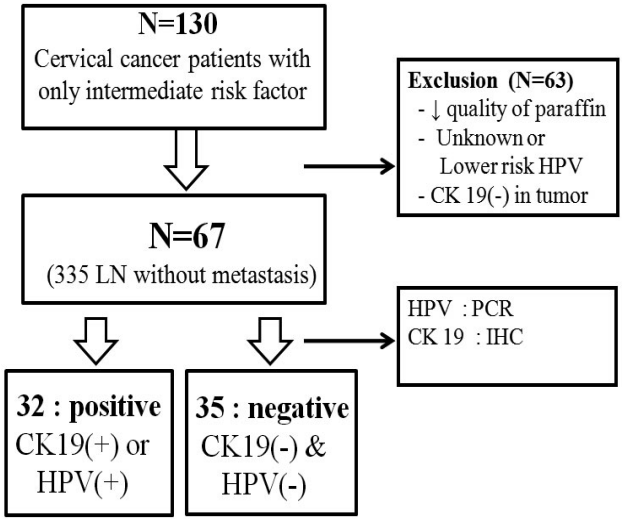


Figure 1. — Flow diagram of selection of the study population. CTX: chemotherapy; PCR: polymerase chain reaction.

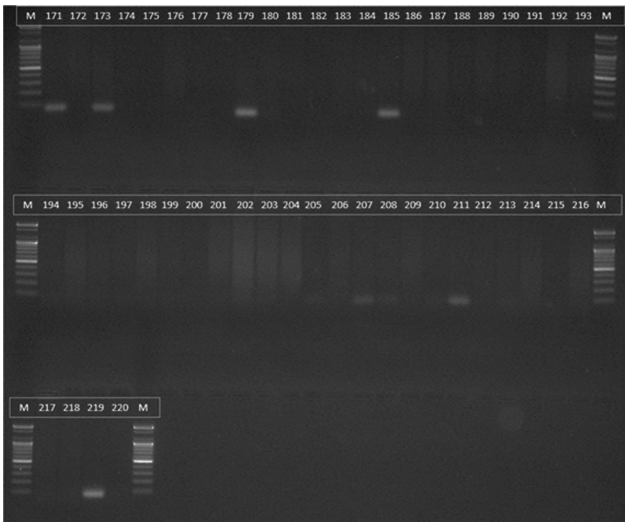


Figure 2. — The strong bands are defined as expression of high-risk HPV DNA. Faint bands are defined as negative expression.

lymphovascular space invasion.

Among the 130 patients, those with poor-quality paraffin block samples, those with unknown or lower risk HPV infection status of the primary tumor before surgery, and those treated with neoadjuvant chemotherapy were excluded. Finally, 335 lymph nodes among 67 patients were analyzed. The lymph nodes were selected among all harvested lymph nodes in each patient. The mean value of selected lymph nodes was five. The selected lymph nodes were located near the primary tumor such as the cardinal or pelvic lymph node. HPV analysis of all primary tumor was performed using Hybrid Capture II or DNA chip. The primary tumor samples were analyzed using immunohistochemical staining for the CK 19. The enrolled 67 patients showed high-risk HPV infection and CK 19 expression in primary tumor. For the selected lymph nodes, the presence of high-risk HPV DNA was determined using conventional polymerase chain reaction (PCR). CK19 expression was analyzed by immunohistochemical staining. Positivity was defined as the presence of CK19 and/or HPV DNA in lymph nodes, while negativity was defined as the absence of both CK19 and HPV DNA (Figure 1).

Paraffin-embedded samples of lymph nodes were cut into ten-μm sections, which were numbered and processed for PCR analysis. DNA extraction was performed using a DNA extraction kit according to the manufacturer's instructions. The purity and concentration of DNA were determined using a spectrophotometer. Extracted DNA samples were kept at −20°C until immediately prior to amplification. HPV DNA was amplified using the short PCR fragment (SPF10) primer set. SPF10 primers amplify a 65-base pair fragment from the L1 region of the HPV genome as previously described [11]. A short-fragment PCR assay was designed to discriminate high risk HPV types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52,53,56, 58, 59, 66,68, 73, and 82.

Four forward and two reverse primers were used (forward primers: 5'-GCiCAGGGiCACAATAATGG-3', 5'-GCiCAGGGiCATAACAATGG-3', 5'-GCiCAGGGiCATAATAATGG-3', 5'-GCiCAAGGiCATAATAATGG-3'; reverse primers: 5'-GTiGTATCiACAACAGTAACAAA-3', and 5'-GTiGTATCiACTACAGTAACAAA-3'). PCR cycling conditions comprised 60 seconds of initial denaturation at 94°C followed by 35 cycles of denaturation at 94°C for 60 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 30 seconds. Finally, a single extension step of 72°C for ten minutes was performed. PCR products were analyzed by electrophoresis using 2% agarose gels, and bands were visualized by ultraviolet illumination following addition of DNA staining solution. Strong bands of the expected size were defined as HPV-positive specimens, while faint bands were defined as HPV-negative specimens (Figure 2).

Immunohistochemical studies were performed on formalin-fixed paraffin-embedded tumor sections using an autosomal platform system, with an anti-CK19 monoclonal antibody (CK19, Clone RCK108) at a dilution of 1:100. All slides were evaluated by a pathologist (JYP) blinded to the clinicopathological information. Yellow-brown particles in the cytoplasm indicated a positive reaction (Figure 3).

Statistical analyses were performed using the Statistical Package for Social Sciences software version 20. Patient characteristics were compared between the positive group (CK19 and/or HPV DNA detected) and the negative group (both CK19 and HPV DNA undetected).

Mean values were compared between groups using the two-sample *t*-test. Frequency data were compared between groups using the $\chi^2$ test and Fisher's exact test, as appropriate. Pearson's correlation analysis was used for correlation between continuous variables and survival outcomes. Analysis of variance was used to analyze differences between group means. Kaplan–Meier survival analysis and log-rank tests were used to compare survival rates between the two groups. *P*-values < 0.05 were considered statistically significant.

This study was approved by the Institutional Review Board, and was supported by a Kyungpook National University Bio-Medical Research Institute grant for the enhancement of academic research.

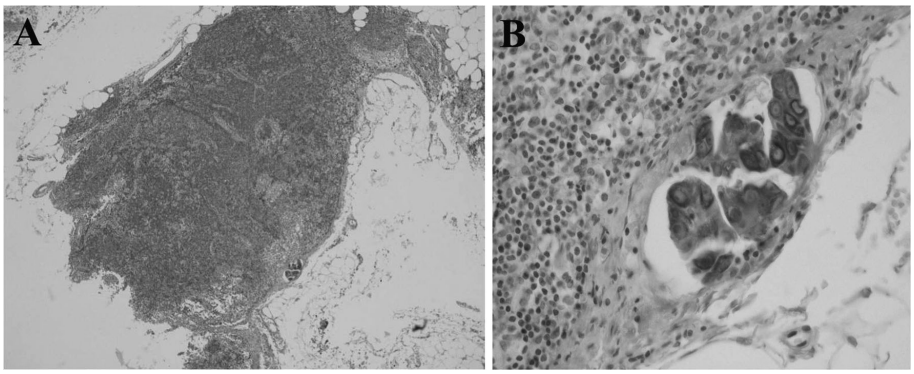


Figure 3. — CK19 expression in a pathologically certified non-metastatic lymph node. There is aggregation of epithelial cells in the subcapsular sinus of lymph node. The yellow brown particles in the cytoplasm represent positive reaction (A) immunohistochemistry ×40, (B) immunohistochemistry ×400.

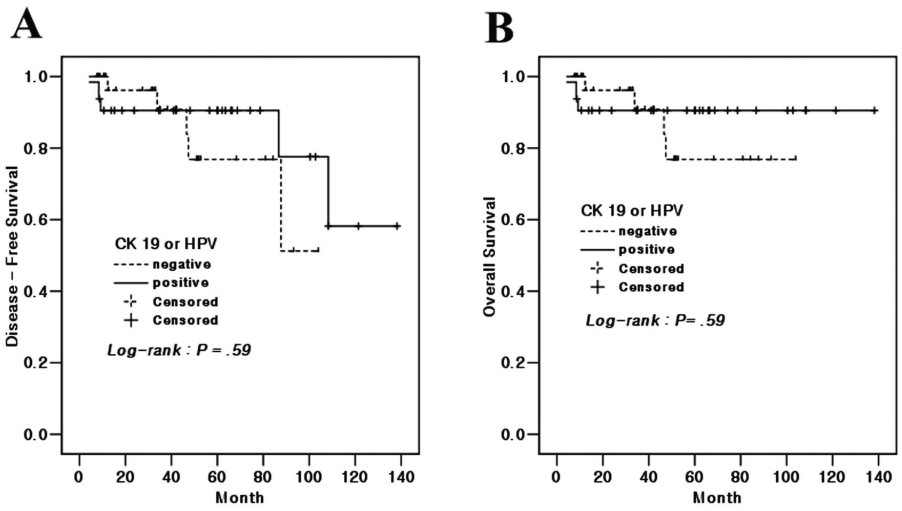


Figure 4. — A) five-year disease-free survival in CK19/HPV DNA positive group and negative group, B) Five-year overall survival in CK19/ HPV DNA positive group and negative group.

## Results

Both groups were similar with respect to age ($p = 0.98$), body mass index (BMI, $p = 0.43$), longest tumor diameter ($p = 0.87$), percentage of stromal invasion ($p = 0.70$), number of lymph-vascular space invasion ($p = 0.19$), adjuvant treatment ($p = 0.29$), and radiation therapy ($p = 0.35$). The predominant International Federation of Gynecology and Obstetrics (FIGO) Stage was IB1 in both groups ($p = 0.93$). Squamous cell carcinoma was the most prevalent histological cancer type found in both groups ($p = 0.95$). There were five cases of recurrence in each group ($p = 0.71$). Three patients died in the positive groups while four patients died in the negative group ($p = 1.00$). The mean follow-up period was statistically different between groups (55.53 ± 36.74 months in the positive group *vs*. 38.49 ± 27.20 months in the negative group, $p = 0.03$). The five-year disease-free and overall survival rates were 90.5% in the positive group and 76.8% in the negative group. There were no statistically significant differences in disease-free survival ($p = 0.47$) and overall survival ($p = 0.59$) between groups (Table 1; Figure 4). HPV DNA was expressed in 23.5% (79/335) of individual lymph nodes and CK19 was detected in 2.7% (9/335) of lymph nodes. HPV DNA was expressed by 46.3% (31/67) of individual patients and 7.4% (5/67) of patients were positive for CK19.

There was no statistically significant correlation between disease-free survival and clinical parameters, such as CK19 positivity, HPV DNA detection, age, BMI, cancer stage, histology, tumor size, tumor invasion, lymphovascular space invasion, adjuvant treatment, or radiation therapy (Table 2). Five patients in each group developed recurrence, including pelvic recurrence in five patients and distal metastasis in another five patients. The sites of recurrence in the positive group were: multiple sites within the vaginal cuff, lung, and peritoneum (n = 1), right pelvic sidewall near the ureter (n = 1), left obturator lymph node (n = 1), and para-aortic lymph node (n = 2). The sites of recurrence in the negative group were: multiple sites in the lung, mediastinum, and skull (n = 1), right pelvis (n = 1), bone (n = 1), and vaginal cuff (n = 2) (Table 3).

Table 1. — *Characteristics of patient groups.*

| | Positive group n=32 | Negative group n=35 | *p* |
|---|---|---|---|
| Age (years) | 49.28±11.20 | 49.34±10.41 | 0.981† |
| BMI (Kg/m$^2$) | 24.38±4.05 | 23.61±3.71 | 0.426† |
| Stage (FIGO) (n) | | | 0.929† |
| Ia1 | 0 | 1(2.9) | |
| Ia2 | 1(3.1) | 1(2.9) | |
| Ib1 | 24(75.0) | 25(71.4) | |
| Ib2 | 2 (6.3) | 4 (11.4) | |
| IIa1 | 5 (15.6) | 3 (8.5) | |
| IIa2 | 0 | 0 | |
| IIB | 0 | 1(2.9) | |
| Histology (n) | | | 0.951† |
| Squamous | 22 (68.7) | 27(77.2) | |
| Adenocarcinoma | 8 (25.0) | 6 (17.1) | |
| Adenosquamous | 2 (6.3) | 2 (5.7) | |
| Diameter (longest, cm) | 2.13±1.34 | 2.05±1.97 | 0.870† |
| Invasion (%) | 67.15±22.60 | 69.21±18.17 | 0.702† |
| LVSI (n) | 16 (50.0) | 12 (34.3) | 0.193‡ |
| Adjuvant treatment(n) | 17 (53.1) | 23 (65.7) | 0.294‡ |
| Radiation (n) | 11(34.4) | 16(45.7) | 0.345‡ |
| Recurrence (n) | 5 (15.6) | 5(14.3) | 0.880§ |
| Deaths (n) | 3 (9.3) | 4(11.4) | 1.000§ |
| Follow up (month) | 55.53±36.74 | 38.49±27.20 | 0.034† |
| DFS (5 years, %) | 90.5% | 76.8% | 0.466¶ |
| OS (5 years, %) | 90.5% | 76.8% | 0.592¶ |

Data are mean ± SD values or number (%). †: two sample *t*-test; ‡: $\chi^2$ test; §: Fisher's exact test; ¶: Kaplan-Meier and log-rank test.
BMI: body mass index; FIGO: International Federation of Gynecology and Obstetrics; LVSI: lymph-vascular space invasion; DFS: disease free survival; OS: overall survival. Adjuvant treatment includes chemotherapy only, radiation therapy only, or chemoradiation therapy.

Table 2. — *The correlation of disease free survival with other parameters.*

| | Sub group | Number of patient | *p* |
|---|---|---|---|
| CK 19 positivity | Positive | 5(7.4) | 0.920 † |
| | Negative | 62(92.6) | |
| HPV DNA | Positive | 31(46.3) | 0.827 † |
| | Negative | 36 (53.7) | |
| Age | | | 0.985§ |
| BMI | | | 0.123§ |
| Stage | Ia1 | 1 (1.5) | 0.612§ |
| | Ia2 | 2(3.0) | |
| | Ib1 | 49(73.1) | |
| | Ib2 | 6 (9.0) | |
| | IIa1 | 8 (11.9) | |
| | IIa2 | 0 | |
| | IIb | 1(1.5) | |
| Histology | Squamous | 49(73.1) | 0.952¶ |
| | Adenocarcinoma | 14 (21.0) | |
| | Adenosquamous | 4 (5.9) | |
| Diameter | | | 0.808§ |
| Invasion | | | 0.158§ |
| LVSI | Positive | 28(41.8) | 0.709‡ |
| | Negative | 39(58.2) | |
| Adjuvant treatment | Yes | 40 (59.7) | 0.508‡ |
| | No | 27 (40.3) | |
| Radiation | Yes | 27 (40.3) | 0.150‡ |
| | No | 40 (59.7) | |

Data are number (%). †: Kaplan-Meier and log-rank test; ‡: two sample *t*-test, §: Pearson's correlation analysis, ¶: ANOVA.
BMI: body mass index; FIGO: International Federation of Gynecology and Obstetrics; LVSI: lymph-vascular space invasion. Adjuvant treatment includes chemotherapy only, radiation therapy only, or chemoradiation therapy.

Table 3. — *Recurrence after radical hysterectomy in patients with intermediate risk factors.*

| | Positive group (n=5) | Negative group (n=5) |
|---|---|---|
| Site (n) | Vaginal cuff, lung, pericardium (1) | Lung, mediastinum, skull (1) |
| | Right pelvis (ureter) (1) | Right pelvis (1) |
| | Left obturator lymph node (1) | Bone (1) |
| | Para-aortic lymph node (2) | Vaginal cuff (2) |

## Discussion

Surgical intervention following detection of early-stage cervical cancer with intermediate risk factors generally has a favorable prognosis. Because postoperative adjuvant therapies such as radiation, chemotherapy, and chemoradiation can have considerable acute and/or chronic effects, identifying risk factors and determining an appropriate post-surgical treatment, if any, is important to prevent over-treatment and minimize side effects [12]. These underlying issues have been previously examined by a clinical trial for risk grouping of patients with intermediate risk factors which aimed to develop new criteria to better predict prognosis for this category of patients [13]. In addition to the efforts towards classification of prognostic factors using clinical parameters, other trials have continued the analysis through laboratory assessment. The most intensive study has been towards identifying markers of occult metastasis or micrometastasis. Micrometastasis is defined as the presence of scattered early cancer cells or cell clusters that cannot be detected by using conventional pathological techniques. The goal of early micrometastasis detection is to help formulate a reasonable treatment strategy and to provide appropriate postoperative adjuvant treatment. However, evidence of a close relationship between micrometastasis and recurrence and prognosis is lacking.

Cytokeratins belong to the intermediate filament protein family and are present within the cytoplasm. Type 8, 18, and 19 cytokeratins are the most abundant cytokeratins in simple epithelial cells and are the most frequently expressed in malignant tumors. Additionally, CK19 is expressed specifically by the epithelial cells of female genital ducts, but not by lymphocytes, meaning that it should be absent in normal lymph nodes. As CK19 is present in tumor

tissue, it has become the most frequently used molecular marker for detection of lymph node micrometastasis of cervical cancer [7,14].

CK19 was detected in at least one lymph node from 7.4% (5/67) of individuals with cervical cancer, which is lower than the prevalence reported in previous studies. This low number of cases limits the present analysis of the association between CK19 expression and clinical outcomes. Zhang *et al.* identified CK19 in 46.6% of pathologically-certified non-metastatic cervical cancer patients, and 24.6% of all lymph nodes analyzed expressed CK19 [15]. Similarly, Van Trappen *et al.* showed via Reverse Transcriptase –PCR (RT-PCR) that 44% of histologically uninvolved lymph nodes were positive for CK19 mRNA, while only 32% of lymph nodes from patients with benign disease were positive at a low CK19 transcription level [16]. Wang *et al.* detected micrometastasis at rates of 43% and 20% using RT-PCR and immunohistochemistry, respectively. The current study population exhibited a significantly lower positive rate than previous studies [7]. Lentz *et al.* analyzed 132 early-stage (Stages Ia2-Ib2) cervical cancer patients with histologically-negative 3016 lymph nodes with cytokeratin AE-1 and CAM 5.2 to detect micrometastases. Approximately 15% of patients and 0.9% of lymph nodes were positive for AE-1 and/or CAM 5.2 [17].

Since the first report describing the identification of HPV DNA in a lymph node affected by cervical cancer metastasis [18], multiple studies have confirmed that pelvic lymph nodes with metastases tend to be HPV-positive [8]. Therefore, detection of HPV DNA has been used to identify micrometastasis in pathologically-certified non-metastatic lymph nodes.

HPV DNA has previously been found in 25–100% of metastatic lymph nodes in cervical cancer. In contrast, only 8.8–90.1% of non-metastatic lymph nodes are positive for HPV [8]. Several researchers have suggested that the presence of HPV DNA in lymph nodes may not be as important as once thought, because such DNA may result from free DNA particles from immune cells or from tumor cells destroyed by immune cells [19, 20]. Additionally, Füle *et al.* found that cervical cancer patients with non-metastatic lymph nodes had similar disease-free survival rates regardless of whether these nodes had detectable HPV [21]. Because the aforementioned studies are not standardized in technique and patient selection, it is difficult to draw conclusions from the overall data.

Compared with previous reports, in this study, HPV DNA and CK19 prevalence in lymph nodes were lower, at both the patient and individual lymph node levels. One potential explanation for the lower prevalence of CK19 expression is that the authors employed immunohistochemistry, and not PCR, to detect CK19 expression. Immunohistochemical staining was used to detect CK19 at protein level and to prevent false positivity at the transcription level [16]. Second, the enrolled patients already had a low risk of metastasis, as indicated by the absence of high-risk factors, which include metastasis to the lymph nodes. Moreover, previous studies analyzed only pathologically-certified non-metastatic lymph nodes regardless of the metastatic status of the patient. It is reasonable to assume that non-metastatic lymph nodes from patients with other metastatic nodes are more likely to be HPV DNA-positive. Third, all the harvested lymph nodes could not be used for the study, mainly, because of poor quality paraffin blocks. The harvested lymph nodes were collected without sentinel lymph node concept, which was recently applied for the operation fields. The number of average harvested pelvic lymph nodes was 23 in this study. Of these lymph nodes, five (21.7%) were used for PCR and immunohistochemistry. Previous studies have focused on the relationship between metastatic status and HPV detection and genotype within the lymph node. However, few studies have investigated the relationship between HPV DNA/CK19 expression and survival outcomes. Furthermore, these studies did not demonstrate any prognostic significance of HPV DNA/CK19 detection, because confounding factors, such as disease severity and presence of metastatic lymph nodes, were not controlled for [21].

In the present study, the authors attempted to determine the prognostic significance of HPV DNA/CK19 presence in non-metastatic lymph nodes from patients with intermediate-risk factors for cervical cancer by conducting survival analyses. HPV DNA was not associated with prognosis in patients with intermediate risk factors. However, owing to the small number of positive cases, this study could not determine the prognostic significance of CK19 in non-metastatic lymph nodes. Given the HPV DNA detection rate of 80% and the death of one of five CK19-positive patients, CK19 expression may have some prognostic significance and should be evaluated by larger-scale studies.

A diagnosis of micrometastasis is made using indirect evidence such as the presence of particular markers rather than by direct histological cancer cell detection. However, the detection of these associated markers does not imply clinically significant metastasis. Much controversy still surrounds the clinical significance of micrometastasis, as not all micrometastases will progress to form new tumors. While the authors anticipated worse survival outcomes for the positive group, this group actually showed a higher survival rate, although it was not a statistically significant difference. These results warrant further investigation into the underlying mechanisms. There is no evidence that the detection of HPV DNA in a pathologically-certified non-metastatic lymph nodes is directly related to cancer cell metastasis. HPV DNA may stem from free DNA particles or a precursor event to metastasis. The presence of HPV DNA may induce an immune defense mechanism without progressing to cancer cell metastasis, which may play a role in suppressing HPV-related oncogenesis, metastasis, or cancer cell attachment. This hypothesis requires further investigation in future studies [22].

Although the role of HPV DNA and CK19 in pelvic lymph nodes of cervical cancer patients has been recently reviewed and summarized all patients with high, intermediate or no risk [10], this study focused on the patients with only intermediate risk factors. The present study, however, was flawed as its sample size is lacking, with generalization of this study results and authors could not found the usefulness of CK19 in patients with intermediate risk factors.

## Conclusion

For cervical cancer patients with only intermediate risk factors, the detection of high-risk type HPV DNA in lymph nodes is not useful as a micrometastatic marker, but the value of CK19 expression requires clarification in further large studies.

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