Expression of high-risk HPV DNA and CK19 in pelvic lymph nodes in stage Ia-IIa cervical cancer and their clinical value

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Received November 23, 2011; Accepted February 2, 2012

DOI: 10.3892/or.2012.1708

Abstract. The aim of this study was to investigate the detection rate and methods of micrometastases in early-stage cervical cancer by detecting the expression of high-risk HPV DNA and CK19 in pelvic lymph nodes. A total of 104 lymph nodes with/without pathologically confirmed metastases, from 28 patients with early-stage cervical cancer, were included for detection of high-risk HPV DNA and CK19 expression using *in situ* hybridization and immunohistochemistry, respectively. The detection rate of high-risk HPV DNA and CK19 in lymph nodes in patients with pathologically-confirmed lymph node metastases was higher compared to that in lymph nodes in patients without pathologically-confirmed lymph node metastases (P<0.001). In all 80 pathologically-negative lymph nodes, the positivity rates of high-risk HPV DNA and CK19 detection were 45 and 25%, respectively. In 57 lymph nodes in patients without pathologically-confirmed lymph node metastases the positivity rates of high-risk HPV DNA and CK19 detection were 43.5 and 24.6%. The detection rate of high-risk HPV DNA and CDK19 in 15 patients without pathologically-confirmed lymph node metastases were 60 and 46.6%, respectively. The detection rates of high-risk HPV DNA and CK19 in 104 lymph nodes were 56.7 and 41.3% (KI=0.46). The results of the two detection methods showed good consistency. Both detection of high-risk HPV DNA by *in situ* hybridization, and CK19 by immunohistochemical method detected lymph node micrometastases in early-stage cervical cancer. As a method of detection on the molecular level, *in situ* hybridization was more sensitive for the detection of lymph node micrometastases in early-stage cervical cancer.

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Key words: human papillomavirus, cervical cancer, lymph node micrometastases, *in situ* hybridization, immunohistochemistry, cytokeratin

Introduction

Cervical cancers, which severely threaten women's health are the only kind of human malignancies whose incidence rate and motality rate can be reduced by medical interventions (1). In China, surgery is the major therapy for early stage cervical cancer, and its standard procedure includes radical hysterectomy plus pelvic lymph node dissection. One of the most important prognostic factors for cervical cancer is lymph node metastases. Studies note that patients with pathologically confirmed nodal metastases can reduce their postoperative recurrence rate as well as increase survival rate by receiving postoperative adjuvant radiotherapy or/and chemotherapy (2). Therefore, the treatment strategy which further affects prognosis directly depends on the identification of regional lymph node metastases in patients with early-stage cervical cancer.

Micrometastases refer to the scattered isolated cancer cells that can hardly be detected by common pathological examination. Along with the development of immunochemistry and molecular biology, it is now possible to detect single and insidious cancer cells that are concealed under hematoxylin and eosin (H&E) stain in conventional pathology, providing new detection methods for studying lymph node micrometastases in cervical cancer. Cytokeratin (CK) is mainly used as a detection index in former studies on lymph node micrometastases in cervical cancer, yet detection rate of micrometastases in pathologically negative lymph nodes varies greatly by different methods: 8-15% (3) by immunohistochemical (IHC), and 44% by polymerase chain reaction (PCR) with the micrometastases rate of 50% (4). Besides, high-risk human papilloma virus (HPV) DNA which is integrated into cervical cancer cell nucleus is also applied for micrometastasis detection, and by detection of HPV 16 DNA using PCR the micrometastases rate of cervical cancer with pathologically negative lymph nodes is 26.3-38% (5,6). The reason for the great differences of these results is due to the disadvantages of both PCR and IHC, the former method can be easily contaminated and have more internal interference, resulting in exceedingly high false-positive rate, while low sensitivity of the latter one can easily increase false-positive rate as well. In this study we employed *in situ* hybridization (ISH), which is more accurate in location and has higher specificity but no internal interference,

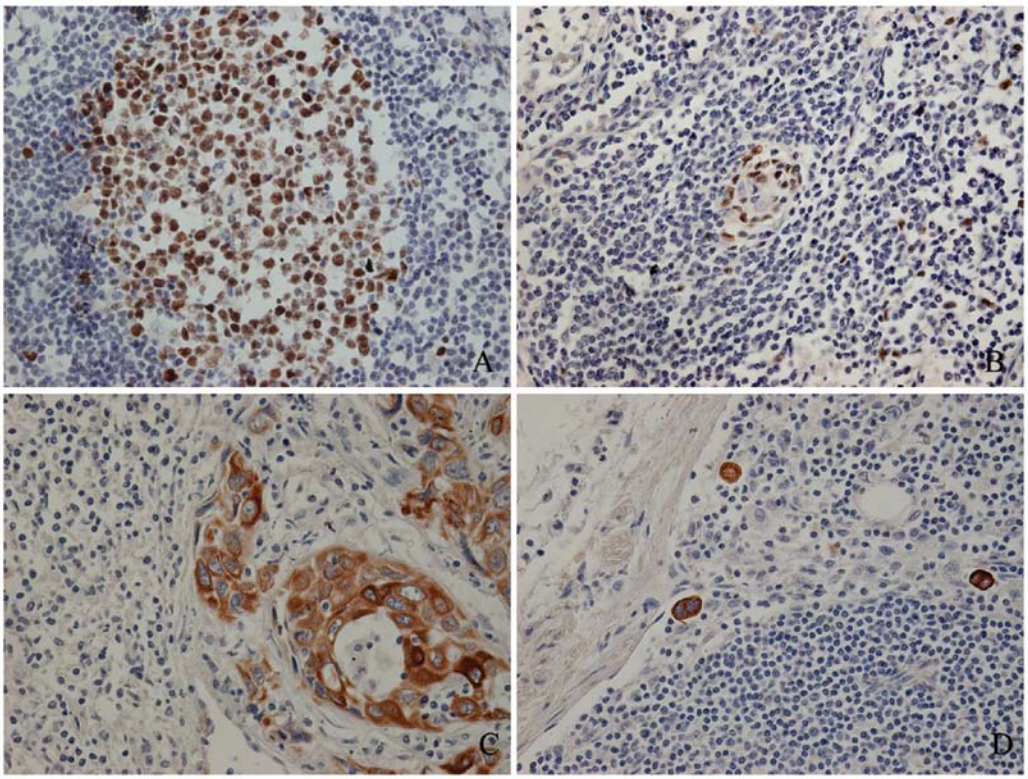

Figure 1. HPV DNA and CK19 expression in lymph nodes. (A) HPV DNA expression in pathologically certified metastatic lymph node. (B) HPV DNA expression in a lymph node from patient without pathologically-confirmed lymph node metastases. (C) CK19 expression in a pathologically certified metastatic lymph node. (D) CK19 expression in a lymph node from patient without pathologically-confirmed lymph node metastases.

for detection of high-risk HPV DNA, combined with IHC for detecting CK19. We investigated detection methods of micrometastasis in lymph nodes in early-stage cervical cancer and its relation with the clinical pathological features, and additionally a comparison between these two detection methods was made.

Materials and methods

Resource of specimens and reagents. We selected 28 paraffin-embedded specimens of cervical cancer operated at China Medical University Affiliated Shengjing Hospital from 2003 to 2005. Patients of these specimens had neither received preoperative adjuvant therapies (radiotherapy or chemotherapy), nor experienced other medical or surgical complications. Based on the International Federation of Gynecology and Obstetrics (FIGO) staging criteria for cervical carcinoma, the patients included 15 cases of stage I and 13 cases of stage II. Of the cases, 26 showed squamous cell carcinoma and two adenocarcinoma. Pathological grading, 7 cases were of high differentiation, 14 of intermediate differentiation, and 7 of low differentiation. The mean age of the patients was 41 years (range, 29-60 years). Thirteen cases had lymph node metastases confirmed by pathological examination, and the other 15 did not. Paraffin-embedded specimens were taken from the primary lesions and lymph nodes in the obturator and internal iliac regions of both sides. A total number of 104 lymph nodes were obtained from these 28 patients, averaging 3 to 4 per patient. According to the pathological metastatic condition, the lymph nodes were divided into three groups: group of metastatic lymph nodes from patients with lymph node metastases, group of non-metastatic lymph nodes from patients with lymph node metastases, and group of lymph nodes from patients without lymph node metastases.

Materials and preparation of sections. High-risk HPV DNA ISH DIG Staining System Kit (FISH 2004 HPV) was purchased from Tianjin Haoyang Biology Corp.; CK19 monoclonal antibody, Ultra SensiveS-P IHC was purchased from Fuzhou Maixin Biotechnology Corp. Histological section of tissues was 4 μm.

Detection of high-risk HPV DNA by ISH. High-risk HPV DNA probes were designed and synthesized (sequences: upstream 5'-CTGTGTAGGTGTTGAGGTAGGTCGTGGTC-3', downstream 5'-ATCATCAACATTTACCAGCCCGACGAGC-3'). This probes could be used in combination to hybridize successfully with positive template sequences of HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. Phosphate-buffered saline instead of probes was added for negative controls, while 10 lesions, which were confirmed pathologically as cervical cancer served as positive controls. Assessment of results, positive hybridization signal was identified as brown yellow particles located in the nucleus.

Detection of CK19 using IHC method. CK19 was detected using IHC method according to the kit directions. Negative controls and positive controls were the same as previously described. Assessment of the result, yellow brown particles in the cytoplasm represented positive reaction.

Table I. Detection rate of HPV DNA and CK19 in three groups of lymph nodes.

Groups of lymph nodes	N	HPV DNA (+)			CK19 (+)		
		n	%	P-value	n	%	P-value
H&E (+) lymph nodes in patients with metastases	24	23	95.8	P<0.001[a]	23	95.8	P<0.001[a]
H&E (-) lymph nodes in patients with metastases	23	13	59.1		6	26.1	
H&E (-) lymph nodes in patients without metastases	57	23	43.5		14	24.6	

[a]Comparison between three groups or two groups are all P<0.001. HPV, human papilloma virus; CK, cytokeratin; H&E, hematoxylin and eosin.

Statistical analysis. All statistical information was analyzed using SPSS 13.0 software. We applied the χ^2 test to compare the differences of detection rate of high-risk HPV DNA and CK19 among these three groups, and Fisher's exact tests to analyze the possible relationship between the positivity rate of high-risk HPV DNA in lymph nodes with clincopathological features including age, tumor stage, pathological grade and lymph node metastases. P-value <0.05 was considered statistically significant. Consistency test was performed to analyze consistency of the results by calculating Kappa index (KI): 0.75>KI>0.4 was defined as good consistency and KI≥0.75 as extremely good consistency.

Results

Expression of high-risk HPV DNA in lymph nodes in three groups and their relation with the clinicopathological parameters of cervical cancer. All 28 primary lesions with cervical cancers displayed positive reaction when detecting the expression of high-risk HPV DNA using ISH. Ten of these 28 cases were selected for positive controls. From 13 patients with lymph node metastases, 23 of 24 pathologically certified metastatic lymph nodes were high-risk HPV DNA positive (95.8%); so were 13 of 23 lymph nodes without pathologically-certified metastases (59.1%) (Fig. 1A). Twenty-three of 57 lymph nodes from 15 patients without pathologically-confirmed nodal metastases revealed the presence of HPV DNA (43.5%). All high-risk HPV DNA-positive lymph nodes were derived from 9 (9/15) patients and the detection rate of micrometastases was 60% (Fig. 1B). Positivity rate of HPV DNA in all pathologically negative lymph nodes was 45%.

Table I shows comparison of the detection rate of high-risk HPV DNA in three groups of lymph nodes. The detection rate of high-risk HPV DNA in group of metastatic lymph nodes from patients with lymph node metastases was obviously higher than that either in group of non-metastatic lymph nodes from patients with nodal metastases, or in the group of lymph nodes from patients without nodal metastases (P<0.001). According to the location, group of lymph nodes from patients without lymph node metastases were further divided into four subgroups such as left obturator lymph nodes, right obturator lymph nodes, left internal iliac lymph nodes, and right internal iliac lymph nodes, whose detection rate of HPV DNA turned out to be 60.0% (9/15), 25.0% (3/12), 46.7% (7/15), 26.7% (4/15), respectively, suggesting no statistical significance (P=0.172). The detection rate of HPV DNA in the left lymph nodes, including left obturator and left internal iliac lymph nodes, was higher than that in the right lymph nodes, including right obturator and right internal iliac lymph nodes (53.3 and 25.9%, respectively) (P=0.035).

As presented in Table II, detection rate of HPV DNA in lymph nodes in FIGO stage I patients was 78.6% (10/14), and 85.7% (12/14) in stage II patients, indicating that the detection rate of HPV DNA showed an upward trend along with increased stage (P>0.05), a downward tendency with increased differentiation degree (P>0.05), and no relation with the patient age (P>0.05).

Expression of CK19 in three lymph node groups and their relationship with the clinicopathological parameters of cervical cancer. All 28 primary lesions with cervical cancer revealed CK19 positive reaction. Twenty-three of 24 pathologically-confirmed metastatic lymph nodes from patients with lymph node metastases were positive for CK19 (95.8%). Six of 23 lymph nodes without pathologically-confirmed metastases were CK19-positive (26.1%) (Fig. 1C). Of the 57 lymph nodes from 15 patients without pathologically-confirmed lymph node metastases, 14 showed CK19 positive (24.6%). As all 14 CK19 positive lymph nodes came from 7 patients, the detection rate of micrometastases was accordingly 46.6% (7/15), (Fig. 1D). Detection rate of CK19 in all pathologically-negative lymph nodes was 25% (20/80).

Comparison of the detection rate of CK19 in the three groups is depicted in Table I. The detection rate of CK19 in metastatic lymph nodes from patients with nodal metastases was remarkably higher than that in the other two groups (P<0.001).

Consistency test of the results of the detection methods. The consistency of the results of two detections methods, IHC method for detecting CK19 and ISH for detecting HPV DNA, was good. For 104 lymph nodes specimens, the detection rate by ISH was 56.7% (59/104), and 41.3% (43/104) by IHC

Table II. Association between HPV DNA and CK19 expression and pathological features in lymph nodes.

Clinical pathological parameters	N	HPV DNA (+)		P-value	CK19 (+)		P-value
		n	%		n	%	
Age (years)							
≤35	9	7	73.7	P=0.703	6	66.7	P=0.944
>35	19	15	88.8		14	73.7	
Stage by FIGO							
I	14	10	78.6	P=0.648	8	57.1	P=0.209
II	14	12	85.7		12	85.7	
Differentiation degree							
High	7	5	71.4	P=0.806	5	71.4	P=0.592
Medium	14	11	78.6		11	78.6	
Low	7	6	85.7		4	57.1	
Lymph node metastases							
+	13	13	100	P=0.018	13	100	P=0.002
-	15	9	60.0		7	46.7	

HPV, human papilloma virus; CK, cytokeratin.

Table III. Relevance of HPV DNA and CK19 detection in lymph nodes.

	High-risk HPV DNA		
	+	-	N
CK19			
+	42	1	43
-	17	44	61
N	59	45	104

HPV, human papilloma virus; CK, cytokeratin.

method. The coincidence of the methods was 71.7%, and KI was 0.46 (Table III).

Discussion

In 1995, WHO and LARC established the significant role of HPV infection in the development of cervical cancer. Molecular biological studies in recent years have found that high-risk HPV DNA, including type-16, 18, 30, 31, 33, 35, 39, 45, 51, 52, 56, 58, 66, 69. exist in the genome of the host cells mainly in integration pattern. Moreover, 120 types of HPV have been identified, of which about 35 types can cause genital duct infection and 20 types are associated with tumors, and HPV infection can be detected in 99.8% of cervical cancers. HPV in the metastatic lesions of cervical cancer always displays the same type as in the primary lesions, and this is also true for the integration pattern when the viral DNA is integrated into cancer cells in the primary lesions. Therefore, it has become a crucial marker of micrometastases to find HPV DNA in pathologically-negative lymph nodes (7). There are some major methods for detecting viral DNA, such as nucleic acid hybridization, hybrid-capture system (HCS), and PCR. In this study we primarily employed ISH, which detects unknown DNA samples on molecular level by identical DNA sequences. As a convenient, technologically mature, simple and quick method, ISH can directly detect the HPV DNA sequences in the tumor tissue as well as be observed in the infectious particles under a microscope, therefore becoming 'gold standard' for HPV identification (8). Apart from its advantageous characteristics of localizability and high specificity, it can additionally avoid contamination resulting from PCR technique to reduce false-positive rate. Although PCR test has been mostly used in recent studies on lymph node micrometastases of cervical cancer, in this study we applied ISH for investigating micrometastases of cervical cancer. The results demonstrated that the detection rate of HPV DNA in all pathologically-negative lymph nodes in patients with and without nodal metastases was 45%, and in patients without pathological nodal metastases 43.5%, both of which were higher than the reported results by Chan *et al* (5). The probable reasons might be: i) the hybridiztion probes in this experiment covered almost all high-risk HPV DNA, while Chan *et al* only detected HPV 16 DNA. ii) We increased the sample size and therefore reduced possible bias. iii) It was possible for the false positive rate to appear in this study for the reason that some scattered high-risk HPV DNA-positive cells were probably macrophage-phagocytized cancer cells, the dead cells that were not micrometastatic cancer cells as they were misleadingly regarded. In this study we did not find significant association between the detection of HPV

DNA and some parameters such as age, stage or grade of tumor, yet the sample size was comparatively small so that we could not draw this conclusion affirmatively. The explanation for the result that the detection rate of high-risk HPV DNA in pathologically-confirmed metastatic lymph nodes using ISH was <100% may be that during the metastases and integration processes of viral DNA, some mutations occurred, which led to exclusion of these genes from the detection scale of the used probes.

Cytokeratin, an index of especial value for oncological diagnosis, belongs to intermediate filament protein family and exists in the cytoplasm to construct reticular cystoskeleton which is responsible for the mechanical intactness of the cells. More than 20 types of different CK have been identified currently, among which type-8, -18 and -19 are most abundant in simple epithelial cells and most frequently expressed in malignant tumors. The expression of CK varies along with the types of epithelial cells, differentiation degree, and tissue development. During the gradual process for a normal cell progressing into a cancer cell, the structure of the CK does not change the characteristic that renders it a pragmatic tumor marker (9). Besides CK19 is expressed specifically in the epithelial cells of female genital duct, but not in lymphocytes, so that it should be absent in normal lymph nodes, therefore becoming the most frequently used molecular marker for lymph node micrometastases of cervical cancer. Thus, CK is still the most commonly applied as monitoring index in studies on micrometastases of cervical cancer. A recent study, in which CK19 was used as an index for detecting micrometastases in sentinal lymph nodes of cervical cancer, by Wang *et al* (10) found that the detection rates of micrometastases were 42.85 and 20% using PCR and IHC, respectively, and suggested that PCR was more sensitive than IHC. Moreover, Van Trappen *et al* (4) also noted that 44% of pathologically-negative lymph nodes expressed CK19, and that early micrometastases occured in 50% of patients with cervical cancer. In this study the detection rate of micrometastases by IHC for detecting CK19 expresssion in pathological lymph nodes of cervical cancer was 24.6%, while Lentz *et al* (11), using similar method, index and materials, found micrometastases occurred in 15% of the patients and that the detection rate of micrometastases in lymph nodes was 0.9%, both of the percentages are lower than ours. The incidence rates of micrometastases in our study detected by ISH and IHC (60 and 46.6%, respectively) were also higher than that reported in other literature. This may be probably due to small sample size and the false-positive rate caused by some cancer cells that had been eliminated by lymphatic system and some dead cancer cells, both of which could be falsely considered as insidious metastatic cancer cells.

Sentinel lymph node (SLN) refers to the first lymph nodes along the metastatic way, which could reflect the lymph drainage condition in the whole region. Localization of SLN of cervical cancer demonstrates that SLN are mainly located in iliac blood vessels (33.3-84.1%) and obturator region (11.5%) (12). We selected lymph nodes in obturator and internal iliac of both sides, expected them, as SLN, to be more accurate in reflecting the lymph node metastases status in pelvic. The results showed that the micrometastases rate in lymph nodes from left obturator and internal iliac region was higher than that in the right side ($P<0.05$), indicating that we should pay more attention to the detection and clearance of the left pelvic lymph nodes to provide evidence for further correct treatment strategy. On the other hand, definite conclusion can not be drawn until more research has been carried out with large sample size.

The metastases of cancer, as habitually understood, starts from one single cell which proliferates into visible metastatic lesions, and those scattered isolated early cancer cells or cell cluster that could hardly be detected by conventional pathological technique are defined as micrometastases. As for the micrometastases in breast cancer, AJCC has set its lower limit as metastatic lesion measuring between 0.2 mm and 2 mm is of grade PN1 (13) and metastatic lesion ≤0.2 mm, such as an isolated tumor cell, is of grade PN0 (14). This opinion was solidified in this experiment as that micrometastases rate in pathologically-negative lymph nodes from patients with nodal metastases was obviously higher than that from patients without nodal metastases ($P<0.001$), suggesting that micrometastases of cervical cancer was an early incident appearing before metastatic lesion could be pathologically observed. Also it indicated that patients could benefit from early detection of micrometastatic lesion, which could help to formulate correctly a reasonable treatment strategy and to promptly and accurately provide postoperative radiotherapy and chemotherapy. These steps are of great significance for patients with cervical cancer to enhance their postoperative survival. Moreover, it was also confirmed that both of the two methods employed in this experiment could be applied for detection of micrometastases in early-stage cervical cancer, while ISH for detection of HPV DNA was more sensitive than IHC method for detecting CK19. However, the clinical significance of micrometastases is still under discussion, for up to now definite evidence is still lacking for proving a close relation between micrometastases and the recurrence and prognosis of cervical cancer. Though most scholars approve association between micrometastases and prognosis of cervical cancer, there is no clear evidence for evaluating the clinical value of micrometastases. We will continue the follow-up of these patients to investigate the possible association between micrometastases and prognosis of cervical cancer.

Acknowledgements

This study was supported by grants from The National Natural Science Foundation of China (nos. 30571958, 30872757, 81072118); Educational Department Science Foundation of Liaoning Province (no. 20121268), Liaoning Natural Science Foundation (no. 20052107); Educational Department Doctor Startup Fund (no. 20070159023); Educational Department Key Laboratory of Liaoning Province (no. 2008S247); Shengjing Freedom Researchers Plan (200807). The Science Foundation of Shenyang (1053125-1-54-1, 2009225007-2).

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