Presence of Human Papilloma Virus DNA in Pelvic Lymph Nodes Can Predict Unexpected Recurrence of Cervical Cancer in Patients with Histologically Negative Lymph Nodes[1]

Yasuaki Kobayashi, Mitsuo Yoshinouchi,[2] Guo Tianqi, Keichiro Nakamura, Atsushi Hongo, Shigehito Kamimura, Yasushi Mizutani, Junichi Kodama, Yasunari Miyagi, and Takafumi Kudo

Department of Obstetrics and Gynecology, Okayama University Medical School, Okayama 700, Japan

ABSTRACT

Patients without any evidence of lymph node metastases are supposed to have a fair prognosis, but some of these patients develop recurrent disease unexpectedly after surgery. The object of this study is to examine whether the detection of human papilloma virus (HPV) DNA could be used as a diagnostic marker to predict such recurrences. Two hundred and thirty-six patients undergoing radical hysterectomy and pelvic lymphadenectomy for stage Ib and II cervical cancer at Okayama University Hospital (Japan) from 1988–1994 were reviewed, and only those cases positive for HPV-16 or HPV-18 in primary sites were included in this survey. The E6–E7 region of the HPV genome was amplified by a sensitive nested PCR from archival pelvic lymph node specimens. HPV sequences identical to those of the primary sites were detected in histologically confirmed negative lymph nodes, regardless of histological type or HPV type of the primary lesion, in 9 of 10 patients who recurred within 4 years of surgery. In contrast, histologically confirmed negative lymph nodes from 12 patients with stage IIb disease without evidence of recurrent disease were all negative for the presence of HPV, except for 1 lymph node. The presence of HPV DNA in histologically negative nodes implies the possibility of early nodal involvement or coexistence of undetectable hematogenic dissemination and could therefore be used as a diagnostic marker to predict the unexpected recurrence of these patients.

INTRODUCTION

With steady progress in the early detection of the preinvasive state, the incidence of invasive cervical cancer is decreasing, leading to a better survival rate. In Japan, radical hysterectomy with pelvic lymphadenectomy is generally reserved for patients with stage Ib and II disease who are in good physical condition. The survival of patients after radical hysterectomy is dependent on several factors, such as nodal status, tumor size, paracervical involvement, depth of myometrial invasion, lymph vascular space invasion, and histological type (1, 2). The prevalence of metastases in pelvic lymph nodes is the main contributing factor to the high mortality rate in cervical cancer. In fact, a favorable survival rate (5-year disease-free survival, 86.0%) has been observed in patients with negative pelvic nodes, whereas a survival rate of 54.2% has been observed in patients with nodal involvement in our hospital. Nevertheless, we cannot eliminate some patients who later present with unexpected recurrence, in spite of the accomplishment of radical hysterectomy and histologically confirmed negative pelvic nodes.

A strong association between specific HPV[3] types and anogenital cancer has been well established (3). HPV viral DNA is identified as the causative agent in at least 90% of cervical carcinomas by means of PCR (4). Viral DNA is integrated into the cellular genome in the majority of malignant tumors (5), and two specific open reading frames, E6 and E7, are consistently transcribed in these tumors (6).

Several studies have demonstrated the presence of HPV DNA in lymph node metastases (7–14). Recently developed PCR has allowed more sensitive and retrospective studies to detect HPV DNA in lymph nodes. However, the prognostic significance of HPV DNA in lymph nodes has not been clearly evaluated. The object of this study is to examine whether the detection of HPV DNA could be used as a diagnostic marker to predict recurrence in patients whose lymph nodes are histologically confirmed as negative. Sensitive PCR detection of HPV DNA in histologically negative lymph nodes may be helpful in planning postoperative therapy.

MATERIALS AND METHODS

Case studies of 236 patients who underwent radical hysterectomy and pelvic lymphadenectomy for stage Ib and II cervical cancer at Okayama University Hospital from 1988–1994 were reviewed. The stage of disease was assessed clinically, based on the International Federation of Gynecology and Obstetrics criteria for cervical carcinoma. Specimens were immediately fixed in 10% neutral-buffered formalin and

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[2] To whom requests for reprints should be addressed, at Department of Obstetrics and Gynecology, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama 700, Japan. Phone: 81-86-235-7320; Fax: 81-86-225-9570; E-mail: m_yoshi@cc.okayama-u.ac.jp.

[3] The abbreviation used is: HPV, human papilloma virus.

Table 1 Clinicopathological characteristics of cervical cancer patients with pelvic lymph node metastases

No.	Age (yr)	Stage	Cell type[a]	HPV type: Primary site	HPV type: Lymph nodes
1	50	IIb	SCC, LNK	18	18+
2	47	IIb	SCC, LNK	16	16+
3	60	IIb	ADE	16	16+
4	57	IIb	SCC, SNK	16	16+
5	55	IIb	SCC, LNK	16	16+
6	59	IIb	SCC, SNK	16	16+
7	59	IIb	SCC, LNK	16	16+
8	39	IIb	ADSQ	18	18+

[a] SCC, squamous cell carcinoma; LNK, large cell nonkeratinizing carcinoma; SNK, small cell nonkeratinizing carcinoma; ADE, adenocarcinoma; ADSQ, adenosquamous cell carcinoma.

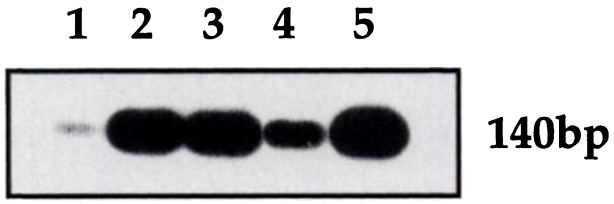

Fig. 1 Nested PCR and sequential Southern blot analysis from archival lymph nodes with histologically confirmed metastases. DNA from archival lymph nodes with definite tumor foci was subjected to nested PCR for HPV-16 sequences and then hybridized with internal-specific oligonucleotide probes. The primary sites of all patients were positive for HPV-16 DNA. One hundred and forty bp of the corresponding fragment were readily amplified and abundantly displayed with a variation of signal intensities. *Lanes 1–5*, patients with stage IIb carcinoma with nodal metastases.

embedded in paraffin at the time of surgery. Primary lesions were screened for the presence of HPV DNA, and only HPV-16- or HPV-18-positive cases were included in the current survey. Eight patients with stage IIb carcinoma were examined by our nested PCR to detect HPV DNA from corresponding lymph nodes with definite tumor nests. These were evaluated as positive controls. In 10 patients with histologically confirmed negative lymph nodes and paracervical involvement, disease recurred within 4 years (range, 5–42 months) after surgery. Corresponding archival pelvic lymph node specimens were available from these patients. Bilateral common iliac, external iliac, and obturator nodes were routinely investigated. When all of these were negative for HPV, the remaining nodes available were further examined. Twelve patients with stage IIb disease with histologically confirmed negative lymph nodes were randomly selected from patients without evidence of disease with a minimum of 36 months of follow-up after surgery.

Nested PCR and Subsequent Southern Blot Analysis. Sections (8-μm thick) were sliced from each block with caution to avoid carry-over contamination. Two or three sections at multiple levels in the lymph node were combined to increase the sensitivity of the procedure. A serial section from the specimen was processed for conventional light microscopy, and histopathology of the section was thoroughly reviewed by an experienced gynecological pathologist. DNA solutions for PCR were prepared with DEXPAT™ (Takara Shuzo Co., Ltd., Kyoto, Japan) according to the manufacturer's protocol. The presence of HPV DNA was first examined by nested PCR with a pair of outer primers, p16-l and pU-2R. These are common primers for HPV-16 and HPV-18. Then, 5 μl of PCR products were subjected to a second PCR with a pair of inner primers, p16-l and p16–2R or p18–2R. p16–2R and p18–2R correspond to the E6 antisense sequence of HPV-16 and HPV-18, respectively. For every set of PCR runs, reaction mixture without template DNA was included as a negative control. The conditions for specific PCR were described elsewhere (15, 16). For further improvement of sensitivity and specificity, final PCR products were Southern-transferred and hybridized with internal specific oligonucleotide probes p16-I or p18-I, as described elsewhere (15, 17). All oligodeoxynucleotides were synthesized using a Model 394 DNA synthesizer (Applied Biosystems, Foster City, CA).

Statistical Analysis. Statistical analysis was performed using Fisher's exact probability test. Probability values less than 0.05 were considered statistically significant.

RESULTS

HPV DNA in Lymph Node Metastases. Clinicopathological characteristics of eight patients with stage IIb cervical carcinoma are summarized in Table 1. The histopathological cell types of the primary sites of these patients included six squamous cell carcinomas (four large cell nonkeratinizing-type carcinomas and two small cell nonkeratinizing-type carcinomas), one adenocarcinoma, and one adenosquamous cell carcinoma. HPV-16 DNA was found in the primary lesions of six patients, and HPV-18 was detected in two patients. Eight lymph nodes with histologically confirmed metastases from each patient were tested for the presence of HPV DNA. In all patients, the corresponding fragment was readily amplified by nested PCR and abundantly displayed by subsequent Southern hybridization with a variation of signal intensities. Representative hybridization signals obtained with HPV-16 probes are shown in Fig. 1. Our PCR and Southern hybridization techniques are capable of detecting HPV DNA from archival pelvic lymph node specimens.

HPV DNA in Histologically Negative Lymph Nodes. Table 2 summarizes the clinicopathological characteristics of 10 patients who experienced unexpected disease recurrence after surgery. Histopathological cell types of the primary sites of these patients included nine squamous cell carcinomas (six large cell nonkeratinizing-type carcinomas and three small cell nonkeratinizing-type carcinomas) and one adenosquamous cell carcinoma. All of these patients received 2500–3000 cGy of ^{137}Cs vaginal cuff irradiation, which is a routine postoperative irradiation therapy in our hospital. Two patients also received 5000 cGy of external pelvic irradiation with a linear accelerator, because their primary lesions were large and showed lymph vascular space invasion, although histopathological analysis had detected neither node metastases nor positive surgical margins. HPV-16 DNA was found in all primary sites, except for one case with adenosquamous cell carcinoma, in which HPV-18

Table 2 Clinicopathological characteristics of cervical cancer patients without pelvic lymph nodes metastases

No.	Age (yr)	Stage	Cell type[a]	Adjuvant therapy[b]	Recurrence	HPV type	
						Primary site	Lymph nodes
1	63	IIb	SCC, SNK	10 MeV, Cs	Bone (6 mo)	16	16+
2	47	IIb	SCC, SNK	Cs	Pelvis (7 mo)	16	16+
3	41	IIb	SCC, LNK	10 MeV, Cs	Bone (42 mo)	16	16+
4	62	Ib	SCC, LNK	Cs	Bone (5 mo)	16	−
5	55	IIb	SCC, SNK	Cs	Bone (34 mo)	16	16+
6	37	IIb	SCC, LNK	Cs	Lung (11 mo)	16	16+
7	45	IIb	ADSQ	Cs	Liver (11 mo)	18	18+
8	70	IIb	SCC, LNK	Cs	DOD[c] (16 mo)	16	16+
9	58	IIb	SCC, LNK	Cs	Pelvis (24 mo)	16	16+
10	52	IIb	SCC, LNK	Cs	Bone (6 mo)	16	16+

[a] SCC, squamous cell carcinoma; LNK, large cell nonkeratinizing carcinoma; SNK, small cell nonkeratinizing carcinoma; ADSQ, adenosquamous cell carcinoma.

[b] 10 MeV, 50 Gy of external irradiation; Cs, ^{137}Cs vaginal cuff irradiation.

[c] DOD, died of disease.

DNA was detected. In 9 of 10 patients, the identical HPV type as in the primary site was displayed in either of these histologically negative lymph nodes, as shown in Fig. 2. In patient 4, because the bilateral common iliac, external iliac, and obturator nodes were all HPV negative, the remaining lymph nodes (including the bilateral suprainguinal, internal iliac, and cardinal nodes) were further investigated for the presence of HPV. However, we did not detect positive signals from any nodes in this patient (data not shown). Fig. 3 shows hybridization signals displayed from each node of patient 2. This Japanese woman presented with a recurrent pelvic mass 6 months after surgery and received external irradiation and systemic chemotherapy. The left-side nodes in *Lanes 1–4* were all negative, whereas the right obturator and internal iliac nodes were apparently positive, and the right common iliac and external iliac nodes also harbored faint bands. Then, the remaining corresponding paraffin-embedded lymph node specimens were thoroughly reviewed microscopically for tiny metastases and confirmed to be negative.

Histologically confirmed negative lymph nodes from 12 patients with stage IIb disease without evidence of disease were analyzed for the presence of HPV. However, except for one lymph node, they did not contain any HPV sequence (data not shown).

There was a significant difference between HPV DNA status in lymph nodes and the patient's disease-free survival ($P = 0.0004$).

DISCUSSION

The principle for treatment of cervical cancer is that the primary lesion and potential sites of spread should be treated. We have two modalities for primary treatment: surgery and radiotherapy. Radiotherapy can be used in all stages of the disease, but surgery alone is limited to patients with stage I and II disease in Japan. The outcome of patients who undergo radical hysterectomy and pelvic lymphadenectomy is affected adversely by several factors, such as nodal status, tumor size, paracervical involvement, depth of myometrial invasion, lymph vascular space invasion, and certain histological types (1, 2). It is now widely known that pelvic lymph node involvement is the main contributing factor to the high mortality rate in cervical cancer (18). In general, several representative sections from a lymph node specimen were processed for conventional light microscopy. It is likely, however, that invading cancer cells may be restricted to tiny areas within the cortex; consequently, they may be missed after arbitrary section cutting. This suggests an inevitable light microscopic limitation in evaluating the small foci of cancer cells or occult metastases. It is also possible that biologically transformed but morphologically nonmalignant cells exist in the lymph nodes. Most patients who receive successful surgery with a confirmation of negative lymph node metastases and surgical margins are supposed to have a favorable prognosis. Therefore, administration of adjuvant therapy including external whole pelvic irradiation and/or systemic chemotherapy is not required for such patients. Nevetheless, some of these patients unexpectedly develop recurrent disease after surgery.

The presence of the HPV genome in lymph node metastases was first described by Lancaster *et al.* (7). They also reported one nonmetastasizing lymph node sample containing HPV sequences. Studies have demonstrated the stable persistence of integrated HPV DNA with invasive tumor cells (8–10). There is a compatible precedent for the presence of HPV DNA sequences in cancer cells that exist within lymph nodes (7–14). PCR allows *in vitro* amplification of specific DNA sequences to easily detectable amounts, such that even very small amounts of DNA present in a paraffin section of fine-needle aspirations can be evaluated (19). Now, one can expect the possible utility of detection of the HPV genome by sensitive PCR as a diagnostic marker to find early nodal metastases and to predict previously unexpected disease recurrence.

First, we tried to detect HPV DNA from lymph nodes with definite positive tumor nests to confirm the capability of our screening procedure. The corresponding fragment of HPV sequences identical to that of the primary site was successfully amplified and readily visualized from metastatic lymph nodes in eight patients, regardless of histological type or HPV type of the primary lesion, without any exceptions (Fig. 1; Table 1). According to Shimada *et al.* (15) and Fujinaga *et al.* (16), the sensitivity of this nested PCR was minimally estimated to be

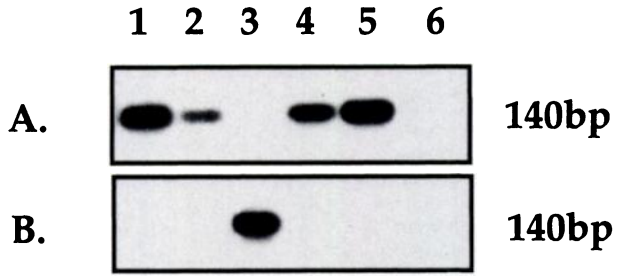

Fig. 2 Nested PCR and sequential Southern blot analysis from archival lymph nodes with histologically confirmed negative metastases. Patients with histologically confirmed negative lymph nodes and paracervical involvement experienced disease recurrence after surgery. The primary sites of these patients were positive for HPV-16 DNA, except for one that was positive for HPV-18 DNA (*Lane 3*). Negative control was loaded in *Lane 6*. DNA from histologically confirmed negative lymph nodes was subjected to nested PCR for HPV-16 sequences (*Lanes 1, 2, 4,* and *5*) or HPV-18 sequences (*Lane 3*). The second PCR products were then hybridized with p16-1 (*A*) or p18-1 (*B*) type-specific probes. Thorough removal of the hybridized probes was performed between each hybridization. HPV sequences identical to those of the primary sites were identified.

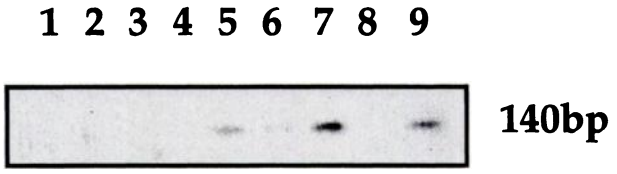

Fig. 3 Nested PCR and sequential Southern blot analysis from all available pelvic lymph nodes of patient 2. All of the dissected pelvic lymph nodes were examined for the presence of HPV-16 DNA. *Lane 1*, left common iliac nodes; *Lane 2*, left external iliac nodes; *Lane 3*, left obturator nodes; *Lane 4*, left suprainguinal nodes; *Lane 5*, right common iliac nodes; *Lane 6*, right external iliac nodes; *Lane 7*, right obturator nodes; *Lane 8*, left suprainguinal nodes; *Lane 9*, right internal iliac nodes.

10^{-7}. Our tools are thus both sensitive and specific enough to demonstrate the presence of metastasizing cells from formalin-fixed paraffin-embedded specimens.

Then, we applied these techniques to survey the presence of HPV DNA in histologically confirmed negative lymph nodes. The bilateral common iliac, external iliac, and obturator nodes were routinely included in this survey, because these nodes are known to be frequently involved (20, 21, 22). Overall, the HPV type identical to that of the primary lesion was identified in these nodes in 9 of 10 patients (Table 2). In the remaining patient, all of the other pelvic lymph nodes were investigated for the presence of HPV DNA. However, we did not obtain a positive signal from any of them. HPV sequences were observed in some nodes but not in others within an individual (Fig. 3). It is postulated that this was reflected by the presence of early and tiny metastases and the level of sensitivity of our method. We can conclude from this study of relatively small numbers that the common iliac, external iliac, and obturator nodes should be investigated to determine whether or not they contain HPV sequences.

Three of nine patients (33%) whose pelvic nodes exhibited HPV sequences but microscopically confirmed metastases were small cell nonkeratinizing carcinomas. There is general agreement that patients with small cell carcinoma have a worse prognosis than those with large cell variants, because small cell carcinomas behave more aggressively and are more likely to give rise to nodal metastases than any other type of squamous neoplasms (23, 24). The high incidence of small cell carcinoma in which HPV DNA was detected in the lymph nodes in the current study strongly supports this conclusion.

It was surprising that a majority of the sites of recurrence in these nine patients were hematogenic (*i.e.*, bone, lung, or liver; 67%). In these patients, oncogenic HPV DNA was amplified from some pelvic lymph nodes. It is likely that small numbers of cancerous cells were present within the cortex but were missed on section cutting. Our results indicate that the presence of HPV sequences in pelvic lymph nodes implies the coexistence of undetectable hematogenic dissemination at the time of surgery. In fact, most patients developed the recurrent lesion within 12 months after surgery. Furthermore, two patients experienced disease recurrence although they received postoperative external irradiation as well as vaginal cuff irradiation. Postoperative radiotherapy might decrease pelvic recurrence and, consequently, improve the survival rate (25). However, radiation plus systemic chemotherapy would be required to prevent unexpected recurrence.

In conclusion, HPV sequences were amplified by our sensitive PCR in histologically negative pelvic lymph nodes in patients who unexpectedly developed recurrent lesions after surgery. The presence of HPV DNA implies early nodal involvement or the coexistence of undetectable hematogenic dissemination and could therefore be used as a diagnostic marker to predict the unexpected recurrence of disease in such patients. An investigation of at least the common iliac, external iliac, and obturator nodes for the presence of HPV DNA identical to that of the primary site would produce much benefit in planning the adjuvant therapy of cervical cancer.

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